Mechanisms of Shiga toxin-mediated signaling and toxicity

Johansson, Karl

2020

Document Version:
Publisher's PDF, also known as Version of record

Mechanisms of Shiga toxin-mediated signaling and toxicity

KARL JOHANSSON
FACULTY OF MEDICINE | LUND UNIVERSITY

Printed by Media-Tryck, Lund 2020 NORDIC SWAN ECOLABEL 3041 0903

LUND UNIVERSITY

FACULTY OF MEDICINE

Lund University, Faculty of Medicine
Doctoral Dissertation Series 2020:4
ISBN 978-91-7619-864-3
ISSN 1652-8220

Mechanisms of Shiga toxin-mediated signaling and toxicity

Karl Johansson
Department of Pediatrics
Clinical Sciences Lund

LUND
UNIVERSITY

DOCTORAL DISSERTATION
by due permission of the Faculty of Medicine, Lund University, Sweden.
To be defended in Belfragesalen, Biomedicinskt Centrum, D15.
Friday the 17th of January 2020, at 9.00

Faculty opponent
Professor Dr. Lambertus van den Heuvel
Dept of Pediatric Nephrology, Radboud University Medical Center,
Nijmegen, The Netherlands

Organization	Document name
LUND UNIVERSITY Department of Pediatrics Clinical Sciences Lund	DOCTORAL DISSERTATION
	Date of issue 17th of January 2020
Author: Karl Johansson	Sponsoring organization

Title and subtitle: Mechanisms of Shiga toxin-mediated signaling and toxicity

Abstract

Shiga toxin (Stx) is the main virulence factor of enterohemorrhagic *Escherichia coli* (EHEC). EHEC strains cause gastrointestinal infection and release Stx that can gain access to the circulation. Patients may develop hemolytic uremic syndrome with extensive kidney damage.

In the first paper we investigated if blood cell-derived microvesicles released during EHEC infection contain Stx. Toxin-containing microvesicles were found in plasma from EHEC-infected patients and mice, and in kidney cells. Toxin transferred within microvesicles retained its toxic potential. This study demonstrated a novel mechanism of Stx delivery to the kidney.

In the second paper the role of the Stx receptor, globotriaosylceramide (Gb3) in toxicity and intracellular transport of Stx delivered via microvesicles was investigated. Stx-positive microvesicles were taken up by both Gb3-positive and Gb3–negative cells but the toxin only exhibited toxicity when the cells expressed endogenous Gb3.

Stx induces calcium influx, cytotoxicity and microvesicle release in cells. In the third paper we found that ATP is released from HeLa cells after Stx stimulation and that ATP functions as a secondary messenger to promote all the above-mentioned toxin effects. Blocking purinergic P2X signaling with NF449 inhibited Stx-induced calcium influx, microvesicle release and protected cells from the cytotoxic effects.

In the fourth paper the role of extracellular ATP was investigated in a murine model of EHEC infection. Infected mice were injected with apyrase to cleave extracellular ATP. Apyrase protected the mice form EHEC-induced intestinal damage and delayed symptoms.

The fifth paper is a review that summarizes the role of extracellular vesicles in normal physiology and kidney diseases.

In summary this thesis defined a novel mechanism of Stx transfer to the kidneys, an ATP-mediated effect of Stx-signaling and the role of Gb3 on the cellular effect of Stx-positive microvesicles.

Key words: EHEC, hemolytic uremic syndrome, Shiga toxin, purinergic signaling, microvesicles

Classification system and/or index terms (if any)

Supplementary bibliographical information		**Language**
ISSN and key title 1652-8220, Medicine Doctoral Dissertation Series 2020:4		**ISBN** 978-91-7619-864-3
Recipient's notes	**Number of pages** 73	Price
	Security classification	

Mechanisms of Shiga toxin-mediated signaling and toxicity

Karl Johansson

LUND
UNIVERSITY

Faculty of Medicine

Department of Pediatrics
Clinical Sciences Lund
Lund University
Sweden
2020

Karl Johansson
Department of Pediatrics
Clinical Sciences Lund
Lund University
221 84 Lund
E-mail: karl.johansson@med.lu.se
Phone: +46 46 222 07 45

Faculty of Medicine, Clinical Sciences Lund, Department of Pediatrics
ISBN 978-91-7619-864-3
ISSN 1652-8220
Printed in Sweden by Media-Tryck, Lund University
Lund 2020

Media-Tryck is an environmentally certified and ISO 14001:2015 certified provider of printed material.
Read more about our environmental work at www.mediatryck.lu.se

MADE IN SWEDEN

Till minnet av min pappa

Table of contents

List of papers

This thesis is based on the following papers:

I. Ståhl AL, Arvidsson I, **Johansson KE**, Chromek M, Rebetz J, Loos S, Kristoffersson A-C, Békassy ZD, Mörgelin M, Karpman D. A novel mechanism of bacterial toxin transfer within host cell-derived microvesicles. *PLoS Pathog* 2015;11:e1004619.

II. **Johansson KE,** Willysson A, Kristoffersson AC, Tontanahal A, Gillet D, Ståhl AL, Karpman D. Shiga toxin-bearing microvesicles exert a cytotoxic effect on recipient cells only when the cells express the toxin receptor. Manuscript.

III. **Johansson KE**, Ståhl AL, Arvidsson I, Loos S, Tontanahal A, Rebetz J, Chromek M, Kristoffersson AC, Johannes L, Karpman D. Shiga toxin signals via ATP and its effect is blocked by purinergic receptor antagonism. *Sci Reports* 2019 9(1):14362.

IV. Arvidsson I, Tontanahal A, **Johansson KE**, Kristoffersson AC, Karpman D. Apyrase treatment delays symptoms in a mouse model of *Escherichia coli* O157:H7 infection. Manuscript.

V. Ståhl AL, **Johansson K**, Mossberg M, Kahn R, Karpman D. Exosomes and microvesicles in normal physiology, pathophysiology, and renal diseases. *Pediatr Nephrol* 2019; 34: 11-30.

The following papers have been published but are not included in the thesis:

Kahn R, Mossberg M, Ståhl AL, **Johansson K**, Lopatko Lindman I, Heijl C, Segelmark M, Mörgelin M, Leeb-Lundberg LM, Karpman D, Microvesicle transfer of kinin B1-receptors is a novel inflammatory mechanism in vasculitis, *Kidney Int*, 2017, doi: 10.1016

Karpman D, Ståhl AL, Arvidsson I, **Johansson K**, Loos S, Tati R, Békássy Z, Kristoffersson AC, Mossberg M, Kahn R, Complement interactions with blood cells, endothelial cells and microvesicles in thrombotic and inflammatory conditions, *Adv Exp Med Biol*, 2015, doi: 10.1007

Abbreviations

A/E	Attaching and effacing
ATP	Adenosine triphosphate
EHEC	Enterohemorrhagic *Escherischia coli*
ER	Endoplasmic reticulum
Gb3	Globotriaosylceramide
Gb4	Globotetraosylceramide
HUS	Hemolytic uremic syndrome
IP3	Inositol triphosphate
LPS	Lipopolysaccharide
OMV	Outer membrane vesicles
PLC	Phospholipase C
PE	Phosphatidylethanolamine
PS	Phosphatidylserine
Stx	Shiga toxin
T3SS	Type three secretion system
TLR	Toll-like receptor

Abstract

Shiga toxin (Stx) is the main virulence factor of enterohemorrhagic *Escherichia coli* (EHEC). EHEC strains cause gastrointestinal infection and release Stx that can gain access to the circulation. Patients may develop hemolytic uremic syndrome with extensive kidney damage.

In the first paper we investigated if blood cell-derived microvesicles released during EHEC infection contain Stx. Toxin-containing microvesicles were found in plasma from EHEC-infected patients and mice, and in kidney cells. Toxin transferred within microvesicles retained its toxic potential. This study demonstrated a novel mechanism of Stx delivery to the kidney.

In the second paper the role of the Stx receptor, globotriaosylceramide (Gb3) in toxicity and intracellular transport of Stx delivered via microvesicles was investigated. Stx-positive microvesicles were taken up by both Gb3-positive and Gb3–negative cells but the toxin only exhibited toxicity when the cells expressed endogenous Gb3.

Stx induces calcium influx, cytotoxicity and microvesicle release in cells. In the third paper we found that ATP is released from HeLa cells after Stx stimulation and that ATP functions as a secondary messenger to promote all the above-mentioned toxin effects. Blocking purinergic P2X signaling with NF449 inhibited Stx-induced calcium influx, microvesicle release and protected cells from the cytotoxic effects.

In the fourth paper the role of extracellular ATP was investigated in a murine model of EHEC infection. Infected mice were injected with apyrase to cleave extracellular ATP. Apyrase protected the mice form EHEC-induced intestinal damage and delayed symptoms.

The fifth paper is a review that summarizes the role of extracellular vesicles in normal physiology and kidney diseases.

In summary this thesis defined a novel mechanism of Stx transfer to the kidneys, an ATP-mediated effect of Stx-signaling and the role of Gb3 on the cellular effect of Stx-positive microvesicles.

Introduction

Enterohemorrhagic *Escherichia coli* (EHEC) are gram-negative bacteria that may lead to disease in humans and in severe cases causes hemolytic uremic syndrome (HUS), characterized by thrombocytopenia, hemolytic anemia and acute kidney injury.[1, 2] EHEC enters the body via ingestion of contaminated food or water and passes through the gastrointestinal tract to the large intestine. EHEC is non-invasive and colonizes the large intestine.[3] Within the intestine EHEC releases several virulence factors, including Shiga toxin (Stx), which is the main virulence factor. Stx must enter the circulation to reach and damage the target organs, including the kidney. [4-7]

Free Stx is scarcely detectable in plasma from hemolytic uremic syndrome (HUS) patients.[8] However, Stx may circulate bound to blood cells. Stx may bind to platelets,[9-11] neutrophils[11-13], monocytes[11, 14] and red blood cells[15] and thus be transported in the circulation, but this does not fully explain how it is transferred to target organ cells such as in the kidney. Stx-binding induces release of microvesicles from platelets, neutrophils, monocytes and red blood cells.[11, 15] Microvesicles are small (100 – 1000 μm) vesicles that are shed directly from the plasma membrane.

The first paper of this thesis investigated the systemic transport of Stx via blood cell-derived microvesicles in the circulation and the delivery of the toxin-bearing vesicles to relevant kidney cells. The potential of toxin within microvesicles to exert a cytotoxic effect was further addressed.

Stx is endocytosed by cells following binding to its receptor, globotriaosylceramide (Gb3).[16] After uptake, Stx is transported via the retrograde pathway to the endoplasmic reticulum (ER).[17] From the ER the enzymatic A subunit of Stx is released into the cell cytosol where it exhibits RNA N-glycosidase activity cleaving an adenine base of the 28S rRNA, causing inhibition of protein synthesis and thereby leads to cell death.[18, 19]

Stx has been detected within cells that lack the Gb3 receptor[20] and may also indirectly affect cells that lack the toxin receptor.[21, 22] This was also demonstrated in murine glomerular endothelial cells in the first paper of this thesis.[23], which may indicate a receptor-independent mechanism of toxin uptake. In the second paper included in this thesis we investigated the importance of endogenous Gb3 in the recipient cell for the toxicity of microvesicle-delivered Stx in Gb3-positive and Gb3-negative cells.

Stx induces calcium influx, protein synthesis inhibition, apoptosis and microvesicle release in stimulated cells.[5, 11, 15, 24-27] In the third paper of this thesis a secondary messenger contributing to these cellular effects of the toxin was identified.

Stx induced release of ATP *in vitro* and *in vivo* and the released ATP activated purinergic receptors on host cells. We showed that pharmacological blockade of purinergic P2X signaling inhibited Stx-mediated calcium influx, microvesicle-release and cytotoxicity *in vitro.*

The fourth paper in the thesis investigated the role of extracellular ATP in an *in vivo* mouse model of EHEC infection using the ATP-hydrolyzing enzyme apyrase. Apyrase-treatment delayed symptoms in mice. Furthermore, goblet cell depletion and intestinal apoptosis were reduced.

The fifth paper focused on describing the role of extracellular vesicles in normal physiology as well as biomarkers of kidney disease. Specifically the review mentions the role of microvesicles in HUS and in inflammatory renal diseases.

In summary, this thesis addresses the transport of Stx within the circulation to the kidneys via microvesicles, the importance of the Gb3 receptor in microvesicle-delivered Stx toxicity, the mechanism by which Stx induces cell signaling and the effect of extracellular ATP during EHEC infections. This thesis thereby provides novel insight in the pathogenesis of EHEC infection and may contribute to identifying possible targets for future therapeutic strategies.

Enterohemorrhagic *Escherichia coli*

EHEC are human pathogens. The bacteria colonize the intestine of graminivores asymptomatically.[28, 29] Human infection occurs via the fecal-oral route by ingestion of contaminated food or water and has a global prevalence.[30] EHEC may infect individuals in all age groups but is especially harmful to infants and the elderly and can in severe cases cause hemolytic uremic syndrome (HUS) leading to acute kidney injury and death.[2] Infectious doses as low as 100 colony-forming units can cause disease symptoms.[31] Ingested EHEC is transported in the gastrointestinal tract to the large intestine, which it colonizes.[32] EHEC has developed efficient acid resistance, which explains its capability to survive the acidic environment of the stomach.[33, 34] EHEC is a non-invasive bacteria and once it has reached the large intestine it colonizes the intestinal epithelium but does not cause bacteremia or septicemia.[3]

There are several pathogenic EHEC serotypes. These strains are named based on the terminal portion of the lipopolysaccharide (O-antigen) and the type of flagella (H-antigen) that the bacteria express. Serotypes associated with human disease include O157:H7, O26:H11, O103:H2, O111:NM, O121:H19 and O145:NM.[35]

EHEC adhesion

The initial EHEC adhesion to the intestinal mucosa is mediated by several factors, such as fimbriae and the type three secretion system (T3SS) (Figure 1).[36] This first adhesion is followed by a more intimate adhesion leading to attaching and effacing (A/E) lesions. A/E lesions are anchoring points that require transfer of bacterial proteins into the host epithelial cell. EHEC encodes the protein complex T3SS that resembles a syringe-like structure. T3SS attaches to host cells, linking the bacteria to the epithelial cell and allows for bacterial protein transfer into the recipient cell membrane. In order to transfer proteins to the host cell EHEC forms a pore in the host membrane composed of EspB and EspD.[37] EHEC protein-transfer includes translocated intimin receptor (Tir) and *E. coli* secreted protein F-like protein from prophage U (EspFu).[38, 39] Tir proteins form transmembrane structures in the epithelial cell that enable binding to intimin that is present on EHEC.[40, 41] EspFu recruits the actin-modulating proteins Wilskott-Aldrich syndrome protein (N-WASP) and Insulin receptor substrate p53 (IRSp53) that connect the Tir

complexes to the actin network of the host cell and also form the pedestal-like actin structures that are typical for A/E lesions.[42]

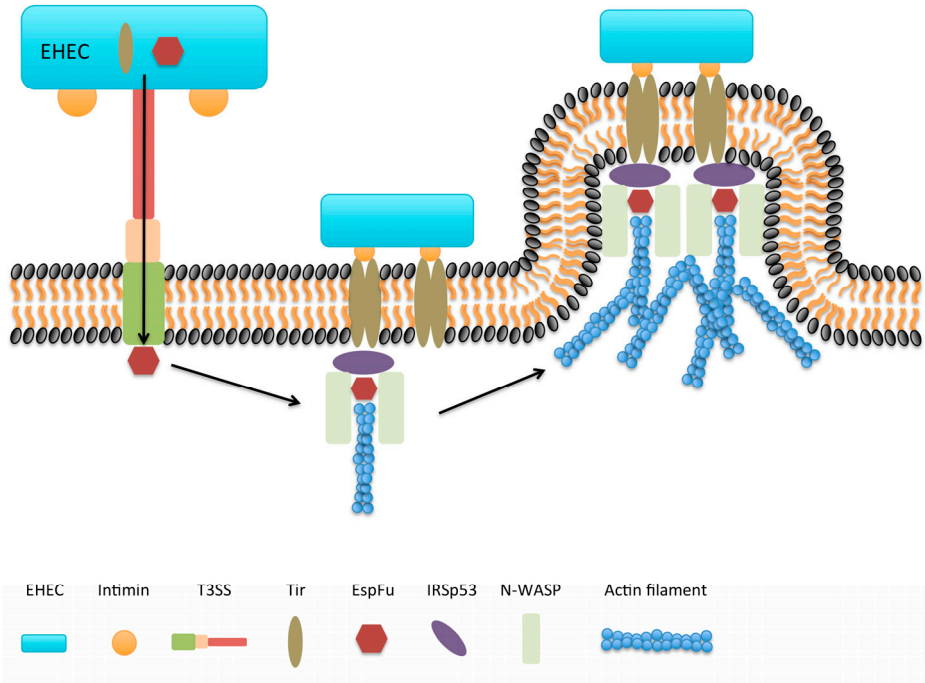

Figure 1. Schematic figure depicting the formation of an attaching/effacing lesion.
EHEC attaches to epithelial cells via the type 3 secretion system (T3SS) and transports Tir and EspFu into the host cell. Tir is inserted into the membrane of the recipient cell and forms dimers that functions as anchoring points for the intimin receptor. EspFu promotes remodelling of the host cell's actin cytoskeleton and subsequently forms pedestal-like structures. This figure was made using PowerPoint software v.14.7.7.

Certain determinants of the T3SS have been associated with bacterial virulence. For example, EspB, has been shown to induce diarrhea in healthy volunteers who ingested a wild-type strain and its isogenic ΔEspB mutant.[43] Intimin was associated with a higher mortality in a mouse model.[44] The presence of intimin and T3SS effectors were associated with a higher symptom score in murine EHEC infection.[45]

EHEC virulence factors

During EHEC infection the bacteria release several virulence factors. The main virulence factor is Shiga toxin (Stx). Other virulence factors include lipopolysaccharide (LPS), EHEC-hemolysin and subtilase.

Shiga toxin

Stx is the major virulence factor of EHEC. The toxin is named after the Japanese physician Kiyoshi Shiga who discovered *Shigella dysenteriae*, a Stx-producing bacteria that may cause dysentery.[46] Shiga toxin is an AB5 toxin, consisting of an enzymatically active A-subunit and a binding, pentameric B-subunit.[47]

Release and transport of Shiga toxin

Stx is released from EHEC via outer membrane vesicles (OMVs) or by bacterial lysis. It is released in the intestine and is essential for intestinal damage and hemorrhagic colitis as shown in animal models.[45, 48] For the toxin to exert its systemic effect it needs to be transported from the intestine into the circulation. It is not completely known how Stx is transported across the intestinal epithelium; however, several mechanisms have been suggested.

The tight proximity of intestinal cells was shown to be disrupted in response to EHEC infection *in vivo* and *in vitro*.[49, 50] Furthermore, during EHEC infection, Stx was shown to diffuse between monolayer cultures of the intestinal epithelial cell line T84 cells, a cell line lacking the Stx receptor.[51] The translocation of Stx across intestinal epithelium was enhanced during inflammation with neutrophil extravasation into the intestinal lumen.[52]

Stx may be taken up by intestinal cells by macropinocytosis, followed by release on the basal side via transcytosis.[20] Free toxin has also been shown to cross monolayer cultures of T84 cells without disrupting the barrier function of the culture. The translocation of the toxin was shown to be energy-dependent, indicating a cell-mediated transport.[53]

Uptake of Stx by intestinal cells may occur via receptor-mediated endocytosis, followed by subsequent release through cell death induced by the toxin. Human intestinal epithelial cells normally only express trace amounts of the Stx receptor.[54] The fatty acid butyrate have been shown to increase expression of the toxin receptor in intestinal epithelial cells *in vitro,* providing a possible mechanism for Stx uptake and translocation. [55]

Regardless of the mechanism through which Stx is translocated over the intestinal epithelial cell layer the toxin also needs to cross the lamina propria (a layer of connective tissue) to reach blood vessels. Stx has been found in the lamina propria in a study using *in vitro* intestine organ cultures.[56] Access to the bloodstream may be a result of the massive inflammation and damage of the intestine. Once Stx has gained access to blood vessels it can interact with and damage intestinal endothelial cells.[57]

The transport of Stx in the circulation was a focus of this thesis. This aspect is of interest because only trace amounts of free Stx have been found in patient blood.[8] Stx can bind to platelets,[11] monocytes,[11] red blood cells, [15] and neutrophils.[11, 12] Binding may lead to cellular uptake, as has been shown in platelets and monocytes.[10, 58] Cells that bind Stx shed microvesicles, which will be described below.

From the circulation Stx targets various organs, such as the kidney[59, 60] and the central nervous system.[61] Target cells in the kidney include glomerular endothelial cells, tubular cells, mesangial cells and podocytes.[4-7, 62-64]

Shiga toxin 1 and 2

Stx is a collective name for Stx1 and Stx2. While Stx1 has an almost identical amino acid sequence to the Stx produced by *Shigella dysenteriae*, save for one amino acid, Stx1 and Stx2 share only 56% amino acid homology.[65, 66] Both Stx1 and Stx2 are released by EHEC but Stx2 has been associated with more severe disease during HUS.[67] The two toxins share the same receptors but with different affinities, as will be described below. There are currently three known variants of Stx1: Stx1a, Stx1c and Stx1d and seven variants of Stx2: Stx2a-g.[68, 69]

The Shiga toxin receptor

Stx binds to the neutral glycosphingolipids globotriaosylceramide (Gb3) or globotetraosylceramide (Gb4) (Figure 2).[70] There is also evidence for binding of Stx2e to globopentaosylceramide.[71] Neutral glycosphingolipids consist of a ceramide base with carbohydrates attached to the hydrophilic part. Ceramide in turn consists of sphingosine with an attached fatty acid that varies in length. The first step in Gb3 biogenesis is attachment of glucose (Glu) to ceramide via glucosylceramide synthase, producing glucosylceramide. This is followed by addition of two galactose (Gal) molecules to produce Gb3. An additional addition of N-acetylgalactosamine results in Gb4. The enzyme responsible for the final step in Gb3 synthesis is lactosylceramide 4-α-galactosyltransferase and the presence of this enzyme is a critical determinant in cellular Stx-sensitivity.[72]

Glycosphingolipids are present in the membrane of most mammalian cells and are enriched in membrane domains known as lipid rafts.[73, 74] Lipid rafts, or detergent-resistant membrane domains, are dynamic structures of the membrane with increased concentrations of long-tailed lipids, cholesterol and trans-membrane proteins. These structures promote endocytic events and are involved in cell signal transduction.[74]

Stx1 and Stx2 both have affinity for Gb3 and Gb4.[70] However, Stx1-binding requires the saccharide part of the glycosphingolipid, as the soluble glycan part of

Gb3 (Glu-Gal-Gal) is sufficient for Stx1-binding. In contrast Stx2, also utilizes the lipid residues for binding.[70] Stx1 binds well to purified Gb4 but Stx2 require additional factors for Gb4-binding, such as cholesterol and phosphatidylcholine.[70] The side-chain of the ceramide residue also affects Stx1- and Stx2-binding. Stx1 has increased affinity for side-chains with 20 or 22 carbons while Stx2 has optimal affinity for 18-carbon side-chains.[75]

Each part of the pentameric B-subunit of Stx has three binding sites, resulting in 15 possible binding sites for the receptor per Stx molecule.[76, 77] This induces clustering of Gb3 upon Stx binding and decreases the probability of dissociation.

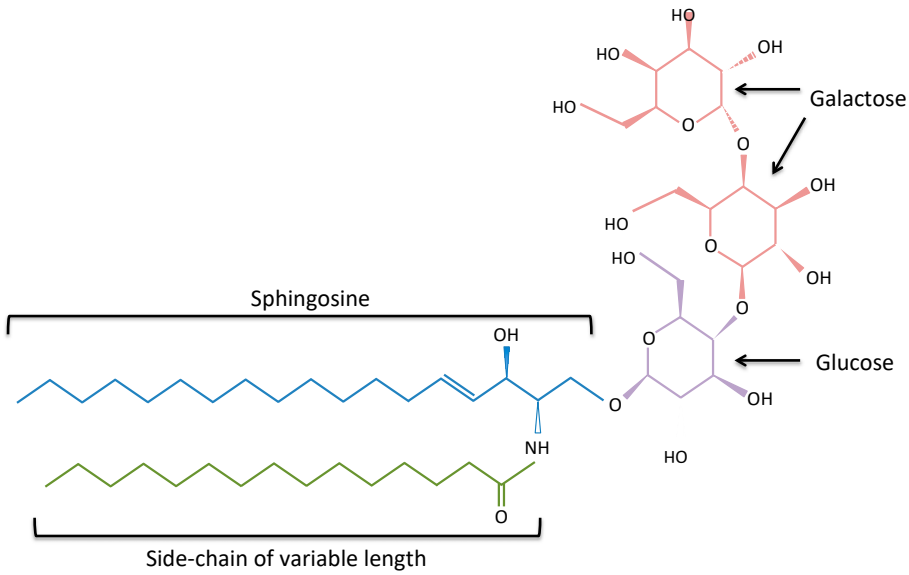

Figure 2. Schematic drawing of the Shiga toxin receptor globotriaosylceramide
The Shiga toxin receptor globotriaosylceramide (Gb3) consists of a sphingosine backbone (blue), a side chain that varies in the number of carbons (green), a glucose attached to the sphingosine (purple) and two galactoses (red). Sphingosine together with a side-chain forms ceramide.

Endocytosis and intracellular transport of Shiga toxin

After binding of Stx to Gb3 and Gb4 in the outer plasma membrane leaflet, the toxin is internalized by the cell. Stx uptake has been shown to occur through several pathways, involving both clathrin-dependent and –independent mechanisms.[78, 79] Upon binding, several Stx molecules cluster together and induce mechanical membrane invaginations in the plasma membrane.[80] These invaginations are recognized and stabilized by the BAR-domain protein endophilin-A2 that together with actin and dynamin induce scission, producing

intracellular vesicles.[81] The early vesicular structures fuse with early endosomes and are sorted and further transported to the trans-golgi network and the endoplasmic reticulum (ER) (Figure 3).[58, 82]

The transport route of the Stx is dependent on if the Gb3 that is encountered at the plasma membrane is associated with a detergent-resistant membrane environment. Interestingly, in human macrophages and dendritic cells Gb3 is separated from detergent-resistant membranes, leading to lysosomal transport of the toxin, while in HeLa cells, where Gb3 is associated with lipid rafts, Stx is transported to the ER.[58] Furthermore, in HeLa cells Stx seems to be associated with detergent-resistant membranes throughout the retrograde transport.[58]

During the transport route the A-subunit of the toxin is enzymatically cleaved, mainly by the protease furin, into Stx-A_1 and -A_2 moieties that are held together by disulfide bonds.[83] In the ER the disulfide bonds are reduced and the A_1-moiety is unfolded by the ER-chaperone Binding immunoglobulin Protein (BiP).[84] BiP associates with an ER-translocase and refolds and translocates the A_1-moiety over the ER membrane and into the cell cytosol.[19, 84]

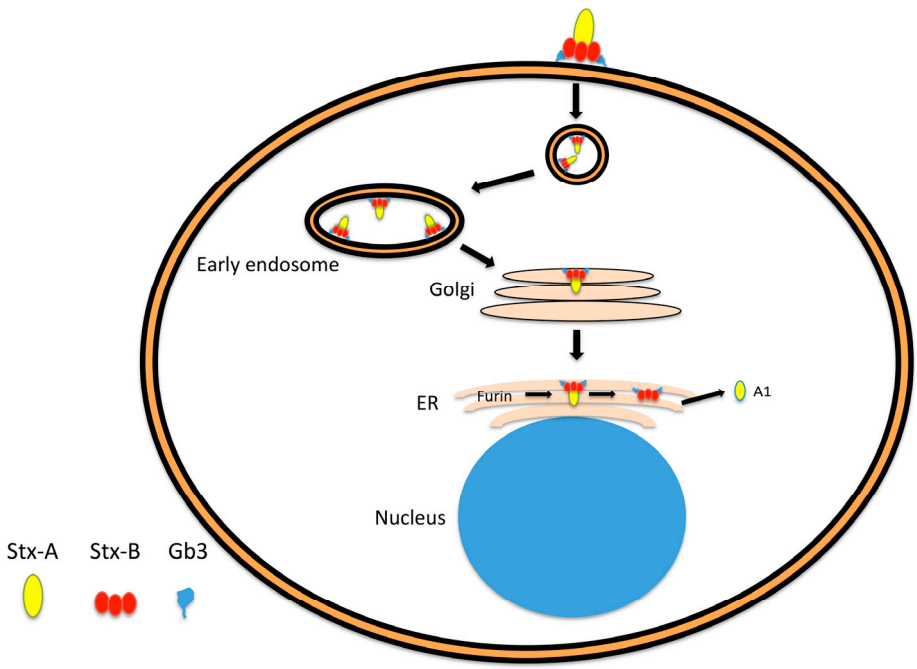

Figure 3. Retrograde transport of Shiga toxin.
The B-subunit of Shiga toxin (Stx-B) binds to Gb3 and the toxin is taken up by the cell. The toxin is transported to the early endosome, followed by the Golgi apparatus and the endoplasmic reticulum (ER). In the ER the A-subunit of Stx (Stx-A) is cleaved by furin and a part of the A-subunit (A1) is released into the cytosol.

Protein synthesis inhibition and apoptosis

Stx induces inhibition of protein synthesis in several mammalian cell types. Once the A_1-moiety of Shiga toxin is transferred into the cytosol it depurinates an adenine base of the 28S rRNA in the 60S subunit of the ribosome and thereby inhibits mRNA translation.[26, 85]

Stx causes apoptosis in a range of different cell types, such as intestinal mucosal cells, renal cortical cells and epithelial cells.[5, 27, 86] Stx-induced apoptosis involves activation of the initiator caspases, caspase-8 and -9.[27, 87] These are in turn is induced by Calpain and Cytochrome C. Cytochrome C is released from the mitochondria via pores consisting of Bak/Bax and Bak has been shown to be upregulated by Stx2. [88, 89] Caspase-8 and -9 both activate the executioner caspases, caspase-3 and -7, which eventually executes the apoptotic program.[89]

The mechanism for how Stx induces apoptosis is not completely understood, however multiple contributing pathways have been suggested. Upon Gb3-binding, Stx induces cellular activation, characterized by calcium influx from extracellular sources.[25, 90] This activation seems to be dependent on cross-linking of Gb3, as an IgM antibody, targeting several Gb3s, was shown to give rise to a similar response, while the corresponding IgG, targeting one Gb3, did not.[25] The B-subunit of Stx1 alone has been shown to induce apoptosis in Gb3-positive cell lines.[91]

Stx induces ER stress signaling in target cells. When Stx reaches the ER it is recognized by the chaperone BiP as an incorrectly folded protein.[84] BiP acts as a surveyor of protein folding in the ER. Under normal conditions BiP inhibits proteins that control ER cell stress responses. When incorrectly folded proteins are present in the ER, BiP associates with these and thereby releases its inhibiton of ER stress proteins leading to ER stress signaling.[92 93]

Stx-mediated inhibition of protein synthesis induces the ribotoxic stress response and may cause apoptosis via activation of p38 mitogen activated protein kinase.[94] Interestingly, it is not the halted protein synthesis that gives rise to this response, but rather the site where the depurination occurs, as indicated by the lack of stress response caused by cycloheximide, a mechanistically distinct protein synthesis inhibitor.[93]

Taken together, Stx-induced apoptosis may depend on several cell stress pathways.

Shiga toxin-induced signaling

Stx-binding to its receptor initiates signaling in the cell. The signaling is quite unique given that the glycosphingolipid receptor, to which Stx binds, lack means for signal transduction into the cell on its own. The mechanism for how Stx

initiates signaling through binding of membrane lipids is unknown. One theory is that the clustering of Gb3 that occurs through the multiple binding sites of the Stx B-subunit and subsequent clustering of Stx mechanically forces proximity and activation of adjacent receptors.

Stx1 binding leads to calcium influx in a range of cell types, including endothelial and epithelial cells.[24, 25] The exact mechanism has not been elucidated but several factors have been shown to be involved in the signaling. The response seems to be linked to the phospholipase C (PLC) pathway, as Stx1-induced calcium influx in human umbilical vein endothelial cells was almost completely abrogated when treated with a PLC inhibitor.[24]

The origin of the increased cytosolic calcium in response to Stx1-binding seems to come mainly from extracellular sources. Stx1-binding to HeLa cells in calcium-free solutions resulted in minor cytosolic calcium increases.[25]

Not much is known about Stx2-induced signaling. Stx2 binding induces calcium influx in murine neurons[95] but not in human endothelial cells.[24] Stx2 does, however, activate protein kinase A (PKA) in endothelial cells, preceding release of von Willebrand factor.[24] The activation occurs via an unconventional mechanism, as it is not preceded by elevation in cylic AMP, which is the canonical pathway for PKA activation.[24]

Lipopolysaccharide

LPS is an endotoxin found in almost all gram-negative bacteria.[96] As the name suggests it consists of a lipid anchor, connecting it to the bacterial membrane, and a saccharide part that extends out from the membrane. The saccharide component of LPS is divided into a core region, closest to the lipid anchor, and the O-antigen, which constitutes the most distal part of LPS.[97] Soluble LPS that has been released from the bacterial membrane is highly immunogenic. The core region of LPS binds to LPS-binding protein that in turn binds to and forms a complex with CD14. This complex facilitates activation of TLR4, starting a signaling via the adaptor protein myeloid differentiation primary response 88 (MyD88), that results in cytokine release and activation of the innate immune system.[98]

Patients with EHEC-induced HUS have LPS bound to circulating blood cells and blood cell-aggregates.[11, 99] LPS induce release of tissue factor from whole blood.[11] MyD88 knock-out mice exhibit more severe symptoms and have a higher bacteria burden compared to wild-type mice in response to EHEC infection, suggesting a role for LPS.[100]

EHEC-hemolysin

EHEC-hemolysin is a pore-forming toxin that is mainly released associated with OMVs.[101] The toxin is delivered into target cells and translocates to the mitochondria. The toxin is inserted into the mitochondrial membrane followed by subsequent oligomerization.[101] Clustered EHEC-hemolysin subunits cause pore-formation and lead to unregulated flow of ions and other small molecules across the mitochondrial membrane, ultimately leading to apoptosis. The importance of this phenomenon *in vivo* is unknown. EHEC-hemolysin may also be released in soluble form from the bacteria and has been shown *in vitro* to bind to and lyse red blood cells.[102]

Subtilase

Subtilase is an AB5 toxin that was identified following an outbreak of HUS that was caused by *E. coli* O113:H21.[103] It was later identified in other EHEC strains.[104] The B-subunit of the toxin binds to glycans with terminal N-glycolylneuraminic acid, which is not naturally produced in human cells, but may be incorporated following ingestion of red meat and dairy products.[105] Subtilase causes vascular injury, thrombosis and mortality when injected into mice.[103] The cellular damage is mediated through cleavage of the ER protein BiP by the A-subunit of subtilase,[106] which induces ER stress and subsequent cell death.[107]

Outer membrane vesicles

OMVs are membrane-derived vesicles released from Gram-negative bacteria.[108] Their diameter differs between different types of bacteria and ranges between 20 and 100 nm for vesicles released by *E. coli* O157:H7.[109] OMVs are released in response to various physiological stimuli, such as temperature change, which leads to loss of cross-linking between the proteoglycan layer and the outer membrane and disruption of the outer membrane fluidity.[110-112] OMVs released by EHEC carries Stx, LPS and hemolysin. OMV-delivered Stx and hemolysin were shown to be taken up by both intestinal epithelial cells and renal microvascular endothelial cells, leading to cell death in these cells.[101] In a mouse model, intraperitoneal injection of purified OMVs, derived from *E. coli* O157:H7, containing both Stx2 and LPS were shown to induce HUS-like symptoms.[113]

Host response during EHEC infection

Interaction of EHEC with intestinal epithelium

EHEC colonization causes an inflammatory response in the intestine.[114] The initial response is mediated by the innate immune system through recognition of pathogen associated molecular patterns (PAMPs) such as LPS and flagellin that are present on the bacteria.[100, 115] PAMP recognition initiates signaling cascades, mainly induced by toll-like receptors (TLR)-4 and -5. The early inflammatory response in the intestine is enhanced by release of the pro-inflammatory cytokines IL-8 and TNF-α.[116, 117] IL-8 is a chemoattractant that recruits neutrophils. Neutrophils infiltrate the colonic mucosa layer during EHEC infections, possibly by IL-8-mediated chemotaxis.[118] Furthermore, high levels of IL-8 in the intestine are associated with a worse outcome during EHEC infection.[114]

In the intestine, the mucosal layer represents a major barrier during infection. The mucosal layer consists of epithelial cells and a layer of mucus, mainly produced by goblet cells.[119] In addition there are antimicrobial peptides, such as cathelicidin, play a role in the preservation of the mucosal layer. Cathelicidin knock-out mice have a thinner mucosal layer and are more susceptible to EHEC infection compared to wild-type mice. [120] During EHEC infection in humans there is massive damage and inflammation in the intestinal mucosal layer.[45] Goblet cell depletion has been reported in EHEC infected mice.[121, 122] EHEC secretes a mucinase termed StcE that cleaves components of the intestinal mucus, thinning the mucus layer and increases bacterial adhesion.[123]

Blood cell response to Shiga toxin

Stx may lead to both cell death and cellular activation, depending on the cell type to which it binds. Stx is non-toxic to monocytes and neutrophils but induces activation in these cells.

During EHEC-induced HUS, Stx is bound to platelets in the circulation.[11] *In vitro* the toxin binds to Gb3 and a glycolipid called 0.03.[9] However, Stx binding to platelets have been shown to occur only when the platelets are activated and this

seems to be dependent on how exposed the Gb3 on the platelet is.[124] Following binding, Stx is taken up by the platelet.[10]

Stx1-binding to isolated human monocytes causes activation and release of several cytokines, such as IL-1β, IL-8 and TNFα.[14, 125] In addition, both Stx1 and Stx2 induce release of IL-1β, IL-8 and TNFα from a monocytic cell line *in vitro*, with Stx2 being a greater inducer.[126] Stx2-stimulation of monocytes cells leads to release of cytokines that in turn activates platelets.[125] Importantly, both TNFα and IL-1β have been linked to increased Gb3 expression in endothelial cells and may play an important role in the EHEC pathogenesis.[127] Stx2 activates the inflammasome complex in monocytic cells.[128] Activation of the inflammasome complex requires a dual response in which ATP signaling constitutes one of the activating events.[129] The inflammasome is important for IL-1β and caspase-1 maturation and pyroptosis, a caspase-1-dependent apoptotic-like process that has been implicated in Stx2 toxicity.[128, 130, 131]

In neutrophils Stx2 has been shown to reduce cell death via activation of intracellular signaling pathways.[132] Stx2 also induces release of reactive oxygen species in neutrophils, which may exacerbate EHEC infection.[133]

Stx1 and Stx2 induce the release of von Willebrand factor from endothelial cells.[24] von Willebrand factor is important in platelet adhesion to sites of endothelial injury and promotes platelet aggregation. von Willebrand may drive platelet consumption and thrombi formation during EHEC infection.[2]

Clinical manifestations of EHEC infection

After oral EHEC ingestion clinical symptoms appear between 3 and 8 days and usually manifest as abdominal cramps and watery diarrhea and in some cases bloody diarrhea.[134] EHEC infection may resolve spontaneously but about 15 % of the patients develop HUS, typically 2 – 12 days after the start of diarrhea.[2] HUS manifests as the simultaneous occurrence of thrombocytopenia, hemolytic anemia and acute kidney injury.[135] Patients may exhibit high blood pressure, jaundice, pallor and symptoms of renal failure.

Thrombocytopenia likely occurs as a consequence of damaged endothelium. Stx activates platelets in the presence of LPS or certain cytokines. Damaged endothelium leads to exposure of fibrinogen, collagen and von Willebrand factor, resulting in pro-thrombotic activation of platelets.[2] The activation drives consumption of platelets and results in the formation of microthrombi. Interestingly, no major consumption of coagulation factors is seen in HUS.

Hemolytic anemia in HUS is non-immune, indicating that it is not induced by antibodies. The main hypothesis regarding the mechanism is that red blood cells are damaged as a result of microthrombi in the blood vessels, narrowing their path and mechanically rupturing them. This is supported by the presence of schistocytes (fragmented red blood cells) in blood smears from HUS patients.[136] In addition, evidence for decreased membrane fluidity in the membrane of red blood cells, as a consequence of oxidative stress, has been described.[137, 138] This may cause cell rigidity and consequently higher risk of rupture.

During EHEC-induced HUS the kidney undergoes extensive injury to the renal cortex.[5] This is mediated by several mechanisms, including formation of microthrombi that occlude the glomerular capillaries, detachment of glomerular endothelial cells, thickening of the vessel walls and severe tubular damage.[5]

Purinergic signalling

Purinergic signaling comprises a family of receptors that bind and respond to extracellular nucleotides, such as adenosine triphosphate (ATP) or adenosine diphosphate (ADP), and adenosine. Purinergic receptors are expressed in most, if not all, cell types in the body. ATP is the main energy source for mammalian cells. Various stimuli or mechanical injury may cause release of ATP from cells. Extracellular ATP is an important damage-associated molecular pattern ligand and its signaling via purinergic receptors is involved in many different processes, of which inflammation is the most prominent.[139]

Receptor types

Purinergic receptors are divided into three different receptor classes: the metabotropic P1 and P2Y receptors and the ionotropic P2X receptors (Figure 4).[140]

P1 receptors

There are four different types of P1 receptors, all of which utilize adenosine as their main endogenous ligand. These receptros are expressed in the central nervous system, heart, kidney and adipose tissue.[141] P1 receptors mainly, but not exclusively, have inhibitory effects on the cells on which they are expressed.[142]

P2Y receptors

P2Y receptors are present in almost all cells in the body and eight different types of P2Y receptors have been identified to date. P2Y receptors mainly bind ADP as endogenous ligands but can also use other adenine or uracil nucleotides.[143]

P2X receptors

Just as P2Y receptors, P2X receptors are expressed in most cell types in the body. There are seven different P2X receptor subtypes ($P2X_{1\text{-}7}$) that form homo- or heterotrimeric complexes, utilizing ATP as the sole endogenous ligand.[144] P2X

receptors are ionotropic and in the open state conformation they allow passage of specific ions, depending on the P2X receptor, in a concentration-dependent manner. The $P2X_1$ receptor is the only known P2X receptor present on platelets and has a high specificity for calcium ions.[145]

Release of ATP

There are three main mechanisms of ATP release: passive release following mechanical insult to the plasma membrane, exocytosis of endosomally stored ATP and release of cytosolic ATP via transmembrane channels.[146, 147]

The most studied pathway of ATP-release is via transmembrane channels, such as the pannexin family. The pannexin 1 channel has been shown to open and release ATP in response to activation of the ER-associated calcium channel inositol triphosphate (IP3) receptor.[148, 149] This is believed to occur through PLC-mediated cleavage of phosphatidylinositol 4,5-bisphosphate into diacylglycerol and IP3 and subsequent binding of IP3 to IP3R (Figure 4).[150] In addition, depletion of ER-stored calcium activates the ER-associated calcium sensor protein STIM1 that in turn opens several membrane channels, including calcium channels and possibly also ATP-conducting channels.[151] This mechanism is believed to be important for re-stocking of calcium in the ER.[152] Several studies have demonstrated a direct association between PLC-activation and ATP release and this pathway likely represents the current most well studied pathway of ATP-release.[148, 153-155]

ATP is present at millimolar concentrations in the cell cytosol. Most ATP-activated P2X receptors respond in the micromolar range of ATP concentrations.[156] Under normal conditions released ATP may only reach micromolar ranges in the vicinity of the zone of release, pointing at auto- and paracrine signaling. However, during systemic inflammation a general increase in plasma ATP levels is present.[139]

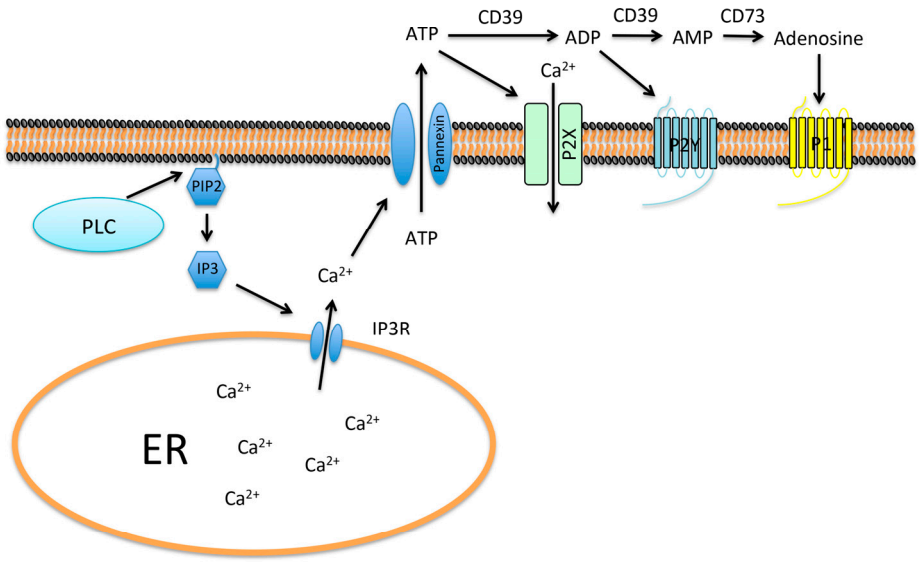

Figure 4. Schematic depiction of ATP-release via membrane channels.
Activated phospholipase C (PLC) cleaves the membrane bound phosphatidylinositol 4,5-bisphosphate (PIP2) into inositol trisphosphate (IP3) and diacylglycerol. Released IP3 binds to the IP3 receptor in the membrane of the endoplasmic reticulum (ER), causing release of ER-stored calcium. This is turn opens Pannexin-channels in the plasma membrane and allows transport af adenosine triphosphate (ATP) out of the cell. Extracellular ATP activates P2X receptors on the cell from which it was released and on neighbouring cells, which results in influx of ions, such as calcium. ATP is degraded by the ectonucleosidase CD39 into adenosine diphosphate (ADP) that binds and activates P2Y channels. ADP is further degraded by CD39 into AMP, which is ultimately cleaved by CD73 into adenosine that binds to P1 receptors.

ATP release via bacterial factors

There are various modes by which bacterial factors may induce release of ATP from host cells. Direct interaction of virulence factors at the cell membrane may stimulate release of ATP by induction of intracellular signaling. In immune cells LPS mediates release of ATP via activation of TLR4.[157] Other bacterial toxins may allow ATP release by forming a membrane pore. For example, HokB from *Escherichia coli* forms a transmembrane pore leading to ATP leakage.[158] Similarly, EHEC-hemolysin and leukotoxin A from *Aggregatibacter actinomycetemcomitans* were incorporated in lipid vesicles allowing ATP release via toxin pores.[159]

Degradation of extracellular ATP

Enzymes that degrade ATP and other purinergic nucleotides are called ectonucleotidases. There are two different ectonucleotidases in humans, namely CD39 and CD73. CD39 and CD73 are membrane bound enzymes that are mainly expressed on vascular endothelial cells and lymphocytes.[160] CD39 cleaves off terminal phosphate groups of ATP and ADP and CD73 cleaves off the final phosphate group of AMP to form adenosine (Figure 4).[160] The expression of CD39 and CD73 is inducible and has been indicated as a mechanism for regulatory T-cells and neutrophils to regulate the immune response.[161] Continuous stimulation with ATP quickly desensitizes P2X receptors.[156] Ectonucleotidases are thus crucial in order to keep purinergic receptors sensitive to stimuli and to regulate the levels of extracellular ATP.

Apyrase is a nucleotidase that cleaves off phosphate groups of ATP and ADP.[162] In contrast to the endogenous membrane bound ectonucleotidases, apyrase is a soluble enzyme. In a murine model for LPS-induced systemic inflammation, excessive levels of ATP was shown to drive tissue damage and increase the risk of mortality in mice and exogenously administrated apyrase displayed a protective effect.[139] One of the focuses of this thesis was to investigate the effect of exogenously administrated apyrase in a mouse model of EHEC infection.

The role of extracellular ATP in blood cells

ATP is involved in platelet aggregation. ATP-release has been suggested to be a major route for auto- and paracrine amplification of calcium influx leading to aggregation.[163] Collagen and thrombin have been shown to induce ATP release from platelets and blockade of the ATP-signaling, either via degradation of ATP or inhibition of the P2X1 receptor, was shown to reduce both the calcium response and platelet aggregation.[148, 164] Platelets from P2X1-deficient mice exhibit reduced adhesion and thrombus formation *ex vivo* in response to collagen stimulation.[165]

In monocytes ATP release and stimulation plays a major role in the release of IL-1β. ATP comprises an important step in assembly and activation of a complex called the inflammasome. The inflammasome is necessary for IL-1β maturation and release. Both LPS and the complement factor C3a were shown to induce release of IL-1β from monocytes only during simultaneous presence of extracellular ATP.[166, 167] Apyrase treatment and P2X7 receptor blockade inhibited the C3a-induced release of the cytokines.[167]

Induction of chemotaxis of neutrophils through IL-8 has been shown to be dependent on stimulation with extracellular ATP.[168] Furthermore, neutrophils

release ATP from the site in which the chemotactic stimulant has a high concentration in order to amplify their own response and increase their motility to reach chemoattractants. [169]

Extracellular vesicles

Extracellular vesicles comprise a collective name for spherical vesicles that are released from most, if not all, cells in the body. Extracellular vesicles include exosomes, microvesicles and apoptotic bodies. These three classes of vesicles have been separated based on several factors, such as size, cellular markers and specific lipids in their outer leaflet. However, as more knowledge has emerged about extracellular vesicles it has become apparent that the distinction between the different populations is not as clear as was previously thought. Currently the only accurate definition seems to be based on their mechanism of release.

One of the first recorded observations of extracellular vesicles were seen during red blood cell maturation and were believed to be residual waste, discarded by the blood cells.[170] With time they have become regarded as a major means of cell communication with functions in immune response, cellular repair and reprogramming and coagulation, to name a few areas.[171-173] In this thesis we have been investigating the role of microvesicles in systemic transport of Shiga toxin.

Exosomes

Exosome biogenesis takes place within the cell via inward budding of the membrane of late endosomes. Endosomes containing intraluminal vesicles are referred to as multivesicular bodies. When the multivesicular bodies fuse with the plasma membrane the intraluminal vesicles are released, at which stage they are termed exosomes.[174] Exosomes are generally smaller than the other populations of extracellular vesicles, ranging between 30 and 100 nm in diameter. Exosomes are the most abundant extracellular vesicle in the circulation and may carry various forms of cargo, including microRNAs, mRNAs, lipids and proteins.[175, 176]

Microvesicles

Microvesicles are shed directly by outward budding and scission of the plasma membrane. Microvesicles are larger than exosomes, with a diameter between 100 to 1000 nm. The vesicles are released constitutively but during cellular activation

their release is increased and their contents and composition is altered. As in the case of exosomes, microvesicles have been shown to transport several types of cargo to other cells and also have an important role in coagulation and thrombosis which will be described below.[177]

Apoptotic bodies

Apoptotic bodies are the largest kind of extracellular vesicles with a diameter between 1 and 5 μm. Apoptotic bodies are shed during cellular breakdown of apoptotic cells. Apoptotic bodies may therefore contain genomic DNA and organelles.[178] Apoptotic bodies may limit inflammatory and apoptotic responses in neighboring cells by phagocytic uptake by immune cells.[179]

Release mechanism of microvesicles

Microvesicles shed from the cell membrane upon activation were the focus of this thesis. The release of microvesicles have been closely linked to cleavage of cortical actin, which separates the actin-mediated anchoring of the plasma membrane, and loss of membrane lipid asymmetry.[180, 181]

Cells have an asymmetrical distribution of lipids in the plasma membrane whereby phosphotidylserine (PS) and phosphatidylethanolamine (PE) are mainly located on the inner leaflet of the membrane.[182] This asymmetry is regulated by ATP-dependent flippases and floppases. Flippases are active during normal levels of intracellular calcium and move certain lipids, such as PS and PE, from the outside to the inside of the membrane, against their gradient.[183] Floppases are inactive during normal intracellular calcium levels and become activated upon increased intracellular calcium levels, allowing them to move lipids from the inside to the outside of the membrane.[184] A third class of intramembraneous lipid-moving enzymes are the scramblases, which are activated in the presence of high levels of intracellular calcium.[185] During increased cytosolic calcium concentration levels, flippases are inactivated and ceases to internalize PS and PE, while floppases and Scramblases are activated. This leads to translocation of PS and PE to the outer membrane leaflet.[186]

Lipids in the plasma membrane come in various geometrical shapes, which, in part, dictate their distribution. Focal congregation of certain lipids are known to initiate budding via strict geometrical remodeling of the plasma membrane and are of vital importance for endocytosis.[187] The same phenomenon has been described for shedding of microvesicles. In a study in a *C. elegans* model it was shown that

the loss of flippase led to a significant increase in shedding of microvesicles. The proposed mechanism was that the lack of inward flipping of PE led to clustering of the inverted cone-shaped lipid, creating highly curved membrane patches, that provided a basis for microvesicle release. [181]

The plasma membrane is anchored and stabilized by the cortical actin network. In order to release parts of the plasma membrane in the form of microvesicles this actin network needs to be disrupted. During elevated calcium levels certain actin degrading enzymes, such as calpain, are activated. Calpain cleaves actin-anchoring proteins and disrupts the cortical actin-based cytoskeleton.[180]

Several mechanisms may induce budding of microvesicles, but a scission step is also necessary to complete the process. This is mainly mediated via the ESCRT machinery.[188] The ESCRT machinery consists of four different protein complexes, ESCRT-0, -I, -II and –III. The ESCRT machinery is vital in most processes that require scission of membranes that bud away from the cytosol, such as cytokinesis.[189]

Uptake of microvesicles

Released microvesicles may be taken up by neighboring or distant cells through a variety of uptake mechanisms including phagocytosis, macropinocytosis, clathrin-dependent and –independent endocytosis and membrane fusion. The mode of uptake depends on several factors, such as the composition and origin of the microvesicle and the type of and level of activation the recipient cell.

The main mechanism of vesicle uptake is phagocytosis. [190] Phagocytosis is a receptor-mediated uptake process whereby the cell extends its membrane and engulfs another cell or larger particle, delivering these to phagosomes in the cell. [191] Phagocytosis of microvesicles is not restricted to immune cells, but the rate of the uptake is dependent on the phagocytic capabilities of the recipient cell.[191] Phagocytosis by immune cells such as monocytes is believed to be largely a clearance mechanism. Microvesicles generally expose PS on their outer membrane leaflet, which is a potent signal for phagocytosis.[192]

Macropinocytosis is a type of phagocytosis but instead of uptake of large particles, several smaller particles or solutes are engulfed and taken up. This has been shown to include uptake of microvesicles.[193]

Microvesicles can also be taken up by clathrin-independent uptake such as caveolin- and lipid raft-mediated uptake. This uptake process is often initiated by receptor binding and has been implicated in endocytosis of extracellular vesicles in both endothelial and epithelial cells.[193, 194]

Clathrin-dependent endocytosis has been shown to play a role in uptake of microvesicles.[195] Clathrin promotes endocytosis following ligand binding to a membrane receptor. During the inward budding of the cell membrane clathrin forms a coat around the vesicle, which is then uncoated and transported to the early endosome.[196] Clathrin-mediated uptake of extracellular vesicles have been shown in bone marrow-derived mesenchymal stromal cells.[195]

Extracellular vesicles may fuse with the plasma membrane and thereby empty their content into the recipient cell.[175] This is, however, believed to be one of the less common uptake pathways of extracellular vesicles, as it requires higher membrane fluidity that is present in acidic environments, which generally only is present in tumors.[197]

The function of microvesicles

Microvesicles have been implicated in numerous cellular processes, such as cell signaling, transfer of RNAs, proteins and receptors leading to coagulation, thrombosis and inflammation. They may also be used as biomarkers for diagnostic purposes and as vehicles for drug-delivery. The most important function of microvesicles is intracellular communication by transport of proteins, lipids, carbohydrates and RNAs. As microvesicles may contain cargo they can transport molecules that would otherwise be phagocytosed or degraded.

Microvesicles can induce cell signaling in recipient cells by membrane-bound ligands that bind directly to receptors on the surface of the target cell.[198] Furthermore, microvesicles may fuse with the target cell membrane. Microvesicles may deliver receptors directly to the plasma membrane of the recipient cell. For example, functional CCR5 or bradykinin B1 receptor may be transferred to recipient cells thus promoting infection or inflammation.[199, 200]

Microvesicles can regulate protein expression in target cells by delivery of microRNAs, which interfere with mRNA translation.[201] In the same fashion, coding mRNAs can be delivered via microvesicles, followed by subsequent translation of the mRNAs into proteins. In a study on microvesicles derived from glioblastoma cells, approximately 27,000 unique mRNAs were found within the vesicle population. Recipient cells were able to translate the mRNAs following vesicle uptake.[202]

Microvesicles have been shown to play an important role in coagulation and thrombosis by several mechanisms. Microvesicles derived from platelets, endothelial cells and monocytes carry tissue factor.[11, 203, 204] Additionally, stimulation of monocytes with LPS increases the amount of tissue factor that these vesicles carry.[205] Tissue factor forms a complex with the activated form of

coagulation factor FVII and constitutes the extrinsic pathway of the coagulation cascade. The Tissue factor/FVIIa catalyzes cleavage and activation of FX. Another mechanisms of microvesicle-mediated coagulation is via exposure of PS. PS is an important activator of factor V and factor VII in the coagulation cascade.[206] Platelet-derived microvesicles have also been shown to bind and recruit platelets to sites of endothelial injury, mediated via binding of matrix components, in a rabbit model.[207]

Microvesicles may promote both pro- and anti-inflammatory responses. ATP-stimulation of LPS-primed monocytic cells was shown to induce shedding of microvesicles containing the pro-inflammatory cytokine IL-1β.[208] Leukocyte-derived microvesicles have been shown to induce release of cytokines from endothelial cells.[209] Extracellular vesicles from platelets can inhibit production of inflammatory cytokines in regulatory T cells.[210]

As microvesicles carry surface markers and cargo from their cell of origin they may serve as biomarkers for disease. This has been exploited for diagnostic purposes in different types of cancer. Microvesicles from glioblastoma cells were shown to contain abundant levels of a certain miRNA that could be correlated with disease progression.[211]

Extracellular vesicles have been implicated in drug-delivery, as they are endogenous, may conceal the drug from the immune system and may cross biological barriers, as the blood-brain barrier.[212]

Microvesicles in hemolytic uremic syndrome

Patients with EHEC-induced HUS have been shown to have elevated levels of microvesicles.[213, 214] These microvesicles are derived from platelets, monocytes and neutrophils, determined by labeling with specific markers. Furthermore, the microvesicles exhibited pro-thrombotic properties, as they were positive for tissue factor and PS in the outer membrane leaflet. *In vitro* studies have shown that whole blood stimulated with Stx2 gave rise to similar results as in HUS patients, with increased levels of microvesicles from platelets, monocytes, neutrophils and also from red blood cells.[11, 15] These vesicles were also positive for tissue factor and exposed PS.[11] The presence of circulating prothrombotic microvesicles likely contributes to the platelet consumption and thrombus formation in HUS patients.

Microvesicles from platelets and monocytes from HUS patients and from Stx-stimulated whole blood carry complement factors C3 and C9.[213] Furthermore, red blood cell-derived microvesicles from HUS patients and Stx-stimulated whole blood are positive for C3, C9 and C5b-9.[15] Whether these complement factors bind to blood cells and are shed via microvesicle release or are deposited after the

vesicles are released is unclear. However, microvesicles may provide a mechanism for clearance of complement factors.

In this thesis the role of microvesicles in HUS has been further explored as potential mediators of disease.

The present investigation

The overall aim of this thesis was to investigate a mechanism of systemic Shiga toxin transport to the kidney and to assess the importance of the toxin receptor and intracellular pathways of toxin signaling. Shiga toxin is of major importance for the virulence of EHEC strains and therefore a better understanding of its transport and signaling is imperative to the development of future therapeutic strategies.

Specific aims

1. To study if Stx is shed within blood cell-derived microvesicles in the systemic circulation, if the toxin retains its toxicity within microvesicles and if this may provide a mechanism for toxin delivery to the kidney.

2. To study if endogenous Gb3 is essential for Shiga toxin, delivered within microvesicles, to exert its cytotoxic effect intracellularly or if Gb3-negative cells can also take up toxin within microvesicles and be affected.

3. To study if Shiga toxin utilizes ATP and purinergic signaling to induce its effect on cells and how blockade of purinergic receptors affects Stx-mediated calcium influx, toxicity and microvesicle release.

4. To study the importance of ATP signaling in EHEC infection *in vivo* by treating EHEC-infected mice with apyrase.

5. To review the role of extracellular vesicles in normal physiology and kidney diseases.

Methods

The following is a brief description of the methods used in the papers included herein. For a more in-depth description, please see the Material and Methods section of each respective paper.

Ethical considerations

Human samples were used in papers **I**, **II**, **III** and **IV**. The samples were obtained from patients, pediatric controls and healthy adult volunteers with written informed consent from the subjects or their parents. The studies were performed with permission from the Ethics Committee of the Medical Faculty, Lund University.

Animal models were used in papers **I**, **II** and **IV**. All animal experiments were approved by the animal ethics committee of Lund University in accordance to the guidelines of the Swedish National Board of Agriculture and the EU directive for the protection of animals used in science.

Patients and control samples

Samples from 16 children with EHEC-associated HUS, one adult patient with HUS, one adult patient with EHEC infection and four pediatric controls, with conditions that were unrelated with HUS, were available in paper **II**. In addition, a renal biopsy from a kidney of a 13 year old boy with HUS was available.

Flow cytometry

In papers **I**, **III** and **IV** flow cytometry was used to analyze cells or microvesicles. Flow cytometry is a common research tool that flows cells and particles through a laser. Forward and side scattering of the laser gives information about the volume and granularity of the particle and the method is often combined with fluorescent labelling of the particles that are analyzed. In this thesis flow cytometry was used for antibody-labelled microvesicles, to determine their cellular origin. Stx in microvesicles was labeled in combination with a mild detergent, to allow permeabilization of the microvesicles.

Cell culture and transfection

Several cell lines were used in the studies included in this thesis. Immortalized glomerular endothelial cells were used in paper **I**, cervical epithelial (HeLa) cells were used in papers **II** and **III**, and Chinese hamster ovarian (CHO) and colonic epithelial cells were used in paper **II**. In paper **II**, CHO cells were transfected with a plasmid carrying coding DNA for the Gb3 synthase, A4GALT and a resistance

gene. The transfected cells were made into a stable cell line, using negative selection for the cells that did not express the resistance gene. In paper **III**, HeLa cells were transfected with small interfering RNA to silence the expression of the P2X1 receptor. The transfections were carried out on sub-confluent cells using a liposome-based delivering system.

Transmission electron microscopy

Transmission electron microscopy (TEM) of cell cultures and renal biopsy was used in paper **I**. In TEM, the sample is irradiated with electrons. Depending on the density of the sample, electrons will pass through in different amounts, resulting in a highly resolved image of the sample. Gold particles coupled to antibodies may be used to label specific antigens in the sample. In paper **I**, TEM was used to image kidney tissue from a HUS patient and cultured glomerular cells incubated with microvesicles containing Stx. The samples were labelled with gold-conjugated anti-StxB antibodies together with cell-specific markers for microvesicles to enable identification of their parent cells and, in the kidney tissue, for early endosomes and ribosomes.

Viability assay

Viability assays were carried out in papers **I**, **II** and **III**. In paper **I** the viability of cultured glomerular cells was determined based on detachment of dead cells and subsequent staining with crystal violet of remaining, viable, cells. In papers **II** and **III** the viability of cells was measured based on their metabolic activity. Alamar blue was used, a substance that shifts color in response to reduction by cellular NADH and NADPH. The viability measurements were carried out on cells treated with or without the P2X1 inhibitor NF449 and exposed to free Stx or microvesicles with or without Stx.

Protein synthesis assay

In papers **I**, **II**, and **III** the protein synthesis of cultured cells was assayed in cells treated with or without NF449 and exposed to free Stx or microvesicles with or without Stx. In papers **I** and **III** protein synthesis was measured by incorporation of radioactively labelled methionine and in paper **II** a methionine analogue containing an alkyne group, which binds a fluorescent dye. The incorporated methionine or methionine analogue were measured using scintillation or detection of fluorescence, respectively, giving signals proportional to the amount of synthesized proteins.

Caspase activity assay

The caspase activity test was used in paper **III**. The assay measures the activity of the terminal caspases 3 and 7 by incubating cells with a membrane permeable substrate that is cleaved by activated caspase 3 and 7. When the substrate is

cleaved in gives off a green fluorescence when excited with blue light. Green-fluorescent cells indicate ongoing apoptosis. This assay was used on HeLa cells that had been treated with or without NF449 and incubated with Stx.

Nanoparticle tracking analysis

Nanoparticle tracking analysis (NTA) was used for detection of microvesicles in paper **II**. In NTA, particles are flowed under a camera and the size of the particles is determined based on Brownian motion. Suspensions of microvesicles derived from whole blood that had been stimulated with or without Stx were analyzed in order to determine their size distribution.

Thin layer chromatography

In paper **II** the glycosphingolipid content of HeLa, CHO and DLD-1 cells and blood cell-derived microvesicles was analyzed by thin layer chromatography (TLC). In TLC a mixture of substances, such as lipids, in a solvent are separated on a polar surface, exploiting the capillary force. The mixture is separated based on their solubility and polarity.

ATP determination

ATP measurements, using the luciferase activity method, were performed in papers **III** and **IV**. Luciferase is an enzyme from fireflies that catalyzes cleavage of ATP in the presence of D-luciferin and oxygen. This reaction leads to emission of light, that is proportional to the amount of ATP that is present. ATP was measured in cell culture supernatants in paper **III** and in feces from EHEC infected mice in paper **IV**.

Calcium imaging

In paper **III** HeLa cells and platelets were loaded with Fluo-4. Fluo-4 is cell permeable, but is cleaved once it enters cells, causing it to lose its cell permeability. Fluo-4 is a calcium sensitive fluorescent dye that becomes brighter at high calcium concentrations. Following loading with Fluo-4, HeLa cells were stimulated with Stx and platelets were stimulated with LPS and Stx and calcium influx was measured based on their fluorescence.

In vivo models

In vivo models were used in paper **I**, **III**, and **IV**. In paper **I, III** and **IV**, Balb/c mice were treated with streptomycin, to reduce their normal flora and to improve colonization. The mice were fasted for 16 h followed by inoculation with streptomycin-resistant *E. coli* O157:H7. Weight changes, bacterial colonization and disease symptoms were monitored daily. Upon development of disease symptoms, weight loss of 20% or at the predetermined end-point, the mice were sacrificed and blood, kidneys and intestines were collected.

In paper **I** and **III** Balb/c mice were given intraperitoneal injections of Stx2. The mice were monitored and sacrificed as before and blood, kidneys and intestines were collected.

TUNEL assay

In paper **IV** colons taken from EHEC-infected mice that had been treated with apyrase or PBS were stained for apoptotic cells by incorporation of alkyne tagged deoxyuridine triphosphate into fragmented DNA of apoptotic cells, followed by labelling of the alkyne group with a fluorescent probe. The tissues were mounted on slides and analysed by fluorescence microscopy.

Results and discussion

Paper I

In this study we investigated if circulating microvesicles can contain Stx2 and thereby transport the toxin systemically.

Results

Patients with EHEC-associated HUS had elevated levels of circulating microvesicles compared to patients in recovery, as was shown by flow cytometry. These microvesicles carried Stx2 on the vesicle inside, as toxin only could be detected in the presence of a membrane permeabilizing detergent. The microvesicles were derived mainly from platelets, but also from monocytes, neutrophils and red blood cells. Similarly, elevated levels of microvesicles from platelets, neutrophils and monocytes were found in plasma from mice infected with EHEC and these microvesicles also contained Stx2. Infection with an isogenic Stx2-negative bacterial strain displayed similar levels of platelet-derived microvesicles that were negative for the toxin. Intraperitoneal injection of mice with Stx2 also led to elevated levels of platelet-derived microvesicles containing Stx2. Electron microscopy showed that platelet- and leukocyte-derived microvesicles with Stx2 were detected in glomerular and peritubular capillary endothelial cells in a kidney biopsy from a patient with EHEC-associated HUS. In EHEC-infected mice platelet- and leukocyte-derived microvesicles were demonstrated in glomerular and peritubular capillary endothelium and in podocytes and tubular epithelial cells. Furthermore, Stx2-containing microvesicles were observed in glomerular and tubular basement membranes. Stx2 stimulated the release of toxin-containing microvesicles from platelets, monocytes, neutrophils and red blood cells from human whole blood *in vitro.* Immortalized glomerular endothelial cells took up platelet- and leukocyte-derived toxin-

containing microvesicles *in vitro*. The microvesicles were transported to the early endosome, released the toxin in the cytosol and the toxin was ultimately transported to the ribosomes. In addition, Stx2-contained within blood cell-derived microvesicles was able to induce protein synthesis inhibition and cell death in immortalized glomerular endothelial cells.

Discussion

This study is the first to demonstrate the presence of Stx2 within circulating blood cell-derived microvesicles in patients with HUS and in EHEC-infected mice. Stx2-containing microvesicles were demonstrated in glomerular and peritubular capillary cells in both patients and mice. Murine glomerular endothelial cells in mice are Gb3-negative, thus showing a receptor-independent uptake of Stx2.[215] Stx2-positive microvesicles were taken up by glomerular cells and were shown to locate to the early endosome. Stx2 was released from the microvesicles and subsequently translocated to the ribosomes to induce protein synthesis inhibition and cell death, indicating that Stx2 that is delivered via microvesicles retains its cytotoxic potential and can follow the retrograde transport pathway following intracellular delivery. This study is the first to demonstrate uptake and transport of a bacterial toxin within host blood cell-derived microvesicles, a mechanism that may provide host response evasion of Stx.

These results provide a novel mechanism of systemic Stx transport to kidney cells.

Paper II

The glycosphingolipids Gb3 are crucial for Stx binding, uptake and toxicity.[70] However, paper **I** demonstrates the presence of Stx2 in Gb3-negative murine glomerular endothelial cells. Here we investigated if Stx2 delivered via microvesicles that were positive for Gb3 could be taken up and have a toxic effect in Gb3-negative cells. Furthermore, we investigated the intracellular transport route of microvesicle-delivered Stx2 in Gb3-positive cells.

Results

Blood cell-derived microvesicles possess Gb3. Microvesicles derived from human whole blood containing Stx2 were highly toxic to CHO cells that had been transfected with Gb3 synthase (CHO-Gb3), but not to Gb3-negative CHO cells that had been transfected with a vector control (CHO-vector). Gb3-negative CHO-native and DLD-1 cells could internalize toxin-positive vesicles but the toxin did not exert a cytotoxic effect in these cells. Stx2 delivered via microvesicles induced protein synthesis inhibition in Gb3-positive CHO-Gb3 and HeLa cells but not in CHO-vector and DLD-1 cells or in HeLa cells that had been treated with an inhibitor of the Gb3-precursor glucosylceramide. CHO-Gb3 cells that were treated with the retrograde transport inhibitor Retro 2.1 were protected against the protein

synthesis inhibition that was induced by Stx2-positive microvesicles. DLD-1 cells that were incubated with exogenous Gb3 were shown to bind free Stx2, but remained resistant to the toxin.

Discussion

This study highlights the importance of Gb3, not only for Stx2 uptake, but also for its intracellular transport and toxicity. The transport of the microvesicle-delivered toxin was shown to go through the retrograde pathway, as indicated by the reduced ribotoxicity following pre-treatment with Retro 2.1, an inhibitor of retrograde transport. Furthermore, we provide a mechanism for the presence of Stx2 in Gb3-negative cells, such as demonstrated in murine endothelial cells in paper I. Importantly, we showed that microvesicles derived from blood cells are positive for Gb3, to which the toxin likely is bound. Despite being Gb3-positive, the microvesicles did not induce toxicity in Gb3-negative cells, indicating that endogenous Gb3 must be present if Stx2 is to retain its toxicity. It is unclear if the uptake of Stx2-containing microvesicles in Gb3-negative cells serves a purpose, but it may be a mechanism through which Stx2 is cleared via degradation in the lysosomes or the vesicles may be transported through the cells via transcytosis.

Paper III

Stx-binding leads to influx of calcium, protein synthesis inhibition, cell death and shedding of microvesicles in different cells. [5, 11, 15, 24-27] The mechanism of Stx-induced cellular activation is not known. In this paper we investigated cellular release of ATP following stimulation of Stx. Furthermore we investigated if released ATP may act as a second messenger by signaling via purinergic receptors and the role of this in Stx-mediated calcium influx, protein synthesis inhibition, apoptosis and microvesicle release.

Results

Stx1 was shown to induce release of ATP *in vitro* from HeLa cells and *in vivo* in a murine model of intraperitoneal injection of Stx2. Stx1-induced calcium influx in HeLa cells was inhibited by both the P2X1 receptor inhibitor NF449 and by the unspecific P2 receptor inhibitor suramin, suggesting involvement of purinergic signaling. In human platelets both Stx1 and Stx2 in combination with LPS induced calcium influx. The influx of calcium was inhibited by treatment with NF449. NF449 did not affect the transport of Stx1 to the ER in HeLa cells, but did protect the cells from Stx1-induced protein synthesis inhibition, caspase activation and Stx1- and Stx2-induced toxicity. The protection from Stx1-induced apoptosis by NF449 was confirmed by P2X1 silencing in HeLa cells leading to reduced caspase activation. Stx1 and Stx2 induced release of toxin-containing microvesicles from HeLa cells and platelets. NF449 inhibited the release of toxin-containing

microvesicles in both HeLa cells and platelets *in vitro*. Mice infected with EHEC or injected intraperitoneally with Stx2 had elevated levels of platelet-derived microvesicles and microvesicles that contained Stx2 compared to controls. Treatment with suramin lead to significantly lower levels of platelet-derived and Stx2-containing microvesicles. Taken together, Stx induces release of ATP from HeLa cells and in mice. ATP in turn causes calcium influx via purinergic signaling. Inhibition of purinergic signaling is protective against Stx-induced toxicity in HeLa cells and inhibits the release of Stx-containing HeLa cell- and platelet-derived microvesicles.

Discussion

In this study we show for the first time that Stx1 induces release of ATP both *in vitro* and *in vivo*. ATP may act as a second messenger and signal via purinergic receptors in an autocrine and paracrine manner. The involvement of purinergic signaling was further strengthened by the abrogation of Stx-induced calcium influx in HeLa cells and platelets by inhibition with NF449 and suramin. Microvesicles may transport and conceal Stx in the circulation. The release of microvesicles may be a mechanism of ridding the cell of the toxin, but in extension leads to increased spread of Stx. Thus, blockade of microvesicle uptake and specific targeting of extracellular ATP may have a therapeutic application during EHEC infections and should be further explored.

Paper IV

Based on our previous observations of ATP release in response to Stx binding in paper III, the importance of systemic purinergic signaling was addressed in this study. EHEC-infected mice were injected with the ATP- and ADP-degrading enzyme apyrase. The mice were monitored with regard to weight loss and development of disease and intestinal tissues from the mice were analyzed for apoptotic markers and mucosal damage. Furthermore, fecal samples form the mice were analyzed for ATP- and Stx2-content.

Results

Mice were inoculated with EHEC and injected intraperitoneally with apyrase or PBS on day 0 and 2 after inoculation. Infected PBS-treated mice developed signs of disease staring three days after inoculation. A significant difference was observed in EHEC-infected mice treated with apyrase, in which development of disease symptoms was observed first five days after inoculation. Apyrase-treatment also had a protective effect with regard to weight loss in the infected mice. Intestinal tissues from mice that had been sacrificed two days after inoculation with EHEC were analyzed for histopathology, goblet cell depletion and apoptosis. EHEC-infected and PBS-treated mice exhibited apoptotic cells on

the luminal side of the intestine, in contrast to apyrase-treated mice. Apyrase-treated mice had significantly more intact goblet cells and significantly less apoptotic cells in the intestine compared to PBS-treated mice. Fecal samples taken one day post-inoculation contained significantly less ATP and exhibited a tendency of lower Stx2 concentrations in apyrase-treated compared to PBS-treated mice, although this effect was not significant.

Discussion

In this study we show that removal of extracellular ATP had a protective effect on mice during EHEC infection. Apyrase delayed the development of disease in the mice by two days and protected intestinal tissues from apoptosis and goblet cell depletion. Even though only partial protection was obtained, this study provides evidence for involvement of extracellular ATP during EHEC infection. The protective effects may also be improved by optimization of the concentration and timing of the apyrase treatment. The mechanism by which apyrase was protective in this study remains to be determined. Apyrase may affect EHEC directly or act via the host-response. The lower fecal ATP levels in apyrase-treated mice indicate that the treatment was able to reach the intestine. A previous study has shown that extracellular ATP is involved in goblet cell depletion *in vitro*.[216] Apyrase may thus protect goblet cells by degrading ATP and maintain the mucosal barrier. Furthermore, purinergic signaling was shown to be involved in Stx-induced apoptosis of HeLa cells in paper **III** of this thesis. Apyrase-mediated degradation of ATP may block purinergic signaling in the intestine and could thereby be protective in a similar mechanism in this study.

Paper V

Paper V is a review, in which the role of extracellular vesicles in normal physiology and renal and inflammatory-mediated pathological conditions are described. The review summarizes the literature concerning the biogenesis, release, uptake, function in normal physiology and detection of exosomes, microvesicles and apoptotic bodies. Paper V focuses on the role of extracellular vesicles as biomarkers and promoters of disease in vasculitis and EHEC-induced HUS. Furthermore, the review investigates the role of these vesicles in kidney regeneration and their possibilities as vehicles for drug-delivery.

Conclusions and future perspectives

Conclusions

- Stx2 induces release of blood cell-derived microvesicles that are positive for the toxin. Stx2-containing microvesicles circulate and are delivered to the kidney in EHEC-HUS. Stx2-containing blood cell-derived microvesicles are internalized by renal glomerular cells followed by inhibition of protein synthesis and subsequent cell death.

- Stx2 can be taken up by Gb3-positive and Gb3-negative cells within microvesicles. Stx2 delivered by microvesicles is toxic to Gb3-positive but not to Gb3-negative cells.

- Stx induces the release of ATP and subsequent purinergic receptor activation. Purinergic receptor signaling is involved Stx-mediated calcium influx, microvesicle release, protein synthesis inhibition and apoptosis.

- Removal of extracellular ATP by apyrase degradation is partially protective in EHEC-infected mice and protects the intestine from damage.

Future perspectives

The mechanism of Stx-transfer to the kidney via blood cell-derived microvesicles provides a model for how the toxin reaches and damages the kidney during EHEC-induced HUS. Interventions that target either the release or the uptake of microvesicles during EHEC infections could thus be protective and should be further explored. However, microvesicles have implications in several physiological roles that must be taken into consideration in such studies. The involvement of purinergic signaling in Stx-mediated microvesicle release indicates that targeting of extracellular ATP may inhibit Stx-mediated microvesicle release and could provide a more specific target, as to not interfere with the positive aspects of circulating microvesicles.

Purinergic signaling is also involved in Stx-mediated calcium influx in human platelets. Platelet activation and thrombus formation contributes to the pathogenesis during HUS. Inhibition of purinergic signaling may counteract thrombus formation during EHEC-infection.

The effects of apyrase-treatment during EHEC infections should thus be further studied.

Populärvetenskaplig sammanfattning

Enterohemorrhagisk *Escherichia coli* (EHEC) är en bakterie som är sjukdomsframkallande i människor. EHEC är en del av normalfloran hos idisslare, så som kor, och sprids till människor främst genom förtäring av förorenad mat eller dryck. Efter intag färdas bakterien genom mag- och tarmkanalen och fäster sig främst till slemhinnan i tjocktarmen, varpå den börjar växa. EHEC orsakar inflammation och skada i tarmen och kan leda till blodiga diarréer och i vissa fall hemolytiskt uremiskt syndrom, vilket kännetecknas av blodbrist, låga nivåer av blodplättar och akut njurskada och kan vid allvarliga fall leda till död.

EHEC är en icke-invasiv bakterie, vilket betyder att den stannar kvar i tarmen och släpper där ifrån ut olika toxiner (gifter) som går in i cirkulationen. Det viktigaste toxinet som släpps ut av bakterien heter Shiga toxin (Stx).

Stx binder till en receptor som heter globotriaosylceramid (Gb3) som finns på vissa celler. Efter att Stx bundit till sin receptor tas toxinet upp av cellen och förstör cellens produktion av äggviteämnen (proteiner), vilket så småningom leder till celldöd. Stx färdas till målorgan, så som njuren och det centrala nervsystem, och orsakar där skador.

Stx kan också binda till och tas upp av olika blodceller i blodbanan utan att orsaka celldöd. När toxinet binder till blodceller påverkas dessa istället genom att producera och frisätta olika proteiner som orsakar inflammation och genom att frisätta mikrovesikler. Mikrovesikler är mycket små blåsor som består av membran som kommer ifrån cellen som de frisatts ifrån.

Det är inte känt hur Stx tar sig till sina målorgan. Toxinet finns endast i mycket små nivåer i blodomloppet. Blodceller som binder Stx kan transportera runt toxinet i blodet men hur det sen frisläpps från cellen och tar sig till njuren är okänt.

I det första delarbetet undersöktes huruvida mikrovesikler som släpps ut från blodceller innehåller Stx och om dessa mikrovesikler kan tas upp av och orsaka skada i njurceller. Blodplasma från patienter med pågående EHEC-infektion visade sig innehålla mikrovesikler från blodplättar och röda och vita blodceller och en del as dessa vesikler innehöll också toxinet. Dessa vesikler kunde även hittas i njurvävnad både bundna till och inuti celler. En intressant observation var att mikrovesikler med Stx även kunde hittas i celler som saknade receptorn till vilken Stx binder. Mikrovesikler som innehöll Stx togs upp av odlade njurceller och

orsakade skada i dessa. Vi presenterade genom det här delarbetet en modell för hur Stx kan ta sig genom blodbanan till njuren via mikrovesikler och generellt hur bakterier kan använda sig av mikrovesikler för att undgå att upptäckas av kroppens immunförsvar.

I det andra delarbetet undersöktes vikten av Stx-receptorn, Gb3, för upptag och toxicitet av Stx som levererats via mikrovesikler. Normalt sett tas Stx endast upp av och är skadligt för celler som har Gb3. I det första delarbetet hittade vi emellertid Stx inuti njurceller som saknar Gb3. Utifrån den observationen skapade vi celler med och utan Gb3 och tillsatte mikrovesikler som innehöll Stx. Både celler med och utan Gb3 tog upp Stx-innehållande mikrovesikler, men endast celler med Gb3 skadades av toxinet. Vi fann även att toxinet skadade dessa celler genom att stoppa deras proteinproduktion, vilket är den huvudsakliga mekanismen för hur Stx orsakar toxicitet. Dessa resultat indikerar att Stx kan tas upp av celler utan Gb3, genom överföring i mikrovesikler, men att Gb3 krävs för att toxinet ska kunna utföra sin skadliga funktion.

I det tredje delarbetet undersökte vi hur Stx orsakar signalering, toxicitet och fritsättning av mikrovesikler från celler. Vi fann att Stx orsakar frisättning av ATP från celler. ATP är den viktigaste energimolekylen i celler. Frisatt ATP kan binda till olika receptorer och på så sätt aktivera celler. När vi blockerade bindningen av ATP till dessa receptorer så hämmades signaleringen och toxiciteten från Stx och antalet frisatta mikrovesikler minskade. Utifrån dessa resultat föreslår vi att effekterna av blockerad ATP-signalering under pågående EHEC-infektion borde undersökas.

I det fjärde delarbetet undersökte vi hur ATP utanför celler i cirkulationen påverkar EHEC-infektioner. Detta undersöktes genom att klyva ATP med hjälp av ett protein som heter apyrase. Apyrase skyddade tarmceller mot celldöd och bevarade även den skyddande slemhinnan som finns i tarmen. Vi fann också att apyrase fördröjde sjukdomsförloppet vid EHEC-infektion.

Det femte arbetet är en litteraturstudie som sammanfattar betydelsen av olika vesikler som släpps ut från celler, så som mikrovesikler, under normala omständigheter och vid olika njursjukdomar.

Acknowledgements

I would like to thank everyone who, in one way or another, has made this thesis possible.

Diana, min handledare, tack för din ständiga uppmuntran och ditt pådrivande. Utan din kunskap och vägledning hade jag aldrig hittat rätt genom den snåriga akademin. Du har verkligen gjort det möjligt för en yr doktorand att hitta fokus under de här åren.

Anne-lie, min bihandledare, tack för alla samtal, för allt stöd och uppmuntran och framför allt för din vänskap. Du har varit en fantastisk rumskompis som alltid tagit dig tiden att svara på mina helt slumpmässiga frågor.

Ann-charlotte och Ida, tack för alla skratt, diskussioner, stöd, vägledning, sånger, åsikter, ja listan kan göras lång. Ni har hjälpt mig så mycket med allt här i labbet och det har varit en trygghet att veta att man alltid kan svänga förbi ert kontor för att få råd eller bara för lite småprat.

Annie, tack för all märkliga diskussioner vid morgonkaffet, för alla falska sångduetter i cellabbet, för alla konstiga metoder vi försökte oss på, för ditt ordningssinne som nästan smittade av sig på mig och för att du alltid tagit dig tid för att hjälpa till med både forskning och uppmuntran.

Ashmita, thanks for being a source for optimism and smiles, for all the little talks before leaving the lab, for always being more than happy to drain me on blood and for introducing me to weird podcasts.

Milan, tack för din obotliga optimism under den tiden vi jobbade tillsammans. Jag hoppas mycket på att vi kommer hamna i samma labb igen någon gång.

Ingrid, Sunna, Zivile, Maria och Sebastian, tack till er kära kollegor för många trevliga stunder, roliga samtal, intressanta diskussioner och för att ni hjälpt mig att förstå ”läkarbiten”.

Niklas och Hilda, tack för den visserligen korta, men mycket trevliga, tiden vi har jobbat tillsammans. Jag önskar er ett stort lycka till med er forskning.

Johan, tack för att du handledde mig under min första tid på labbet, för din kunskap och uppmuntran när du jobbade på vårt labb och för hjälp och trevliga samtal i lunchrummet nu när du är vår granne.

Karina, tack för att du har tagit dig tid att svara på mina frågor, lånat ut material, gett tips på labbutrustning och varit så snäll och glad.

Martin, Jill, Linn, Anja, Philaiphon, Abdul, Jennifer and Annika, a big thanks for being such nice neighbors, sharing your knowledge and brightening up the lunch room with your presence.

Eva, tack till dig för att du är en så bra granne, alltid är glad och hjälpsam och för trevliga samtal i fikarummet.

Mattias, Daniel, Ludger and Robin, a big thanks to my co-authors. Without you this thesis would not have been possible.

Vänner, tack för att ni finns där när jag behöver er som mest.

The hockey team, thanks for helping me taking my mind off research now and then and for being such amazing people.

Emma, bästa syster, tack. Jag vet att du alltid ställer upp vad som än skulle hända och att du alltid bryr dig.

Mamma och Lars, tack för att ni är så fantastiska föräldrar och förebilder och alltid finns till hand oavsett vad.

References

1. Karmali MA, Steele BT, Petric M*, et al.* Sporadic cases of haemolytic-uraemic syndrome associated with faecal cytotoxin and cytotoxin-producing *Escherichia coli* in stools. *Lancet* 1983; **1:** 619-620.
2. Karpman D, Loos S, Tati R*, et al.* Haemolytic uraemic syndrome. *J Intern Med* 2017; **281:** 123-148.
3. McKee ML, O'Brien AD. Investigation of enterohemorrhagic *Escherichia coli* O157:H7 adherence characteristics and invasion potential reveals a new attachment pattern shared by intestinal E. coli. *Infect Immun* 1995; **63:** 2070-2074.
4. Obrig TG, Karpman D. Shiga toxin pathogenesis: kidney complications and renal failure. *Curr Top Microbiol Immunol* 2012; **357:** 105-136.
5. Karpman D, Håkansson A, Perez MT*, et al.* Apoptosis of renal cortical cells in the hemolytic-uremic syndrome: *in vivo* and *in vitro* studies. *Infect Immun* 1998; **66:** 636-644.
6. Robinson LA, Hurley RM, Lingwood C*, et al. Escherichia coli* verotoxin binding to human paediatric glomerular mesangial cells. *Pediatr Nephrol* 1995; **9:** 700-704.
7. Obrig TG, Louise CB, Lingwood CA*, et al.* Endothelial heterogeneity in Shiga toxin receptors and responses. *J Biol Chem* 1993; **268:** 15484-15488.
8. Brigotti M, Tazzari PL, Ravanelli E*, et al.* Clinical relevance of shiga toxin concentrations in the blood of patients with hemolytic uremic syndrome. *Pediatr Infect Dis J* 2011; **30:** 486-490. .
9. Cooling LL, Walker KE, Gille T*, et al.* Shiga toxin binds human platelets via globotriaosylceramide (Pk antigen) and a novel platelet glycosphingolipid. *Infect Immun* 1998; **66:** 4355-4366.
10. Karpman D, Papadopoulou D, Nilsson K*, et al.* Platelet activation by Shiga toxin and circulatory factors as a pathogenetic mechanism in the hemolytic uremic syndrome. *Blood* 2001; **97:** 3100-3108.
11. Ståhl AL, Sartz L, Nelsson A*, et al.* Shiga toxin and lipopolysaccharide induce platelet-leukocyte aggregates and tissue factor release, a thrombotic mechanism in hemolytic uremic syndrome. *PLoS One* 2009; **4:** e6990.

12. Brigotti M, Caprioli A, Tozzi AE*, et al.* Shiga toxins present in the gut and in the polymorphonuclear leukocytes circulating in the blood of children with hemolytic-uremic syndrome. *J Clin Microbiol* 2006; **44:** 313-317.

13. Te Loo DM, van Hinsbergh VW, van den Heuvel LP*, et al.* Detection of verocytotoxin bound to circulating polymorphonuclear leukocytes of patients with hemolytic uremic syndrome. *J Am Soc Nephrol* 2001; **12:** 800-806.

14. van Setten PA, Monnens LA, Verstraten RG*, et al.* Effects of verocytotoxin-1 on nonadherent human monocytes: binding characteristics, protein synthesis, and induction of cytokine release. *Blood* 1996; **88:** 174-183.

15. Arvidsson I, Stahl AL, Hedstrom MM*, et al.* Shiga toxin-induced complement-mediated hemolysis and release of complement-coated red blood cell-derived microvesicles in hemolytic uremic syndrome. *J Immunol* 2015; **194:** 2309-2318.

16. Jacewicz M, Clausen H, Nudelman E*, et al.* Pathogenesis of S*higella diarrhea*. XI. Isolation of a shigella toxin-binding glycolipid from rabbit jejunum and HeLa cells and its identification as globotriaosylceramide. *J Exp Med* 1986; **163:** 1391-1404.

17. Sandvig K, Garred O, Prydz K*, et al.* Retrograde transport of endocytosed Shiga toxin to the endoplasmic reticulum. *Nature* 1992; **358:** 510-512.

18. Endo Y, Tsurugi K, Yutsudo T*, et al.* Site of action of a Vero toxin (VT2) from *Escherichia coli* O157:H7 and of Shiga toxin on eukaryotic ribosomes. RNA N-glycosidase activity of the toxins. *Eur J Biochem* 1988; **171:** 45-50.

19. Tam PJ, Lingwood CA. Membrane cytosolic translocation of verotoxin A1 subunit in target cells. *Microbiology* 2007; **153:** 2700-2710.

20. Malyukova I, Murray KF, Zhu C*, et al.* Macropinocytosis in Shiga toxin 1 uptake by human intestinal epithelial cells and transcellular transcytosis. *Am J Physiol Gastrointest Liver Physiol* 2009; **296:** G78-92.

21. Morigi M, Galbusera M, Gastoldi S*, et al.* Alternative pathway activation of complement by Shiga toxin promotes exuberant C3a formation that triggers microvascular thrombosis. *J Immunol* 2011; **187:** 172-180.

22. Petruzziello-Pellegrini TN, Yuen DA, Page AV*, et al.* The CXCR4/CXCR7/SDF-1 pathway contributes to the pathogenesis of Shiga toxin-associated hemolytic uremic syndrome in humans and mice. *J Clin Invest* 2012; **122:** 759-776.

23. Ståhl AL, Arvidsson I, Johansson KE*, et al.* A novel mechanism of bacterial toxin transfer within host blood cell-derived microvesicles. *PLoS Pathog* 2015; **11:** e1004619.

24. Liu F, Huang J, Sadler JE. Shiga toxin (Stx)1B and Stx2B induce von Willebrand factor secretion from human umbilical vein endothelial cells through different signaling pathways. *Blood* 2011; **118:** 3392-3398.

25. Klokk TI, Kavaliauskiene S, Sandvig K. Cross-linking of glycosphingolipids at the plasma membrane: consequences for intracellular signaling and traffic. *Cell Mol Life Sci* 2016; **73:** 1301-1316.

26. Obrig TG, Moran TP, Brown JE. The mode of action of Shiga toxin on peptide elongation of eukaryotic protein synthesis. *Biochem J* 1987; **244:** 287-294.

27. Fujii J, Matsui T, Heatherly DP*, et al.* Rapid apoptosis induced by Shiga toxin in HeLa cells. *Infect Immun* 2003; **71:** 2724-2735.

28. Naylor SW, Low JC, Besser TE*, et al.* Lymphoid follicle-dense mucosa at the terminal rectum is the principal site of colonization of enterohemorrhagic *Escherichia coli* O157:H7 in the bovine host. *Infect Immun* 2003; **71:** 1505-1512.

29. Cray WC, Jr., Moon HW. Experimental infection of calves and adult cattle with *Escherichia coli* O157:H7. *Appl Environ Microbiol* 1995; **61:** 1586-1590.

30. Nguyen Y, Sperandio V. Enterohemorrhagic *E. coli* (EHEC) pathogenesis. *Front Cell Infect Microbiol* 2012; **2:** 90.

31. Tuttle J, Gomez T, Doyle MP*, et al.* Lessons from a large outbreak of *Escherichia coli* O157:H7 infections: insights into the infectious dose and method of widespread contamination of hamburger patties. *Epidemiol Infect* 1999; **122:** 185-192.

32. Lewis SB, Cook V, Tighe R*, et al.* Enterohemorrhagic *Escherichia coli* colonization of human colonic epithelium in vitro and ex vivo. *Infect Immun* 2015; **83:** 942-949.

33. Barnett Foster D. Modulation of the enterohemorrhagic *E. coli* virulence program through the human gastrointestinal tract. *Virulence* 2013; **4:** 315-323.

34. Lin J, Smith MP, Chapin KC*, et al.* Mechanisms of acid resistance in enterohemorrhagic Escherichia coli. *Appl Environ Microbiol* 1996; **62:** 3094-3100.

35. Karmali MA, Mascarenhas M, Shen S*, et al.* Association of genomic O island 122 of *Escherichia coli* EDL 933 with verocytotoxin-producing Escherichia coli seropathotypes that are linked to epidemic and/or serious disease. *J Clin Microbiol* 2003; **41:** 4930-4940.

36. Farfan MJ, Cantero L, Vidal R*, et al.* Long polar fimbriae of enterohemorrhagic *Escherichia coli* O157:H7 bind to extracellular matrix proteins. *Infect Immun* 2011; **79:** 3744-3750.

37. Wolff C, Nisan I, Hanski E*, et al.* Protein translocation into host epithelial cells by infecting enteropathogenic *Escherichia coli*. *Mol Microbiol* 1998; **28:** 143-155.

38. Garmendia J, Phillips AD, Carlier MF*, et al.* TccP is an enterohaemorrhagic *Escherichia coli* O157:H7 type III effector protein that couples Tir to the actin-cytoskeleton. *Cell Microbiol* 2004; **6:** 1167-1183.

39. Vingadassalom D, Kazlauskas A, Skehan B*, et al.* Insulin receptor tyrosine kinase substrate links the *E. coli* O157:H7 actin assembly effectors Tir and EspF(U) during pedestal formation. *Proc Natl Acad Sci U S A* 2009; **106:** 6754-6759.

40. Deibel C, Kramer S, Chakraborty T*, et al.* EspE, a novel secreted protein of attaching and effacing bacteria, is directly translocated into infected host cells, where it appears as a tyrosine-phosphorylated 90 kDa protein. *Mol Microbiol* 1998; **28:** 463-474.

41. Luo Y, Frey EA, Pfuetzner RA*, et al.* Crystal structure of enteropathogenic *Escherichia coli* intimin-receptor complex. *Nature* 2000; **405:** 1073-1077.

42. de Groot JC, Schluter K, Carius Y*, et al.* Structural basis for complex formation between human IRSp53 and the translocated intimin receptor Tir of enterohemorrhagic *E. coli*. *Structure* 2011; **19:** 1294-1306.

43. Tacket CO, Sztein MB, Losonsky G*, et al.* Role of EspB in experimental human enteropathogenic *Escherichia coli* infection. *Infect Immun* 2000; **68:** 3689-3695.

44. Frankel G, Phillips AD, Novakova M*, et al.* Intimin from enteropathogenic *Escherichia coli* restores murine virulence to a *Citrobacter rodentium* eaeA mutant: induction of an immunoglobulin A response to intimin and EspB. *Infect Immun* 1996; **64:** 5315-5325.

45. Bekassy ZD, Calderon Toledo C, Leoj G*, et al.* Intestinal damage in enterohemorrhagic *Escherichia coli* infection. *Pediatr Nephrol* 2011; **26:** 2059-2071.

46. Trofa AF, Ueno-Olsen H, Oiwa R*, et al.* Dr. Kiyoshi Shiga: discoverer of the dysentery bacillus. *Clin Infect Dis* 1999; **29:** 1303-1306.

47. Fraser ME, Chernaia MM, Kozlov YV*, et al.* Crystal structure of the holotoxin from *Shigella dysenteriae* at 2.5 A resolution. *Nat Struct Biol* 1994; **1:** 59-64.

48. Fontaine A, Arondel J, Sansonetti PJ. Role of Shiga toxin in the pathogenesis of bacillary dysentery, studied by using a Tox- mutant of *Shigella dysenteriae* 1. *Infect Immun* 1988; **56:** 3099-3109.

49. Roxas JL, Koutsouris A, Bellmeyer A*, et al.* Enterohemorrhagic *E. coli* alters murine intestinal epithelial tight junction protein expression and barrier function in a Shiga toxin independent manner. *Lab Invest* 2010; **90:** 1152-1168.

50. Philpott DJ, McKay DM, Mak W*, et al.* Signal transduction pathways involved in enterohemorrhagic *Escherichia coli*-induced alterations in T84 epithelial permeability. *Infect Immun* 1998; **66:** 1680-1687.

51. Philpott DJ, Ackerley CA, Kiliaan AJ*, et al.* Translocation of verotoxin-1 across T84 monolayers: mechanism of bacterial toxin penetration of epithelium. *Am J Physiol* 1997; **273:** G1349-1358.

52. Hurley BP, Thorpe CM, Acheson DW. Shiga toxin translocation across intestinal epithelial cells is enhanced by neutrophil transmigration. *Infect Immun* 2001; **69:** 6148-6155.

53. Acheson DW, Moore R, De Breucker S*, et al.* Translocation of Shiga toxin across polarized intestinal cells in tissue culture. *Infect Immun* 1996; **64:** 3294-3300.

54. Holgersson J, Jovall PA, Breimer ME. Glycosphingolipids of human large intestine: detailed structural characterization with special reference to blood group compounds and bacterial receptor structures. *J Biochem* 1991; **110:** 120-131.

55. Jacewicz MS, Acheson DW, Mobassaleh M*, et al.* Maturational regulation of globotriaosylceramide, the Shiga-like toxin 1 receptor, in cultured human gut epithelial cells. *J Clin Invest* 1995; **96:** 1328-1335.

56. Schuller S, Frankel G, Phillips AD. Interaction of Shiga toxin from *Escherichia coli* with human intestinal epithelial cell lines and explants: Stx2 induces epithelial damage in organ culture. *Cell Microbiol* 2004; **6:** 289-301.

57. Jacewicz MS, Acheson DW, Binion DG*, et al.* Responses of human intestinal microvascular endothelial cells to Shiga toxins 1 and 2 and pathogenesis of hemorrhagic colitis. *Infect Immun* 1999; **67:** 1439-1444.

58. Falguieres T, Mallard F, Baron C*, et al.* Targeting of Shiga toxin B-subunit to retrograde transport route in association with detergent-resistant membranes. *Mol Biol Cell* 2001; **12:** 2453-2468.

59. Uchida H, Kiyokawa N, Horie H*, et al.* The detection of Shiga toxins in the kidney of a patient with hemolytic uremic syndrome. *Pediatr Res* 1999; **45:** 133-137.

60. Chaisri U, Nagata M, Kurazono H*, et al.* Localization of Shiga toxins of enterohaemorrhagic *Escherichia coli* in kidneys of paediatric and geriatric patients with fatal haemolytic uraemic syndrome. *Microb Pathog* 2001; **31:** 59-67.

61. Obata F, Tohyama K, Bonev AD*, et al.* Shiga toxin 2 affects the central nervous system through receptor globotriaosylceramide localized to neurons. *J Infect Dis* 2008; **198:** 1398-1406.

62. Warnier M, Romer W, Geelen J*, et al.* Trafficking of Shiga toxin/Shiga-like toxin-1 in human glomerular microvascular endothelial cells and human mesangial cells. *Kidney Int* 2006; **70:** 2085-2091.

63. Van Setten PA, van Hinsbergh VW, Van den Heuvel LP*, et al.* Verocytotoxin inhibits mitogenesis and protein synthesis in purified human glomerular mesangial cells without affecting cell viability: evidence for two distinct mechanisms. *J Am Soc Nephrol* 1997; **8:** 1877-1888.

64. Locatelli M, Buelli S, Pezzotta A*, et al.* Shiga toxin promotes podocyte injury in experimental hemolytic uremic syndrome via activation of the alternative pathway of complement. *J Am Soc Nephrol* 2014; **25:** 1786-1798.

65. Calderwood SB, Auclair F, Donohue-Rolfe A*, et al.* Nucleotide sequence of the Shiga-like toxin genes of *Escherichia coli*. *Proc Natl Acad Sci U S A* 1987; **84:** 4364-4368.

66. Newland JW, Strockbine NA, Neill RJ. Cloning of genes for production of *Escherichia coli* Shiga-like toxin type II. *Infect Immun* 1987; **55:** 2675-2680.

67. Lee JE, Reed J, Shields MS*, et al.* Phylogenetic analysis of Shiga toxin 1 and Shiga toxin 2 genes associated with disease outbreaks. *BMC Microbiol* 2007; **7:** 109.

68. Shridhar PB, Siepker C, Noll LW*, et al.* Shiga Toxin Subtypes of Non-O157 *Escherichia coli* Serogroups Isolated from Cattle Feces. *Front Cell Infect Microbiol* 2017; **7:** 121.

69. Karve SS, Weiss AA. Glycolipid binding preferences of Shiga toxin variants. *PLoS One* 2014; **9:** e101173.

70. Gallegos KM, Conrady DG, Karve SS*, et al.* Shiga toxin binding to glycolipids and glycans. *PLoS One* 2012; **7:** e30368.

71. Muthing J, Meisen I, Zhang W*, et al.* Promiscuous Shiga toxin 2e and its intimate relationship to Forssman. *Glycobiology* 2012; **22:** 849-862.

72. Okuda T, Tokuda N, Numata S*, et al.* Targeted disruption of Gb3/CD77 synthase gene resulted in the complete deletion of globo-series glycosphingolipids and loss of sensitivity to verotoxins. *J Biol Chem* 2006; **281:** 10230-10235.

73. Kasahara K, Sanai Y. Possible roles of glycosphingolipids in lipid rafts. *Biophys Chem* 1999; **82:** 121-127.

74. Kasahara K, Sanai Y. Functional roles of glycosphingolipids in signal transduction via lipid rafts. *Glycoconj J* 2000; **17:** 153-162.

75. Kiarash A, Boyd B, Lingwood CA. Glycosphingolipid receptor function is modified by fatty acid content. Verotoxin 1 and verotoxin 2c preferentially recognize different globotriaosyl ceramide fatty acid homologues. *J Biol Chem* 1994; **269:** 11138-11146.

76. Ling H, Boodhoo A, Hazes B*, et al.* Structure of the shiga-like toxin I B-pentamer complexed with an analogue of its receptor Gb3. *Biochemistry* 1998; **37:** 1777-1788.

77. Bast DJ, Banerjee L, Clark C*, et al.* The identification of three biologically relevant globotriaosyl ceramide receptor binding sites on the Verotoxin 1 B subunit. *Mol Microbiol* 1999; **32:** 953-960.

78. Torgersen ML, Lauvrak SU, Sandvig K. The A-subunit of surface-bound Shiga toxin stimulates clathrin-dependent uptake of the toxin. *Febs j* 2005; **272:** 4103-4113.

79. Renard HF, Garcia-Castillo MD, Chambon V*, et al.* Shiga toxin stimulates clathrin-independent endocytosis of the VAMP2, VAMP3 and VAMP8 SNARE proteins. *J Cell Sci* 2015; **128:** 2891-2902.

80. Romer W, Berland L, Chambon V*, et al.* Shiga toxin induces tubular membrane invaginations for its uptake into cells. *Nature* 2007; **450:** 670-675.

81. Renard HF, Simunovic M, Lemiere J*, et al.* Endophilin-A2 functions in membrane scission in clathrin-independent endocytosis. *Nature* 2015; **517:** 493-496.

82. Garcia-Castillo MD, Tran T, Bobard A*, et al.* Retrograde transport is not required for cytosolic translocation of the B-subunit of Shiga toxin. *J Cell Sci* 2015; **128:** 2373-2387.

83. Garred O, van Deurs B, Sandvig K. Furin-induced cleavage and activation of Shiga toxin. *J Biol Chem* 1995; **270:** 10817-10821.

84. Johannes L, Romer W. Shiga toxins--from cell biology to biomedical applications. *Nat Rev Microbiol* 2010; **8:** 105-116.

85. Sandvig K, van Deurs B. Endocytosis, intracellular transport, and cytotoxic action of Shiga toxin and ricin. *Physiol Rev* 1996; **76:** 949-966.

86. Békássy ZD, Calderon Toledo C, Leoj G*, et al.* Intestinal damage in enterohemorrhagic *Escherichia coli* infection. *Pediatr Nephrol* 2011; **26:** 2059-2071.

87. Lee SY, Cherla RP, Caliskan I*, et al.* Shiga toxin 1 induces apoptosis in the human myelogenous leukemia cell line THP-1 by a caspase-8-dependent, tumor necrosis factor receptor-independent mechanism. *Infect Immun* 2005; **73:** 5115-5126.

88. Burlaka I, Liu XL, Rebetz J*, et al.* Ouabain protects against Shiga toxin-triggered apoptosis by reversing the imbalance between Bax and Bcl-xL. *J Am Soc Nephrol* 2013; **24:** 1413-1423. doi: 1410.1681/ASN.2012101044. Epub 2012102013 Jun 2012101046.

89. Lee MS, Cherla RP, Tesh VL. Shiga toxins: intracellular trafficking to the ER leading to activation of host cell stress responses. *Toxins (Basel)* 2010; **2:** 1515-1535.

90. Tetaud C, Falguieres T, Carlier K*, et al.* Two distinct Gb3/CD77 signaling pathways leading to apoptosis are triggered by anti-Gb3/CD77 mAb and verotoxin-1. *J Biol Chem* 2003; **278:** 45200-45208.

91. Mangeney M, Lingwood CA, Taga S*, et al.* Apoptosis induced in Burkitt's lymphoma cells via Gb3/CD77, a glycolipid antigen. *Cancer Res* 1993; **53:** 5314-5319.

92. Cyr DM, Hebert DN. Protein quality control--linking the unfolded protein response to disease. Conference on 'From Unfolded Proteins in the Endoplasmic Reticulum to Disease'. *EMBO Rep* 2009; **10:** 1206-1210.

93. Cherla RP, Lee SY, Tesh VL. Shiga toxins and apoptosis. *FEMS Microbiol Lett* 2003; **228:** 159-166.

94. Smith WE, Kane AV, Campbell ST*, et al.* Shiga toxin 1 triggers a ribotoxic stress response leading to p38 and JNK activation and induction of apoptosis in intestinal epithelial cells. *Infect Immun* 2003; **71:** 1497-1504.

95. Obata F, Hippler LM, Saha P*, et al.* Shiga toxin type-2 (Stx2) induces glutamate release via phosphoinositide 3-kinase (PI3K) pathway in murine neurons. *Front Mol Neurosci* 2015; **8:** 30.

96. Alexander C, Rietschel ET. Bacterial lipopolysaccharides and innate immunity. *J Endotoxin Res* 2001; **7:** 167-202.

97. Raetz CR, Whitfield C. Lipopolysaccharide endotoxins. *Annu Rev Biochem* 2002; **71:** 635-700.

98. Dobrovolskaia MA, Vogel SN. Toll receptors, CD14, and macrophage activation and deactivation by LPS. *Microbes Infect* 2002; **4:** 903-914.

99. Ståhl AL, Svensson M, Morgelin M*, et al.* Lipopolysaccharide from enterohemorrhagic *Escherichia coli* binds to platelets through TLR4 and CD62 and is detected on circulating platelets in patients with hemolytic uremic syndrome. *Blood* 2006; **108:** 167-176. Epub 2006 Mar 2002.

100. Calderon Toledo C, Rogers TJ, Svensson M*, et al.* Shiga toxin-mediated disease in MyD88-deficient mice infected with *Escherichia coli* O157:H7. *Am J Pathol* 2008; **173:** 1428-1439.

101. Bielaszewska M, Ruter C, Bauwens A*, et al.* 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* 2017; **13:** e1006159.

102. Bauer ME, Welch RA. Association of RTX toxins with erythrocytes. *Infect Immun* 1996; **64:** 4665-4672.

103. Paton AW, Srimanote P, Talbot UM*, et al.* A new family of potent AB(5) cytotoxins produced by Shiga toxigenic *Escherichia coli*. *J Exp Med* 2004; **200:** 35-46.

104. Paton AW, Paton JC. Multiplex PCR for direct detection of Shiga toxigenic *Escherichia coli* strains producing the novel subtilase cytotoxin. *J Clin Microbiol* 2005; **43:** 2944-2947.

105. Byres E, Paton AW, Paton JC*, et al.* Incorporation of a non-human glycan mediates human susceptibility to a bacterial toxin. *Nature* 2008; **456:** 648-652.

106. Paton AW, Beddoe T, Thorpe CM*, et al.* AB5 subtilase cytotoxin inactivates the endoplasmic reticulum chaperone BiP. *Nature* 2006; **443:** 548-552.

107. Wolfson JJ, May KL, Thorpe CM*, et al.* Subtilase cytotoxin activates PERK, IRE1 and ATF6 endoplasmic reticulum stress-signalling pathways. *Cell Microbiol* 2008; **10:** 1775-1786.

108. Tan K, Li R, Huang X*, et al.* Outer Membrane Vesicles: Current Status and Future Direction of These Novel Vaccine Adjuvants. *Front Microbiol* 2018; **9:** 783.

109. Kolling GL, Matthews KR. Export of virulence genes and Shiga toxin by membrane vesicles of *Escherichia coli* O157:H7. *Appl Environ Microbiol* 1999; **65:** 1843-1848.

110. McBroom AJ, Kuehn MJ. Release of outer membrane vesicles by Gram-negative bacteria is a novel envelope stress response. *Mol Microbiol* 2007; **63:** 545-558.

111. Schwechheimer C, Kulp A, Kuehn MJ. Modulation of bacterial outer membrane vesicle production by envelope structure and content. *BMC Microbiol* 2014; **14:** 324.

112. Bernadac A, Gavioli M, Lazzaroni JC*, et al. Escherichia coli* tol-pal mutants form outer membrane vesicles. *J Bacteriol* 1998; **180:** 4872-4878.

113. Kim SH, Lee YH, Lee SH*, et al.* Mouse model for hemolytic uremic syndrome induced by outer membrane vesicles of *Escherichia coli* O157:H7. *FEMS Immunol Med Microbiol* 2011; **63:** 427-434.

114. Pearson JS, Hartland EL. The Inflammatory Response during Enterohemorrhagic *Escherichia coli* Infection. *Microbiol Spectr* 2014; **2:** Ehec-0012-2013.

115. Miyamoto Y, Iimura M, Kaper JB*, et al.* Role of Shiga toxin versus H7 flagellin in enterohaemorrhagic *Escherichia coli* signalling of human colon epithelium in vivo. *Cell Microbiol* 2006; **8:** 869-879.

116. Dahan S, Busuttil V, Imbert V*, et al.* Enterohemorrhagic *Escherichia coli* infection induces interleukin-8 production via activation of mitogen-activated protein kinases and the transcription factors NF-kappaB and AP-1 in T84 cells. *Infect Immun* 2002; **70:** 2304-2310.

117. Ledesma MA, Ochoa SA, Cruz A*, et al.* The hemorrhagic coli pilus (HCP) of *Escherichia coli* O157:H7 is an inducer of proinflammatory cytokine secretion in intestinal epithelial cells. *PLoS One* 2010; **5:** e12127.

118. Griffin PM, Olmstead LC, Petras RE. *Escherichia coli* O157:H7-associated colitis. A clinical and histological study of 11 cases. *Gastroenterology* 1990; **99:** 142-149.

119. Schroeder BO. Fight them or feed them: how the intestinal mucus layer manages the gut microbiota. *Gastroenterol Rep (Oxf)* 2019; **7:** 3-12.

120. Chromek M, Arvidsson I, Karpman D. The antimicrobial peptide cathelicidin protects mice from *Escherichia coli* O157:H7-mediated disease. *PLoS One* 2012; **7:** e46476.

121. Taguchi H, Takahashi M, Yamaguchi H*, et al.* Experimental infection of germ-free mice with hyper-toxigenic enterohaemorrhagic *Escherichia coli* O157:H7, strain 6. *J Med Microbiol* 2002; **51:** 336-343.

122. Calderon Toledo C, Arvidsson I, Karpman D. Cross-reactive protection against enterohemorrhagic *Escherichia coli* infection by enteropathogenic *E. coli* in a mouse model. *Infect Immun* 2011; **79:** 2224-2233.

123. Yu AC, Worrall LJ, Strynadka NC. Structural insight into the bacterial mucinase StcE essential to adhesion and immune evasion during enterohemorrhagic *E. coli* infection. *Structure* 2012; **20:** 707-717.

124. Ghosh SA, Polanowska-Grabowska RK, Fujii J*, et al.* Shiga toxin binds to activated platelets. *J Thromb Haemost* 2004; **2:** 499-506.

125. Guessous F, Marcinkiewicz M, Polanowska-Grabowska R*, et al.* Shiga toxin 2 and lipopolysaccharide cause monocytic THP-1 cells to release factors which activate platelet function. *Thromb Haemost* 2005; **94:** 1019-1027.

126. Brandelli JR, Griener TP, Laing A*, et al.* The Effects of Shiga Toxin 1, 2 and Their Subunits on Cytokine and Chemokine Expression by Human Macrophage-Like THP-1 Cells. *Toxins (Basel)* 2015; **7:** 4054-4066.

127. van de Kar NC, Kooistra T, Vermeer M*, et al.* Tumor necrosis factor alpha induces endothelial galactosyl transferase activity and verocytotoxin receptors. Role of specific tumor necrosis factor receptors and protein kinase C. *Blood* 1995; **85:** 734-743.

128. Lee MS, Kwon H, Lee EY*, et al.* Shiga Toxins Activate the NLRP3 Inflammasome Pathway To Promote Both Production of the Proinflammatory Cytokine Interleukin-1beta and Apoptotic Cell Death. *Infect Immun* 2016; **84:** 172-186.

129. Mariathasan S, Weiss DS, Newton K*, et al.* Cryopyrin activates the inflammasome in response to toxins and ATP. *Nature* 2006; **440:** 228-232.

130. Conos SA, Chen KW, De Nardo D*, et al.* Active MLKL triggers the NLRP3 inflammasome in a cell-intrinsic manner. *Proc Natl Acad Sci U S A* 2017; **114:** E961-e969.

131. Man SM, Karki R, Kanneganti TD. Molecular mechanisms and functions of pyroptosis, inflammatory caspases and inflammasomes in infectious diseases. *Immunol Rev* 2017; **277:** 61-75.

132. Liu J, Akahoshi T, Sasahana T*, et al.* Inhibition of neutrophil apoptosis by verotoxin 2 derived from *Escherichia coli* O157:H7. *Infect Immun* 1999; **67:** 6203-6205.

133. Ramos MV, Mejias MP, Sabbione F*, et al.* Induction of Neutrophil Extracellular Traps in Shiga Toxin-Associated Hemolytic Uremic Syndrome. *J Innate Immun* 2016; **8:** 400-411.

134. Scheiring J, Andreoli SP, Zimmerhackl LB. Treatment and outcome of Shiga-toxin-associated hemolytic uremic syndrome (HUS). *Pediatr Nephrol* 2008; **23:** 1749-1760.

135. Canpolat N. Hemolytic uremic syndrome. *Turk Pediatri Ars* 2015; **50:** 73-82.

136. Ruggenenti P, Noris M, Remuzzi G. Thrombotic microangiopathy, hemolytic uremic syndrome, and thrombotic thrombocytopenic purpura. *Kidney Int* 2001; **60:** 831-846.

137. Turi S, Nemeth I, Vargha I*, et al.* Oxidative damage of red blood cells in haemolytic uraemic syndrome. *Pediatr Nephrol* 1994; **8:** 26-29.

138. Facorro G, Aguirre F, Florentin L*, et al.* Oxidative stress and membrane fluidity in erythrocytes from patients with hemolytic uremic syndrome. *Acta Physiol Pharmacol Ther Latinoam* 1997; **47:** 137-146.

139. Cauwels A, Rogge E, Vandendriessche B*, et al.* Extracellular ATP drives systemic inflammation, tissue damage and mortality. *Cell Death Dis* 2014; **5:** e1102.

140. Ralevic V, Burnstock G. Receptors for purines and pyrimidines. *Pharmacol Rev* 1998; **50:** 413-492.

141. Sheth S, Brito R, Mukherjea D*, et al.* Adenosine receptors: expression, function and regulation. *Int J Mol Sci* 2014; **15:** 2024-2052.

142. Abbracchio MP, Ceruti S. P1 receptors and cytokine secretion. *Purinergic Signal* 2007; **3:** 13-25.

143. von Kugelgen I, Hoffmann K. Pharmacology and structure of P2Y receptors. *Neuropharmacology* 2016; **104:** 50-61.

144. North RA. P2X receptors. *Philos Trans R Soc Lond B Biol Sci* 2016; **371**.

145. Wang L, Ostberg O, Wihlborg AK*, et al.* Quantification of ADP and ATP receptor expression in human platelets. *J Thromb Haemost* 2003; **1:** 330-336.

146. Dosch M, Gerber J, Jebbawi F*, et al.* Mechanisms of ATP Release by Inflammatory Cells. *Int J Mol Sci* 2018; **19**.

147. Lohman AW, Billaud M, Isakson BE. Mechanisms of ATP release and signalling in the blood vessel wall. *Cardiovasc Res* 2012; **95:** 269-280.

148. Taylor KA, Wright JR, Vial C*, et al.* Amplification of human platelet activation by surface pannexin-1 channels. *J Thromb Haemost* 2014; **12:** 987-998.

149. Locovei S, Wang J, Dahl G. Activation of pannexin 1 channels by ATP through P2Y receptors and by cytoplasmic calcium. *FEBS Lett* 2006; **580:** 239-244.

150. Streb H, Irvine RF, Berridge MJ*, et al.* Release of Ca2+ from a nonmitochondrial intracellular store in pancreatic acinar cells by inositol-1,4,5-trisphosphate. *Nature* 1983; **306:** 67-69.

151. Gudlur A, Zeraik AE, Hirve N*, et al.* Calcium sensing by the STIM1 ER-luminal domain. *Nat Commun* 2018; **9:** 4536.

152. Park CY, Hoover PJ, Mullins FM*, et al.* STIM1 clusters and activates CRAC channels via direct binding of a cytosolic domain to Orai1. *Cell* 2009; **136:** 876-890.

153. Katsuragi T, Matsuo K, Sato C*, et al.* Non-neuronal release of ATP and inositol 1,4,5-trisphosphate accumulation evoked by P2- and M-receptor stimulation in guinea pig ileal segments. *J Pharmacol Exp Ther* 1996; **277:** 747-752.

154. Liu GJ, Werry EL, Bennett MR. Secretion of ATP from Schwann cells in response to uridine triphosphate. *Eur J Neurosci* 2005; **21:** 151-160.

155. Ceriani F, Pozzan T, Mammano F. Critical role of ATP-induced ATP release for Ca2+ signaling in nonsensory cell networks of the developing cochlea. *Proc Natl Acad Sci U S A* 2016; **113:** E7194-e7201.

156. Coddou C, Yan Z, Obsil T*, et al.* Activation and regulation of purinergic P2X receptor channels. *Pharmacol Rev* 2011; **63:** 641-683.

157. Ren H, Teng Y, Tan B*, et al.* Toll-like receptor-triggered calcium mobilization protects mice against bacterial infection through extracellular ATP release. *Infect Immun* 2014; **82:** 5076-5085.

158. Wilmaerts D, Bayoumi M, Dewachter L*, et al.* The persistence-inducing toxin HokB forms dynamic pores that cause ATP leakage. *MBio* 2018; **9**.

159. Skals M, Bjaelde RG, Reinholdt J*, et al.* Bacterial RTX toxins allow acute ATP release from human erythrocytes directly through the toxin pore. *J Biol Chem* 2014; **289:** 19098-19109.

160. Allard B, Longhi MS, Robson SC*, et al.* The ectonucleotidases CD39 and CD73: Novel checkpoint inhibitor targets. *Immunol Rev* 2017; **276:** 121-144.

161. Bono MR, Fernandez D, Flores-Santibanez F*, et al.* CD73 and CD39 ectonucleotidases in T cell differentiation: Beyond immunosuppression. *FEBS Lett* 2015; **589:** 3454-3460.

162. Handa M, Guidotti G. Purification and cloning of a soluble ATP-diphosphohydrolase (apyrase) from potato tubers (Solanum tuberosum). *Biochem Biophys Res Commun* 1996; **218:** 916-923.

163. Mahaut-Smith MP, Jones S, Evans RJ. The P2X1 receptor and platelet function. *Purinergic Signal* 2011; **7:** 341-356.

164. Fung CY, Jones S, Ntrakwah A*, et al.* Platelet Ca(2+) responses coupled to glycoprotein VI and Toll-like receptors persist in the presence of endothelial-derived inhibitors: roles for secondary activation of P2X1 receptors and release from intracellular Ca(2+) stores. *Blood* 2012; **119:** 3613-3621.

165. Hechler B, Lenain N, Marchese P*, et al.* A role of the fast ATP-gated P2X1 cation channel in thrombosis of small arteries in vivo. *J Exp Med* 2003; **198:** 661-667.

166. Netea MG, Nold-Petry CA, Nold MF*, et al.* Differential requirement for the activation of the inflammasome for processing and release of IL-1beta in monocytes and macrophages. *Blood* 2009; **113:** 2324-2335.

167. Asgari E, Le Friec G, Yamamoto H*, et al.* C3a modulates IL-1beta secretion in human monocytes by regulating ATP efflux and subsequent NLRP3 inflammasome activation. *Blood* 2013; **122:** 3473-3481.

168. Kukulski F, Ben Yebdri F, Lecka J*, et al.* Extracellular ATP and P2 receptors are required for IL-8 to induce neutrophil migration. *Cytokine* 2009; **46:** 166-170.

169. Wang X, Chen D. Purinergic Regulation of Neutrophil Function. *Front Immunol* 2018; **9:** 399.

170. Johnstone RM, Adam M, Hammond JR*, et al.* Vesicle formation during reticulocyte maturation. Association of plasma membrane activities with released vesicles (exosomes). *J Biol Chem* 1987; **262:** 9412-9420.

171. Mesri M, Altieri DC. Endothelial cell activation by leukocyte microparticles. *J Immunol* 1998; **161:** 4382-4387.

172. Bussolati B, Camussi G. Renal injury: Early apoptotic extracellular vesicles in injury and repair. *Nat Rev Nephrol* 2017; **13:** 523-524.

173. Owens AP, 3rd, Mackman N. Microparticles in hemostasis and thrombosis. *Circ Res* 2011; **108:** 1284-1297.

174. Hessvik NP, Llorente A. Current knowledge on exosome biogenesis and release. *Cell Mol Life Sci* 2018; **75:** 193-208.

175. Montecalvo A, Larregina AT, Shufesky WJ*, et al.* Mechanism of transfer of functional microRNAs between mouse dendritic cells via exosomes. *Blood* 2012; **119:** 756-766.

176. Street JM, Birkhoff W, Menzies RI*, et al.* Exosomal transmission of functional aquaporin 2 in kidney cortical collecting duct cells. *J Physiol* 2011; **589:** 6119-6127

177. Sinauridze EI, Kireev DA, Popenko NY*, et al.* Platelet microparticle membranes have 50- to 100-fold higher specific procoagulant activity than activated platelets. *Thromb Haemost* 2007; **97:** 425-434.

178. Elmore S. Apoptosis: a review of programmed cell death. *Toxicol Pathol* 2007; **35:** 495-516.

179. Kurosaka K, Takahashi M, Watanabe N*, et al.* Silent cleanup of very early apoptotic cells by macrophages. *J Immunol* 2003; **171:** 4672-4679.

180. Pasquet JM, Dachary-Prigent J, Nurden AT. Calcium influx is a determining factor of calpain activation and microparticle formation in platelets. *Eur J Biochem* 1996; **239:** 647-654.

181. Wehman AM, Poggioli C, Schweinsberg P*, et al.* The P4-ATPase TAT-5 inhibits the budding of extracellular vesicles in *C. elegans* embryos. *Curr Biol* 2011; **21:** 1951-1959.

182. Bretscher MS. Asymmetrical Lipid Bilayer Structure for Biological Membranes. *Nature New Biology* 1972; **236:** 11-12.

183. Tang X, Halleck MS, Schlegel RA*, et al.* A subfamily of P-type ATPases with aminophospholipid transporting activity. *Science* 1996; **272:** 1495-1497.

184. Hamon Y, Broccardo C, Chambenoit O*, et al.* ABC1 promotes engulfment of apoptotic cells and transbilayer redistribution of phosphatidylserine. *Nat Cell Biol* 2000; **2:** 399-406.

185. Zhou Q, Zhao J, Stout JG*, et al.* Molecular cloning of human plasma membrane phospholipid scramblase. A protein mediating transbilayer movement of plasma membrane phospholipids. *J Biol Chem* 1997; **272:** 18240-18244.

186. Daleke DL. Regulation of transbilayer plasma membrane phospholipid asymmetry. *J Lipid Res* 2003; **44:** 233-242.

187. Farge E, Ojcius DM, Subtil A*, et al.* Enhancement of endocytosis due to aminophospholipid transport across the plasma membrane of living cells. *Am J Physiol* 1999; **276:** C725-733.

188. Nabhan JF, Hu R, Oh RS*, et al.* Formation and release of arrestin domain-containing protein 1-mediated microvesicles (ARMMs) at plasma membrane by recruitment of TSG101 protein. *Proc Natl Acad Sci U S A* 2012; **109:** 4146-4151.

189. Elia N, Sougrat R, Spurlin TA*, et al.* Dynamics of endosomal sorting complex required for transport (ESCRT) machinery during cytokinesis and its role in abscission. *Proc Natl Acad Sci U S A* 2011; **108:** 4846-4851.

190. Yanez-Mo M, Siljander PR, Andreu Z*, et al.* Biological properties of extracellular vesicles and their physiological functions. *J Extracell Vesicles* 2015; **4:** 27066.

191. Feng D, Zhao WL, Ye YY*, et al.* Cellular internalization of exosomes occurs through phagocytosis. *Traffic* 2010; **11:** 675-687.

192. Borisenko GG, Matsura T, Liu SX*, et al.* Macrophage recognition of externalized phosphatidylserine and phagocytosis of apoptotic Jurkat cells--existence of a threshold. *Arch Biochem Biophys* 2003; **413:** 41-52.

193. Costa Verdera H, Gitz-Francois JJ, Schiffelers RM*, et al.* Cellular uptake of extracellular vesicles is mediated by clathrin-independent endocytosis and macropinocytosis. *J Control Release* 2017; **266:** 100-108.

194. Svensson KJ, Christianson HC, Wittrup A*, et al.* Exosome uptake depends on ERK1/2-heat shock protein 27 signaling and lipid Raft-mediated endocytosis negatively regulated by caveolin-1. *J Biol Chem* 2013; **288:** 17713-17724.

195. Tian T, Zhu YL, Zhou YY*, et al.* Exosome uptake through clathrin-mediated endocytosis and macropinocytosis and mediating miR-21 delivery. *J Biol Chem* 2014; **289:** 22258-22267.

196. Pearse BM. Clathrin: a unique protein associated with intracellular transfer of membrane by coated vesicles. *Proc Natl Acad Sci U S A* 1976; **73:** 1255-1259.

197. Parolini I, Federici C, Raggi C*, et al.* Microenvironmental pH is a key factor for exosome traffic in tumor cells. *J Biol Chem* 2009; **284:** 34211-34222.

198. Martinez MC, Larbret F, Zobairi F*, et al.* Transfer of differentiation signal by membrane microvesicles harboring hedgehog morphogens. *Blood* 2006; **108:** 3012-3020.

199. Mack M, Kleinschmidt A, Bruhl H*, et al.* Transfer of the chemokine receptor CCR5 between cells by membrane-derived microparticles: a mechanism for cellular human immunodeficiency virus 1 infection. *Nat Med* 2000; **6:** 769-775.

200. Kahn R, Mossberg M, Stahl AL*, et al.* Microvesicle transfer of kinin B1-receptors is a novel inflammatory mechanism in vasculitis. *Kidney Int* 2017; **91:** 96-105.

201. Yuan A, Farber EL, Rapoport AL*, et al.* Transfer of microRNAs by embryonic stem cell microvesicles. *PLoS One* 2009; **4:** e4722.

202. Skog J, Wurdinger T, van Rijn S*, et al.* Glioblastoma microvesicles transport RNA and proteins that promote tumour growth and provide diagnostic biomarkers. *Nat Cell Biol* 2008; **10:** 1470-1476.

203. Combes V, Simon AC, Grau GE*, et al.* In vitro generation of endothelial microparticles and possible prothrombotic activity in patients with lupus anticoagulant. *J Clin Invest* 1999; **104:** 93-102.

204. Del Conde I, Shrimpton CN, Thiagarajan P*, et al.* Tissue-factor-bearing microvesicles arise from lipid rafts and fuse with activated platelets to initiate coagulation. *Blood* 2005; **106:** 1604-1611.

205. Tripisciano C, Weiss R, Eichhorn T*, et al.* Different Potential of Extracellular Vesicles to Support Thrombin Generation: Contributions of Phosphatidylserine, Tissue Factor, and Cellular Origin. *Sci Rep* 2017; **7:** 6522.

206. Lentz BR. Exposure of platelet membrane phosphatidylserine regulates blood coagulation. *Prog Lipid Res* 2003; **42:** 423-438.

207. Merten M, Pakala R, Thiagarajan P*, et al.* Platelet microparticles promote platelet interaction with subendothelial matrix in a glycoprotein IIb/IIIa-dependent mechanism. *Circulation* 1999; **99:** 2577-2582.

208. MacKenzie A, Wilson HL, Kiss-Toth E*, et al.* Rapid secretion of interleukin-1beta by microvesicle shedding. *Immunity* 2001; **15:** 825-835.

209. Mesri M, Altieri DC. Leukocyte microparticles stimulate endothelial cell cytokine release and tissue factor induction in a JNK1 signaling pathway. *J Biol Chem* 1999; **274:** 23111-23118.

210. Dinkla S, van Cranenbroek B, van der Heijden WA*, et al.* Platelet microparticles inhibit IL-17 production by regulatory T cells through P-selectin. *Blood* 2016; **127:** 1976-1986.

211. Akers JC, Ramakrishnan V, Kim R*, et al.* MiR-21 in the extracellular vesicles (EVs) of cerebrospinal fluid (CSF): a platform for glioblastoma biomarker development. *PLoS One* 2013; **8:** e78115.

212. Gudbergsson JM, Jonsson K, Simonsen JB*, et al.* Systematic review of targeted extracellular vesicles for drug delivery - Considerations on methodological and biological heterogeneity. *J Control Release* 2019; **306:** 108-120.

213. Ståhl AL, Sartz L, Karpman D. Complement activation on platelet-leukocyte complexes and microparticles in enterohemorrhagic *Escherichia coli*-induced hemolytic uremic syndrome. *Blood* 2011; **117:** 5503-5513.

214. Ge S, Hertel B, Emden SH*, et al.* Microparticle generation and leucocyte death in Shiga toxin-mediated HUS. *Nephrol Dial Transplant* 2012; **27:** 2768-2775.

215. Psotka MA, Obata F, Kolling GL*, et al.* Shiga toxin 2 targets the murine renal collecting duct epithelium. *Infect Immun* 2009; **77:** 959-969.

216. Merlin D, Augeron C, Tien XY*, et al.* ATP-stimulated electrolyte and mucin secretion in the human intestinal goblet cell line HT29-Cl.16E. *J Membr Biol* 1994; **137:** 137-149.