Interaction of the EHEC virulence factor Shiga toxin 2a with the innate immune system and the role of complement in EHEC-associated hemolytic uremic syndrome

By

Sneha Chatterjee

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Abbreviations

A/E............................................................................ Attaching and effacing
Ab............................................................................. Antibody
aHUS........................................................................ atypical-Hemolytic Uremic Syndrome
AP............................................................................ Alternative Pathway
AP-Conjugated.......................................................... Alkaline-Phosphatase-Conjugated
BSA........................................................................... Bovine Serum Albumin
C1INH....................................................................... Complement 1 Inhibitor
C4Bp........................................................................... Complement 4 Binding protein
CO2............................................................................... Carbon di-oxide
CCP............................................................................. Complement Control Protein
CL-10/CL-L1............................................................. Collectin-10
CL-11/CL-K1............................................................. Collectin-11
CL-12/CL-P1............................................................. Collectin-12
CNS............................................................................ Central Nervous System
CP............................................................................... Classical Pathway
CUB........................................................................... C1r/s, Uegf, Bone,
DAMP........................................................................... Damage Associated Molecular Pattern
DNA........................................................................... Deoxy-ribonucleic Acid
E. col........................................................................ Escherichia coli
ECN........................................................................... European Complement Network
EF............................................................................... Elongation Factor
EHEC................................................................. Enterohemorrhagic Escherichia coli
EHEC-associated Hemolytic Uremic Syndrome
ELISA........................................................................ Enzyme linked Immuno-Sorbent Assay
Esp.............................................................................. Escherichia coli-Secreted Proteins
ER............................................................................... Endoplasmic Reticulum
ERAD................................................................. Endoplasmic Reticulum associated-Protein Degradation
ERK.............................................................................. Extracellular Receptor Kinase
fB............................................................................... Factor B
Fc............................................................................... Fragment crystallizable
fD............................................................................... Factor D
fH............................................................................... Factor H
fi................................................................................ Factor I
GB3............................................................................ Globotriaosylceramide 3
GB4............................................................................ Globotriaosylceramide 4
HCl......................................................Hydrochloric acid
HSPC.............................................Hematopoietic Stem/Progenitor Cell
ICS..................................................International Complement Society
IEC..................................................Intestinal Epithelial Cells
IFN..................................................Interferon
Ig....................................................Immunoglobulin
IL........................................................Interleukin
kDa..................................................kilo-Dalton
LEE..................................................Locus of Enterocyte Effacement
LP......................................................Lectin Pathway
LPS...................................................Lipo-polysaccharide
MAC..............................................Membrane Attack Complex
MAP....................................................Membrane associated-Protein
MAP-1.................................................Mannose Binding Lectin/ficolin-associated protein-1
rMAP-1.............................................recombinant Mannose Binding Lectin/ficolin-associated protein-1
MASP-1.............................................Mannose Binding Lectin/ficolin-associated serine protease-1
rMASP-1............................................recombinant Mannose Binding Lectin/ficolin-associated serine protease-1
MASP-2.............................................Mannose Binding Lectin/ficolin-associated serine protease-2
MASP-3.............................................Mannose Binding Lectin/ficolin-associated serine protease-3
rMASP-3...........................................recombinant Mannose Binding Lectin/ficolin-associated serine protease-3
MASP-HC1/3.......................................Mannose Binding Lectin/ficolin-associated serine protease-Heavy Chain1/3
MBL.....................................................Mannose Binding Lectin
mRNA................................................messenger RNA
NK.....................................................Natural Killer
P........................................................Properdin
PAI.....................................................Pathogenecity Island
PAMP...............................................Pathogen-associated Molecular Patterns
PCR........................................................Polymerase Chain Reaction
PGE2..................................................Prostaglandin E-2
PMSF....................................................Phenylmethylsulfonil Fluoride
PRM....................................................Pattern Recognition Molecule
RNA.....................................................Ribosomal RNA
tRNA..................................................ribosomal ribonucleic Acid
RT-qPCR.........................................Reverse Transcriptase-quantitative Polymerase Chain Reaction
SCR...................................................Short Consensus Repeat
SDS-PAGE.......................................Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SLE.....................................................Systemic Lupus Erythematosus
sMAP..................................................small Mannose Binding Lectin/ficolin-associated protein
STEC..............................................Shiga toxin producing Escherichia coli
Stx....................................................Shiga toxin
Stx2a (BO) ..............................................................................................Shiga toxin (Bologna)
TBS ........................................................................................................Tris-buffered Saline
TBST .....................................................................................................Tris-buffered Saline Tween
TCC ........................................................................................................Terminal Complement Complex
Tir .............................................................................................................Translocated Intimin Receptor
TMA ........................................................................................................Thrombotic Microangiopathy
TNF ........................................................................................................Tumor Necrosis Factor
TTSS .......................................................................................................Type Three Secretory System
VTEC .....................................................................................................Verocytotoxin producing *Escherichia coli*
ABSTRACT

Hemolytic Uremic Syndrome (eHUS) is a severe human disease caused by Enterohemorrhagic Escherichia coli (EHEC). Complement, a component of innate immunity, is believed to play a role in eHUS pathogenesis. The primary EHEC virulence factor, Shiga toxin 2a (Stx2a), activates complement via the alternative pathway (AP) and also binds to factor H [1]. This thesis decodes some of the previously unexplored roles of complement in disease progression.

Discrepant results from an EHEC laboratory in Bologna, Italy, using a different purification protocol than in Innsbruck laboratory (designated as Stx2a throughout the thesis) yielded a particular variety of Stx2a (designated as Stx2a (BO)). This led to a concurrent study (Project I) that compared Stx2a to Stx2a (BO) in their biological properties. Therefore, the experiments in the other two parallel projects, have only been performed using Stx2a and would further require comparative analyses using Stx2a (BO) as well. Project II explores the interaction of Stx2a with pivotal complement proteins C3 and C5. Given the recent affirmation of intracellular complement, Project II also focused on investigating the intracellular synthesis of AP complement components upon stimulation with Stx2a, in various human cell lines. Project III traverses the possible role of lectin pathway (LP) in eHUS pathogenesis by investigating the interaction of Stx2a with the serine proteases (MASP-1 and -3), exclusive to the LP.

Data from the first project suggest that Stx2a and Stx2a (BO) differ in their A-subunit structure leading to previously obtained controversial results and thus assert their critical re-evaluation. The second project reports the intracellular expression of C3 and C5 in gut as well as kidney cells upon stimulation with Stx2a and their binding to Stx2a, corroborating the involvement of complement in eHUS. The third project proclaims that Stx2a binds not only to MASP-1, but more profoundly to MASP-3, proposing a possible involvement of LP in eHUS.
1. INTRODUCTION

1.1 Hemolytic Uremic Syndrome

Hemolytic Uremic Syndrome (HUS) is characterized by clinical manifestations indicating a simultaneous development of non-immune hemolytic anemia, thrombocytopenia and acute kidney failure. It leads to significant morbidity and mortality in the acute phase, where the latter is reported to be between 3% and 5% and is nearly always associated with severe extra-renal disease including severe central nervous system involvement [2]. The two forms of HUS are the diarrhea-positive ‘typical’ HUS associated with infection by Enterohemorrhagic Escherichia coli (EHEC) also called eHUS, and the diarrhea-negative ‘atypical’ HUS viz. aHUS, associated with genetic mutations of certain complement factors or autoantibodies, or lesser common causes including other infections, malignancies, drugs, transplantation, pregnancy, malignant hypertension or other genetic causes (i.e. not affecting the complement system) [3]. Patient investigation should be directed towards comprehensively defining the etiology as the clinical presentation and renal pathology may appear similar regardless the primary cause. Treatment strategies may differ based on the underlying disease pathogenesis. Antibody treatment is controversial, as at least some antibiotics may increase the risk of HUS [4]. Therefore the prognosis of HUS is largely based on its underlying pathology and the scope of appropriate treatment.

1.1.1 Enterohemorrhagic Escherichia coli associated Hemolytic Uremic Syndrome

eHUS is the most frequent cause of acute renal failure in children [5]. Following the initial description of HUS by Swiss physicians in 1955 [6], typical HUS was linked to infection with Shiga toxin (Stx) producing EHEC in early 1980s by Karmali et al. [2, 7-10]. They showed that patients with HUS that was preceded by diarrhea contained E. coli strains in their stools that
produced a toxin which caused irreversible cytotoxic damage to cultured Vero cells obtained from kidney of African green monkey. Thus these bacteria have also been alternatively named as Verotoxin producing *E. coli* (VTEC) or also Shiga toxin producing *E.coli* (STEC). Soon after, O’Brien and LaVeck demonstrated that this toxin associated with HUS, was structurally and antigenically closely related to Stx of *Shigella dysenteriae* type 1 [8]. Gastrointestinal infection with EHEC will develop into eHUS within 2-12 days after the occurrence of diarrhea and may also manifest as hemorrhagic colitis with bloody diarrhea in 15% of the cases [11]. Young children and the elderly are more prone to develop the disease in single cases [11, 12], although there have been reports of eHUS development in middle aged women during the infamous German outbreak of EHEC in 2011 [13]. Although the pathophysiology of eHUS is beginning to be understood, there remains a huge scope of research to be performed for understanding the precise mechanism of cell-organ injury and the outcome of it for superior and specific therapy.

1.1.2 Atypical Hemolytic Uremic Syndrome

aHUS is an ultra-rare disease with an estimated incidence that is most probably between 0.5 and 2 per million [14, 15]. It is associated with an underlying dysregulation of the alternative pathway (AP) of the complement system (1.5.3) usually associated with genetic or acquired defect. The complement abnormality may be a mutation, genetic rearrangement or deletion in a gene encoding a complement factor, or the presence of a homozygous complement gene haplotype or of an autoantibody to complement regulator factor H (fH) [3]. In a number of aHUS patients, an infection (often an upper respiratory or gastrointestinal tract infection) may precede the clinical triad typical for thrombotic microangiopathies (TMA) [16, 17]. Patients who do not develop end-stage renal failure during the first episode tend to relapse with acute symptoms, and the disease may affect several members of the same family [18]. The disease may recur after transplantation.
Plasma exchange combined with immunosuppression as a preliminary therapy [19, 20] and treatment with Eculizumab, complement C5 blocking drug [21-23] have proved to be efficient in therapy.

1.2 Enterohemorrhagic *Escherichia coli*

1.2.1 Serotypes

Over 380 different VTEC serotypes have been isolated from humans and animals, but only a small number of serotypes are linked to human disease [24]. *E. coli* serotype O157:H7 is the prototype and the most common cause of eHUS [25-28] and holds potential for large outbreaks as previously reported [29]. With extensive epidemiological studies, other serotypes such as O26, O111, O103 and O145 have also been described [30, 31] in Europe and elsewhere. Such non-O157:H7 serotypes are also known to cause HUS with similar frequency as O157 strains but show a lower frequency of dysentery [32]. In a prospective study of 394 children with eHUS from Germany and Austria, Gerber and group showed that 43% of the stool samples from these patients yielded serotypes other than O157:H7, including EHEC O26:H11/H− (15%), sorbitol-fermenting (SF) O157:H− (10%), O145:H28/H− (9%), O103:H2/H− (3%), and O111:H8/H− (3%) [33, 34]. The outrageous German outbreak in May 2011 exposed the notorious serotype O104:H4 over the course of three months with 3800 reported cases wherein 800 individuals developed HUS resulting into 54 fatalities [34, 35].

1.2.2 Source and Transmission

Cattle are natural reservoir of EHEC, and approximately 75% of EHEC outbreaks are associated with consumption of bovine-derived products. Despite the source, EHEC colonization in adult ruminants is asymptomatic unlike in humans [36, 37] mainly due to the lack of binding capacity
of the Stx binding globotriaosylceramide 3 (GB3) receptor on their vascular endothelium [38]. This insensitivity in cattle coupled with selective colonization in recto-anal junction unlike the colon as in humans [39] may serve to persist and transmit this pathogen.

EHEC is transmitted from cattle to humans by fecal-oral route through contamination of meat, dairy, farm vegetables, unpasteurized juices and milk and contaminated water or through human interactions of infected individuals [24]. Fecal shedding can harbor viable EHEC for more than seven weeks and up to six months posing long term environmental persistence with increased risk of new infections [40-42]. A small proportion of positive animals, called “super shedders”, excrete more EHEC than others and can account for over 95% of all EHEC shed [43, 44].

1.2.3 Virulence Factors

1.2.3.1 Shiga toxin family

Shiga toxin as detailed in 1.3, is the primary virulence factor associated with eHUS [5, 45] and encoded as two immunologically distinct toxin groups (Table 1.), Stx1 and Stx2, the latter is most commonly associated with clinical isolates of EHEC [29]. Although Stx1a and Stx2a are clinically most relevant subtypes for humans [29], Stx2a is more potent than Stx1a [46] and is more often expressed in affected human isolates.

1.2.3.2 Locus of Enterocyte Effacement (LEE)

LEE is present on a pathogenicity island (PAI) of EHEC that encodes factors mediating A/E (attaching and effacing) lesions [47, 48]. Additionally, LEE also encodes several other virulence factors like the LEE 1, 2 and 3 encodes the Type III Secretion System (TTSS) (1.2.3.3); LEE 4 encodes the *E. coli* secreted proteins (1.2.3.4) EspA, EspB, EspD and EspF [49]. A further operon contains Translocated Intimin Receptor (Tir) (1.2.3.6), its chaperone CesT and intimin
LEE enables bacterial colonization [51] and the Ler locus regulates its several virulence factors [52]. Although quorum sensing, a process by which bacteria regulates their gene expression in response to cell density via auto-inducers, has been shown to be involved in the expression of LEE in EHEC, the low infectious dose of EHEC implies the detection of auto-inducers by commensal *E. coli* to initiate expression of their virulence factors [47].

### 1.2.3.3 The Type III Secretion System (TTSS)

When A/E pathogen enters the host’s gastrointestinal tract, by contact-mediated mechanism with the host, LEE gene is expressed leading to the assembly of TTSS. TTSS then mediates the secretion of specific proteins (EspA, EspB, EspD and Tir) which are essential for the supervision of signal transduction pathways and formation of A/E lesions [49, 53]. In EHEC, TTSS is mainly required for the bacterium’s persistence in the terminal gastrointestinal tract [50].

### 1.2.3.4 *E. coli*-Secreted Proteins (Esp)

Bacterial secreted Esp proteins and TSTT are conserved in EHEC and trigger host cell signal transduction pathways and cytoskeletal rearrangements to mediate intimate bacterial adherence to epithelial cell surfaces *in vitro*. At least 8 Esps have been identified, six of which are encoded by LEE and three of which are secreted by TTSS in significant quantities [54]. In disease progression, secreted proteins EspA and EspB are needed to form A/E lesions *in vivo* (Abe 1998). EspP is known to contribute to pathogenesis of HUS [55, 56].

### 1.2.3.5 Mitochondrion-Associated Protein (MAP)

MAP is a multifunctional protein that targets the mitochondria for loss of membrane potential [47], induces signal that inhibits pedestal formation and appears to be important to guarantee maximal intestinal colonization [51].
1.2.3.6 Translocated Intimin Receptor (Tir)

Tir is secreted by the TTSS into the host membrane [57] and functions as a receptor for bacterial attachment factor, intimin [57, 58]. Tir anchors intimin to host cell actin and other cytoskeletal proteins mediating bacterial pedestal formation, a role which has been shown to be essential for *E. coli* O157:H7’s intestinal colonization in calves [59].

1.2.3.7 Intimin

Intimin facilitates the formation of A/E lesions by binding to Tir and eliciting cytoskeletal rearrangement within the host cell, leading to actin rich-pedestal formation beneath adherent bacteria [60]. At least 17 types of intimin have been identified and intimin-\(\gamma\) is specifically associated with EHEC O157:H7 indicating its tissue specific target in EHEC hosts [49].

1.2.3.8 pO157 Plasmid

The plasmid pO157 is present in almost all EHEC isolates that encode for EHEC hemolysin, a pore forming toxin. This toxin acts on hemoglobin to release free iron that is taken up by the bacteria and thus might contribute to its pathogenesis. Together with Stx gene and intimin, the entero-hemolysin acts as a marker of virulence for most EHEC strains [61]. Another virulence gene, *toxB* present in all O157:H7 isolates is encoded by this plasmid and may influence the LEE encoded TTSS proteins [62].

1.2.3.9 Lipopolysaccharide (LPS)

LPS, an endotoxin, has been suggested to have a synergistic effect with Stx in pathogenesis [35]. It has been reported that compared to healthy volunteers, serum from convalescent patients has significantly higher levels of IgA against O157 LPS. In calves and adult cattle, the serum
antibody response to O157 LPS lasts more than five months [63]. However this immune response is unable to provide protection from reinfection with homologous EHEC strains.

1.2.3.10 EHEC Hemolysin

Hemolysin digests hemoglobin to provide a source of iron to EHEC and is a marker of virulence of most of the O157 and non O157 human isolates [61].

1.2.4 Pathogenesis of EHEC

The infective dose of EHEC O157:H7 required to cause HUS is <50 bacteria and in case of non-O157 strains is even lower with just 10 bacteria per host required to cause potential outbreaks [64]. Upon ingestion, incubation period ranges from 1 to 9 days with diarrhea lasting for 3 to 7.5 days [65]. The bacteria localize in the intestine and initially attaches to the Peyer’s patch of the colon [66]. Mediated by its virulence factors, the bacteria secrete Stx, LPS and other bacterial components into the intestinal lumen and intestinal epithelial and endothelial cells leading to intestinal injury and colitis with bloody diarrhea [67-69], activating inflammatory response. Stx and possibly other virulence factors gain access into the circulation by unknown mechanisms, although binding to monocytes, polymorphonuclear cells, platelets and neutrophils have been suggested [70-74]. Stx binds to GB3 receptors present in microvasculature of kidney, brain and other GB3-endowed organs and induce stimulatory and cytotoxic response causing cell death and organ injury. Stx is thus able to stimulate cells to release cytokines as well as induce cytotoxic cell death by inhibiting protein synthesis [75] or apoptosis [76]. Activated platelets in damaged areas may bind to sub-endothelium and unbound platelet aggregates are removed by reticulo-endothelial cell system thus possibly leading to thrombocytopenia [77]. Glomerular thrombi and tubular epithelial damage [78] will affect glomerular filtration and cause renal failure.
1.3 Shiga toxin

1.3.1 Typing and nomenclature

Shiga toxin from EHEC strains can be broadly classified into two groups Stx1 and Stx2 (now Stx1a and Stx2a), to differentiate them for other toxin subtypes [79], each of which contains the major Stx type and an increasing number of variants as indicated in Table 1.

Table 1: Prototype toxins and strains that produce those toxins [80].

<table>
<thead>
<tr>
<th>Toxin type(s)</th>
<th>Prototype strain used for determination of Stx subtype</th>
<th>Linked with serious human disease; difference(s) from prototype toxin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stx (prototype)</td>
<td>3818T</td>
<td>Yes; N/A</td>
</tr>
<tr>
<td>Stx1a (prototype)</td>
<td>EDL933 (produces Stx1a and Stx2a)</td>
<td>Yes; N/A</td>
</tr>
<tr>
<td>Stx1b</td>
<td>DG131/3</td>
<td>No; immunologically distinct</td>
</tr>
<tr>
<td>Stx1c</td>
<td>MHI813</td>
<td>No; immunologically distinct</td>
</tr>
<tr>
<td>Stx2b (prototype)</td>
<td>EDL933 (produces Stx1a and Stx2a)</td>
<td>Yes; N/A</td>
</tr>
<tr>
<td>Stx2b (originally named VT-2d or Stx2d)</td>
<td>EH250</td>
<td>No; the B subunit gene was not detected by methods used to detect other sx2 B subunit genes</td>
</tr>
<tr>
<td>Stx2c</td>
<td>031</td>
<td>Yes; less toxic to Vero cells and mice</td>
</tr>
<tr>
<td>Stx2d (Stx2dact)</td>
<td>C165-02</td>
<td>Yes; more toxic after incubation with elastase, less toxic to Vero cells</td>
</tr>
<tr>
<td>Stx2e</td>
<td>S1191</td>
<td>No; binds to Gb4, associated with disease in pigs</td>
</tr>
<tr>
<td>Stx2f</td>
<td>T4/97</td>
<td>No; originally isolated in EHEC from pigeons; immunologically distinct</td>
</tr>
<tr>
<td>Stx2g</td>
<td>7v</td>
<td>No; the stx2g gene is not amplified by primers specific for stx2a</td>
</tr>
</tbody>
</table>

Abbreviations: Stx, Shiga toxin; VT, Vero toxin; GB4, globotriaosylceramide 4; EHEC, Enterohemorrhagic Escherichia coli.
1.3.2 Structure

The Stx family share a common AB5 toxin subunit structure wherein the A subunit is approximately 32 kDa and the B subunits are 7.7 kDa each. The A subunit is proteolytically cleaved at a trypsin sensitive region into an enzymatically active 28 kDa A1 subunit and a 4 kDa A2 peptide, the C-termini of which harbors the central pore of pentameric B subunit [81]. The A1-A2 subunits are held together by a disulfide bridge and are responsible for the translocation from endoplasmic reticulum (ER) to the cytosol (Fig. 1). The crystal structure of the Stx1a B subunit pentamer, bound with its receptor, Gb3, shows that each B monomer contains three distinct binding sites for the glycan component of Gb3 [82]. Stx1a and Stx2a have only 56% amino acid similarity [83] and are antigenically distinct [83-85].

Figure 1: Representative figure of the Stx structure. A subunit is subdivided into A1 and A2 and is connected by a disulfide bridge, shown above the protease (furin) sensitive site which overlaps a region important for translocation from the ER to the cytosol. A1 is enzymatically active and is connected to the pentameric B subunit form a central pore which harbors the C-termini of the A subunit. Abbreviations: ER, endoplasmic reticulum. [80]

1.3.3 Stx receptor and cell binding

GB3, a molecule comprised of a lipid or a ceramide component and a trisaccharide, is the primary receptor of Stxs, showcasing high affinity for the molecule due to the presence of at least two and up to three GB3 binding sites per B monomer. In addition, reports suggest that Stx1a and
Stx2a can also weakly bind to GB4 [86]. Individuals with excessive GB3 expression, a condition known as Fabry’s disease, manifest kidney disease among other symptoms.

Gb3 receptors and Stx’s susceptibility is related and have been reported in primary human renal [87] and cerebral microvascular endothelial cells [88, 89] and on neurons within the mouse central nervous system (CNS). Previously, GB3 has been thought to be absent from the colonic tissues but recent reports suggest the expression of GB4 and a smaller fraction of population expressing GB3, in human colonic epithelial HCT-8 cells and that Stx1a and Stx2a do in fact bind to colonic epithelia in fresh tissue sections and to HCT-8 [90]. In addition, both Stx1a and Stx2a cross polarized colonic epithelial T84 cells which effectively lack the GB3 receptor, without disrupting the monolayer [91, 92]. Therefore it can be elucidated that GB3 minus cells may also demonstrate systemic delivery of the toxin. However, GB3 levels on intestinal tissues could have been underestimated as some studies suggest the stimulating role of butyrate on GB3 expression and in turn its sensitivity towards Stx [80, 90, 93] and that the expression is exquisitely sensitive to the absence of that gut metabolite [94].

1.3.4 Stx trafficking in cells and mechanism of action

Stx-Receptor complex enters the cell mainly via clathrin-dependent endocytosis [95] although other mechanisms also exist [96]. The toxin undergoes retrograde transport via early endosomes to traffic into the golgi apparatus [97], where the A subunit is cleaved by furin or other proteases into A1 and A2, but still held together by a disulfide bridge maintaining the AB5 structure. This disulfide bond is reduced once the toxin enters ER, and only the enzymatically active A1 subunit enters the cytosol by subverting the ER-associated protein degradation (ERAD) pathway, to reach the target ribosome [98]. The A1 chain possesses ribosomal ribonucleic acid (rRNA) N-glycosidase activity and as a result removes an adenine from the alpha-sarcin loop in the 28S
ribosomal unit which can no longer associate with the elongation factor 1 (EF1) resulting into protein synthesis inhibition [2]. This has been further supported by studies showing lack of cytotoxicity capability of the active site mutants of Stx. Furthermore, it has been reported that a single molecule of Stx is perhaps sufficient to kill a cell [98].

### 1.3.5 Stx2a association with eHUS

Although the clinical outcome of an infection involving EHEC mainly depends on the type of Stx harbored by the strain, epidemiological data from human diseases indicate Stx2a expressing infectious strains to have a stronger association than Stx1a alone [99-101]. This is further supported by studies indicating that renal microvascular endothelial cells obtained from human glomeruli are about 1000-fold more sensitive to Stx2a than Stx1a [102]. In addition, it has been hypothesized that Stx2a is more toxic than Stx1a from the gut and not just when injected intraperitoneally [45]. The largest outbreak of HUS in Germany in 2011 underscored the strong alliance between Stx2a and the disease.

### 1.4 Immune system

The immune system is an interactive and complex network of lymphoid organs, cells, humoral factors and cytokines that protects higher organisms from diseases. It can detect a variety of pathogens including bacteria, viruses, fungi and parasites as well as macromolecules, such as proteins, polysaccharides, toxins and small chemicals in a very composite order. The degree of disease progression depends on both the pathogenicity of the organism (the virulence factors at its disposal) and the integrity of host defense mechanisms. The vital role of this multiplex system in host defense is best illustrated when it goes wrong, under-activity resulting in the severe infections and tumors of immunodeficiency, over-activity in allergic and autoimmune disease. The detection of infectious agents is very complicated, due to the fact that many pathogens
developed immune-evasion strategies to avoid detection by the immune system [103]. Determined by the speed and specificity of the reaction, this is divided in two parts: the innate and the adaptive (acquired) immunity; although in practice there is much interaction between them.

1.4.1 Innate Immunity

Innate immunity (also called natural or native immunity) provides the early line of defense against microbes. The strength of this system is to be able to take action very quickly and is not specialized for specific pathogens, although it can distinguish self from non-self. The innate immune system of vertebrates uses three strategies of immune recognition that can be described in terms of recognition of “microbial nonself,” recognition of “missing self (markers unique to host and absent from microorganisms),” and recognition of “induced or altered self” [104]. Innate immunity is the phylogenetically the oldest form of host defense, therefore with in-vertebrates; it is the main defense system against infections.

The main elements of the innate immunity are as follows:

- physical and chemical barriers like epithelia and antimicrobial substances produced at epithelial surfaces
- phagocytic cells (neutrophils and macrophages) as well as natural killer cells (NK cells)
- blood proteins including complement proteins and mediators of inflammation
- cytokines that regulate many activities of the cells of innate immunity

The interactions allowing the innate response to eradicate infectious agents, such as phagocytosis, opsonisation, and complement-mediated lysis, require exposure to the surface of the microorganisms, thus largely confined to eradicating extracellular organisms, mostly bacteria.
1.4.2 Adaptive Immunity

Adaptive or acquired immunity is the hallmark of the immune system of higher organisms and consists of antigen-specific reactions through T and B lymphocytes. It is precise in recognizing even closely related molecules in contrast to the innate immunity and can thus take several days or weeks to develop. This response has memory leading to a much more vigorous response upon re-exposure. The unique components of adaptive immunity are lymphocytes and their secreted products, such as antibodies. This system drives targeted immune response in two stages:

- In the first stage, the antigen is presented to and recognized by antigen-specific T or B cell leading to cell priming, activation and differentiation.
- In the second stage, the effector response takes place either due to activated T cells leaving the lymphoid tissue and reaching the disease site or due to the release of antibody from activated plasma B cells into blood and tissue fluids and thus reaches the infection site [105].

Together, innate and adaptive immune responses make an integrated system of host defense in which numerous cells and molecules function in collaboration. Many pathogenic microbes have evolved to resist innate immunity which is the initial line of defense, and their elimination requires more precise and powerful mechanisms of adaptive immunity.

1.5 The Complement System

Complement is a rapid and efficient immune surveillance system directed to intricately discriminate among healthy host tissue, apoptotic cells and foreign intruders and eliminate accordingly orchestrating immune response and inflammatory signals, contributing substantially to homeostasis. Besides its obvious role in eliminating microbes, it also participates in diverse
processes such as synapse maturation, clearance of immune complexes, and mobilization of hematopoietic stem-progenitor cells (HSPCs), tissue regeneration and lipid metabolism [106].

The complement system is composed of up to 50 proteins either found in fluid phase in blood or are membrane associated [107] as inactive precursors. It’s sequential and tightly regulated activation leads to a defined and well-coordinated cascade of enzymatic reactions resulting in a plethora of physiological responses directed towards robust and rapid elimination of pathogens through recognition, opsonization and lysis. Additionally, some evidences have suggested that complement also plays an important role in adaptive immunity involving T and B cells [108] thus maintaining immunologic memory and preventing pathogen re-invasion. Complement can also take actions against healthy cells if not properly controlled.

The first update since 1981 of the nomenclature used in the field of complement was completed by the Complement Nomenclature Committee established under the auspices of the International Complement Society (ICS) and by the boards of the ICS and the European Complement Network (ECN) and reviewed as ’Complement Nomenclature’ in 2014 [109]. Briefly, the classical pathway is numbered from C1-C9 without respecting the activation sequence. The alternative pathway is addressed by its components named as ‘factors’ and denoted by letters such as factor H (fH), factor B (fB), factor D (fD), factor I (fI) and another factor called factor (P) or properdin. Similarly, lectin pathway (LP) components still being discovered and characterized likewise many other components of complement, have distinct nomenclature as described further.

The pathways act as a result of a cascade of enzymatic cleavage of inactive complement proteins. Small, liberated fragments are denoted with an additional "a" (e.g. C3a and C5a) and larger fragments are denoted by a "b" (e.g. C3b), usually being the active component. The only notable
exception is C2a which is a large, active fragment. Upon further inactivation of e.g. C3b, small fragments are generated which are then named with "c" or "d" (C3b → C3c and C3d) [110].

1.5.1 Complement activation and amplification

Complement activation can occur through three different initiation pathways: Classical (CP), Lectin (LP) and Alternative Pathways (AP); depending on the trigger to produce an anticipated result in immune surveillance. All the three pathways converge into a single terminal cascade in an orderly fashion.

1.5.1.1 Classical pathway

The classical pathway is antibody-dependent and is triggered with the recognition of distinct structure on pathogens called pathogen-associated molecular patterns (PAMPs) or apoptotic cells called damage-associated molecular patterns (DAMPs); by the circulating antibodies IgG or IgM. After binding, a rearrangement of the crystallization fragment (Fc)-confirmation enables C1q to bind on to the Fc-region of the antibody. IgM is pentameric and even one molecule is sufficient to activate complement whereas for monomeric IgG, at least two molecules are required. As a part of the C1 complex (C1qr2s2), proteases C1r and C1s are activated upon surface binding of C1q [111, 112]. C1s subsequently cleaves C4 into anaphylatoxins C4a and C4b, the latter exposing a previously hidden thioester and leading to covalent opsonozation of C4b on the pathogen’s surface and in turn activating C2 by cleaving it into C2a and C2b, thus generating the classical pathway C3 convertase (C4b2b). This convertase can then cleave C3 into anaphylatoxin C3a which diffuses away and C3b which binds to C3 convertase to form C5 convertase (C4b2b3b), which proceeds to the terminal cascade which is common for all the three pathways. Along with that, C3b also opsonizes pathogens and therefore promotes phagocytosis.
1.5.1.2 Lectin pathway

The lectin pathway is initiated when pattern recognition molecules (PRMs) such as mannose-binding lectin (MBL), collectin-10 (CL-10 or CL-L1), collectin-11 (CL-11 or CL-K1), ficolin-1 (M-ficolin), ficolin-2 (L-ficolin), and ficolin-3 (H-ficolin or Hakata antigen); bind to PAMPs or DAMPs [113]. Their effector functions are mediated via a set of serine proteases (MASP-1, MASP-2, and MASP-3) originally named after their association with MBL [114], which are found in complexes with the MASPs. Activation of LP leads to cleavage of C4 and C2 and the formation of a C3 convertase (C4b2a), similar to the CP. Only MASP-2 can cleave both C4 and C2, generating the same C3 convertase. Whereas MASP-1 can cleave C2 but not C4, it can supplement the LP response once initiated [115], thereby increasing the efficiency of convertase formation.

Recently, another collectin named collectin-12 (CL-12 or CL-P1) has, in its soluble form, been shown to mediate complement activation [116]. This appears not to involve interaction with the MASPs, but rather with molecules from the alternative pathway. It is suspected that the major role of this pathway takes place during early childhood and in particular during the translational period from the passive immunity provided by the mother’s antibodies to the development of the body’s own mature immunity [110].

1.5.1.2.1 Mannose Binding Lectin (MBL)-associated Serine Protease

MASP1 and MASP2 encode five MASP members by differential splicing. MASP1 encodes for alternative splicing products MASP-1, MASP-3 which are enzymatic and the non-enzymatic MAP-1 whereas MASP2 codes for enzymatic MASP-2 and the non-enzymatic sMAP. MASPs share a similar structure composed of a heavy chain which interacts with the PRMs of the LP and a light chain which comprises of the serine protease domains containing a cleavage site that
separates the heavy and the light chain upon MASP activation. These two chains are joined together by an inter-chain cysteine bridge [113]. The heavy chain is composed from the N-terminus of two CUB domains composed of C1r/s, Uegf, Bone morphogenetic protein separated by an EGF (epidermal growth factor) segment and followed by two CCP (complement control protein) domains. While sMAP contains the first CUB and the EGF domain only, MAP-1 carries most of the heavy chain except for the last CCP domain (Fig. 2) [113]. In a calcium dependent manner, MASPs assemble into homodimers for their interaction with the PRMs.

**Figure 2: Domain and genomic organization of the MASPs.** Domain and exon structure of MASP1 and MASP2 pre-mRNA transcript and alternative splicing products MASP-1, MASP-3, MAP-1, MASP-2, and sMAP. The MASPs are composed of an N-terminal heavy chain (H-chain) and a light chain (L-chain) containing the serine protease domain. The truncated, non-enzymatic MAP-1 carries most of the heavy chain except for CCP2, while sMAP only contains CUB1 and the EGF domain. Abbreviation: MASP, mannose-binding lectin (MBL)/ficolin-associated serine protease; MAP, mannose-binding lectin (MBL)/ficolin-associated protein; CUB, complement C1r/C1s, Uegf, Bmp1; EGF, epidermal growth factor; CCP, complement control protein; PRM, pattern recognition molecule. [113]

### 1.5.1.3 The Alternative Pathway

The alternative pathway activation proceeds through antibody-independent binding of danger signals such as bacteria, yeast, virus infected cells but also C-reactive protein, cobra venom factor, polysaccharides and DAMPs [117, 118]. The tick-over segment keeps complement alert
and allows constant probing of cells [119, 120]. In its native form, C3, the central molecule of the alternative pathway, has few ligands and is relatively inert. However, a small fraction of the C3 molecules are spontaneously hydrolyzed to C3$_{H2O}$, exposing new binding sites; thus constant control by regulatory proteins is required. The fB protease binds hydrolyzed C3 and is cleaved by plasmatic protease fD into Ba and Bb, generating an initial, mainly solvent-based C3 convertase (C3$_{H2O}$Bb) that activates complement by cleaving C3 into its anaphylatoxin C3a and active C3b. Cleavage of C3 exposes a reactive short-lived thioester moiety in C3b, which covalently attaches to amine and carbohydrate groups on the target surface.

Binding of the protein properdin stabilizes the fragment extending the half-life 10 fold. Recently, new data have appeared showing that properdin has two clearly distinct functions: in addition to enhancing AP amplification by stabilization of the C3bBb complex, properdin is a recognition factor directly initiating AP activation [121-124]. Thus, in addition to being an amplification system for all three initial pathways, AP should be regarded as a recognition pathway through properdin, like CP through C1q and LP through MBL and ficolins [121].

This initial tagging is quickly amplified on foreign cells but is immediately regulated on human cells by fH. The cleaved C3b is capable of creating a new C3 convertase with the aid of fB and fD creating an amplification loops, essential for all the three pathways. Binding of C3b to C3 convertase now creates C5 convertase (C3$_{H2O}$BbP3b) that contain an additional C3b molecule and shift the substrate specificity from C3 to C5, which in turn initiates the terminal cascade.

Despite its name, the alternative pathway might account for up to 80–90% of total complement activation, even when initially triggered by the classical pathway or lectin pathway [121].
1.5.1.4 Complement C3

C3 is the central molecule of the complement system (Fig. 3) and supports activation of all the three pathways of complement, as described above. It is a 185 kDa plasma protein at a concentration of approximately 1.2 – 1.3mg/ml in humans. It consists of one $\alpha$-subunit (110 kDa) linked to a $\beta$-subunit (75 kDa) by disulfide bonds. C3 is an ancient molecule with its possible emergence at least 700 million years ago, long before the emergence of immunoglobulins and has been identified in echinoderms and tunicates [125]. It is now well established that C3 functions like a double edged sword, meaning, that it plays a vital role in immunity by promoting phagocytosis, supporting local inflammatory responses against pathogens and instructs adaptive immune response to select the appropriate antigens for a humoral response; whereas in contrast, its unregulated activation leads to host cell damage [125].

The main clinical manifestation of very rare primary C3 deficiency is childhood-onset of recurrent bacterial infections, mainly caused by gram-negative bacteria, such as *Neisseria meningitidis, Enterobacter aerogenes, Haemophilus influenzae,* and *Escherichia coli* although infections with gram-positive bacteria also occur [126]. Infections in the upper and lower respiratory tract, including pneumonia, episodes of sinusitis, tonsillitis and otitis, are the most frequent consequence of the C3 deficiency [126]. Approximately 26% of patients with C3 deficiency develop immune complex-mediated autoimmune diseases resembling systemic lupus erythematosus, and about 26% of patients develop mesangiocapillary or membranoproliferative glomerulonephritis, resulting in renal failure [127].

1.5.1.5 Complement C5

C5 is a plasma glycoprotein of 196 kDa composed of two disulfide-bound polypeptide chains (C5$\alpha$ and C5$\beta$, 115 and 75 kDa, respectively) and is synthesized as an intracellular single chain
Figure 3: Representation of the CP, LP and AP of complement activation, including regulatory molecules. The CP is triggered by binding of C1q to antibody-antigen complexes; the LP by binding of MBL to mannose residues, which activates MASP1 and MASP2 and the AP by spontaneous activation of C3. Activation of these pathways leads to the formation of the TCC/MAC-composed of C5b, C6, C7, C8 and many copies of C9—which results in cell lysis. Abbreviations: CP, classical pathway; LP, lectin pathway; AP, alternative pathway; CFH, factor H; CFI, factor I; DAF, decay accelerating factor; MAC, membrane attack complex; MASP, mannan-binding lectin serine protease; MBL, mannose-binding lectin; MCP, membrane cofactor protein; TAFIa, thrombin-activatable fibrinolysis inhibitor; THBD, thrombomodulin [128].
precursor of 1976 amino acids. Upon activation, the precursor form of C5 is cleaved into two proteolytic fragments: C5a and C5b. C5a is a potent anaphylatoxin that induces smooth muscle contraction, increases vascular permeability, degranulation of basophiles and mast cells and recruitment of lymphocyte to the site of infection. C5b, containing the binding site for complement C6, initiates the lytic pathway as described below. C5 proteolytic fragments have been implicated in damaging inflammatory processes as in sepsis and fetal injury as well as in antiphospholipid syndrome [129].

Dysfunction of the fifth component of complement (C5) was found to be the basis for the deficiency in phagocytosis-enhancing activity of serum present in a proband, her mother, and 15 other relatives [130]. Snyderman et al. in 1979 demonstrated that repeated disseminated gonococcal infection can be associated with C5 deficiency [131]. Another report in 1992 described three generation of individuals from same lineage having C5 deficiencies suffered recurrent meningitis and meningococcemia, as well as recurrent purulent otitis media [132]. One 13-year-old male with evident C5 deficiency had no increased susceptibility to infection [132].

1.5.2 Terminal Complement Complex (TCC)

Terminal Complement Complex (TCC) comprises the Membrane Attack Complex (MAC), assembled on a target membrane, and a similar complex formed in the fluid phase, sC5b-9, both marking the final stages of complement activation. In contrast to the three different upstream paths forming a C5 convertase, only the cleavage of C5 into anaphylatoxin C5a and the active C5b by C5 convertase represents an enzymatic step, while the rest of the cascade is solely an accretion of stable proteins [133]. After C5b associates with C6 in the circulation, the complex may stay there or become inserted into cell membranes, resulting into a hydrophilic complex followed by accretion of C7 which in turn leads to a conformational change by facilitating a
stable linkage due to exposure of lipophilic groups. Attachment with C8 leads to the binding of 10-15 units of C9 to form a lytic pore with a diameter of 10 angstrom. Assembly of the terminal complement complex (C5b–9n) on the membrane may lead to osmotic imbalance through the constant flow of ions, small molecules and water along their concentration gradient, resulting in the lysis of the target cell [133].

Although complement has now been discovered for more than a century ago, the precise mechanism of complement mediated cytotoxicity has been highly debated with two infamous vying hypothesis, one asserting the formation of ‘leaky patches’ caused due to the distortion of phospholipid bilayer by insertion of the polar domains of complement proteins [134] and the other affirming the alternative ‘pore’ model, that proposes the coming together of the polar surfaces of the individual complement components to form a hydrophilic channel through the membrane [135].

1.5.3 Complement Regulation

Soluble and membrane-bound complement regulators (Table 2) help to control complement attack and adjust its severity, propagation and endpoints to the cellular targets. Dysfunction of complement regulation and complement deficiencies are the underlying cause of the pathophysiology of many diseases including HUS, age-related macular regeneration, etc. [136].

1.5.3.1 Factor H

Factor H is found as one of the most abundant human plasma proteins with a concentration of 300 - 800μg/ml in plasma. It is a 150 kDa single chain glycoprotein consisting of 20 repetitive units, termed complement control proteins (CCP) or short consensus repeats (SCRs) which contain 60 amino acids each and are linked together by two disulfide bridges.
Table 2: Complement regulators and receptors for effector proteins [137].

<table>
<thead>
<tr>
<th>Regulator</th>
<th>Point of action</th>
<th>Ligands</th>
<th>Cell surface binding or expression</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Soluble regulators and effectors</strong>&lt;br&gt;Factor H</td>
<td>AP</td>
<td>C3b and C3d</td>
<td>Acquired to surface</td>
<td>Cofactor for factor I and acceleration of AP C3 convertase decay</td>
</tr>
<tr>
<td>FHL1</td>
<td>AP</td>
<td>C3b</td>
<td>Acquired to surface</td>
<td>Cofactor for factor I and acceleration of AP C3 convertase decay</td>
</tr>
<tr>
<td>Properdin</td>
<td>AP</td>
<td>C3</td>
<td>Binds to apoptotic surfaces</td>
<td>Stabilization of AP convertases</td>
</tr>
<tr>
<td>Carboxypeptidase N</td>
<td>CP and LP</td>
<td>C3a, C4a and C5a</td>
<td>NA</td>
<td>Inactivation of anaphylatoxins C3a and C5a</td>
</tr>
<tr>
<td>C4BP</td>
<td>CP and LP</td>
<td>C4</td>
<td>Acquired to surface</td>
<td>Cofactor for factor I and acceleration of CP C3 convertase decay</td>
</tr>
<tr>
<td>C1q</td>
<td>CP</td>
<td>IgG and IgM</td>
<td>Binds to apoptotic surfaces</td>
<td>Activation of the CP</td>
</tr>
<tr>
<td>C1INH</td>
<td>CP and LP</td>
<td>C1r, C1s and MASP-2</td>
<td>NA</td>
<td>Blocks serine protease; is a suicide substrate for C1r, C1s, MASP-2, coagulation factors and C3b</td>
</tr>
<tr>
<td>CFHR1</td>
<td>TP</td>
<td>C5 convertase and TCC</td>
<td>Acquired to surface</td>
<td>Inhibition of C5 convertase and TCC assembly</td>
</tr>
<tr>
<td>Clusterin</td>
<td>TP</td>
<td>C7, C8β, C9 and TCC</td>
<td>NA</td>
<td>Transport of cholesterol, HDL, APOA1 and lipids</td>
</tr>
<tr>
<td>VITRONECTIN</td>
<td>TP</td>
<td>C5b-7 and TCC</td>
<td>NA</td>
<td>Adhesion protein, fibronectin-mediated cell attachment and Arg-Gly-Asp site coagulation in immune defense against Streptococcus spp.</td>
</tr>
<tr>
<td><strong>Surface bound regulators and effectors</strong>&lt;br&gt;CR1</td>
<td>C3</td>
<td>C3b, iC3b, C4b and C1q</td>
<td>Many nucleated cells and erythrocytes, B cells, leukocytes, monocytes and follicular dendritic cells</td>
<td>Clearance of immune complexes, enhancement of phagocytosis and regulation of C3 breakdown</td>
</tr>
<tr>
<td>CR2</td>
<td>C3</td>
<td>C3dg, C3d and iC3b</td>
<td>B cells, T cells and follicular dendritic cells</td>
<td>Regulation of B cell function, B cell co-receptor and retention of C3d tagged immune complexes</td>
</tr>
<tr>
<td></td>
<td>C3</td>
<td>C3b</td>
<td>iC3b and factor H</td>
<td>Monocytes, macrophages, neutrophils, natural killer cells, eosinophils, myeloid cells, follicular dendritic cells, CD4+ T cells and CD8+ T cells</td>
</tr>
<tr>
<td>------------</td>
<td>-------</td>
<td>-----</td>
<td>------------------</td>
<td>--------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>CR3</td>
<td>C3</td>
<td>C3b</td>
<td>iC3b</td>
<td>Monocytes and macrophages</td>
</tr>
<tr>
<td>CR4</td>
<td>C3</td>
<td>C3b</td>
<td>iC3b</td>
<td>Macrophages</td>
</tr>
<tr>
<td>CRIg</td>
<td>C3</td>
<td>C3b</td>
<td>iC3b, C3b, C3c</td>
<td>All cells except erythrocytes</td>
</tr>
<tr>
<td>CD46/MCP</td>
<td>C3</td>
<td>C3b</td>
<td>iC3b</td>
<td>GPI anchor expression by most cell types, including erythrocytes, epithelial cells and endothelial cells</td>
</tr>
<tr>
<td>CD55/DAF</td>
<td>C3</td>
<td>C4b</td>
<td>C4b2b and C3bBb</td>
<td>GPI anchor expression by erythrocytes and most nucleated cells, including renal cells</td>
</tr>
<tr>
<td>CD59</td>
<td>TCC</td>
<td>C8</td>
<td>TCC</td>
<td></td>
</tr>
</tbody>
</table>

**Receptors for complement effector proteins**

<table>
<thead>
<tr>
<th>C3aR</th>
<th>C3</th>
<th>C3a</th>
<th>Neutrophils, monocytes, eosinophils, antigen-presenting cells, T cells, astrocytes, neurons and glial cells</th>
<th>Immune cell recruitment and inflammation</th>
</tr>
</thead>
<tbody>
<tr>
<td>C5aR</td>
<td>C5</td>
<td>C5a</td>
<td>Myeloid cells, monocytes, neutrophils, dendritic cells, antigen-presenting cells, T cells, endothelial cells and renal tubular cells</td>
<td>Immune cell recruitment and inflammation</td>
</tr>
<tr>
<td>C5L2</td>
<td>C5</td>
<td>C5a</td>
<td>Macrophages and neutrophils</td>
<td>Immune cell recruitment and inflammation as a decoy receptor</td>
</tr>
<tr>
<td>C1qR</td>
<td>CP</td>
<td>C1q</td>
<td>Monocytes and B cells</td>
<td>Phagocytosis and cell adhesion</td>
</tr>
<tr>
<td>SIGNR1</td>
<td>CP</td>
<td>C1q</td>
<td>Dendritic cells and microglial cells</td>
<td>Signaling, inflammation and phagocytosis</td>
</tr>
</tbody>
</table>

Abbreviations: APOA1, apolipoprotein A-1; C, complement component; C1INH, C1 inhibitor; C4BP, C4b-binding protein; C5L2, C5a receptor-like 2; CFHR1, complement factor H-related protein 1; CR, complement receptor; CRig, complement receptor of the immunoglobulin superfamily; DAF, complement decay-accelerating factor; FHL1, factor H-like protein 1; TCC, terminal complement cascade; GPI, glycosylphosphatidylinositol; HDL, high-density lipoprotein; MASP-2, mannan-binding lectin serine protease 2; MCP, membrane cofactor protein; NA, not applicable; SIGNR1, a mouse homologue of DC-SIGN, SIGN-related 1.
fH is the main fluid phase regulator and mainly acts on the C3 convertase of the alternative pathway. It has dual functions as follows:

- **Decay-accelerating activity:** fH accelerates the decay of C3 convertase by competitively dissociating Bb from the C3 convertase (C3bBb).
- **Cofactor for FI-mediated inactivation of C3b:** fH serves as a cofactor for the fI-mediated degradation of C3b. When fH binds to C3b, fI can then bind and cleave C3b, which generates iC3b (inactive C3b) [1].

fH has been shown to have a higher binding avidity for C3b bound to non-activators compared to C3b bound to activators, due to the presence of sialic acids or other negative charge clusters which are present on non-activators, such as human cells.

**1.6. Intracellular complement**

Complement proteins C1-C9, factors B, H and I, as well C4BP and C1INH have been known to be produced in the liver and delivered in the systemic blood circulation. However it has been long known that local complement production by monocytes, macrophages, including tissue resident macrophages and non-immune cells such as fibroblasts, epithelial cells, endothelial cells and even placental syncytiotrophoblasts contribute to the local extracellular pool to produce the full array of complement components (Table 3.) [138-141]. Thus complement activation takes place at different location and perhaps it is the location that dictates the function of the complement products. While serum driven complement is evidently extensively involved in its sentinel function of recognizing and removing invading pathogens and irregular self-components, autocrine-functioning complement directs adaptive T cell and antigen presenting cell (APC) responses and tonically active intracellular complement may sustain cell homeostasis [142].
Table 3: Local complement production by myeloid and non-myeloid cells [138, 141].

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Soluble complement components</th>
<th>Complement receptors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hepatocytes</td>
<td>C1r/s, C2-C9, C1NH, C4BP, fB, fH, fI, MBL, Ficolin-2, Ficolin-3, CL-10, CL-11, MASP-1,</td>
<td>C1qR, C3aR, C5aR1/2</td>
</tr>
<tr>
<td></td>
<td>MASP-2, MASP-3</td>
<td></td>
</tr>
<tr>
<td>T cells</td>
<td>C3, C5, P</td>
<td>CR1, CR2, CR4, C3aR, CD46, C5aR1/2</td>
</tr>
<tr>
<td>B cells</td>
<td>C3, C5, H, fI</td>
<td>C1qR, CR1, CR2, CR4, CD46, C3aR</td>
</tr>
<tr>
<td>Monocytes</td>
<td>C1q, C1r/s, C2-C9, C1NH, C4BP, fB, fD, fH, fI, P, MBL, Ficolin-1, CL-11, MASP-1, MASP-2,</td>
<td>C1qR, CR1, CR3, CR4, CD46, C3aR, C5aR1/2</td>
</tr>
<tr>
<td></td>
<td>MASP-3</td>
<td></td>
</tr>
<tr>
<td>Platelets</td>
<td>C1NH, fH, C5-C9</td>
<td>C1qR, CR1, CR4, C3aR, C5aR1/2</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>C3, C6, C7, P, Ficolin-1</td>
<td>C1qR, CR1, CR3, CR4, CD46, C3aR, C5aR1/2</td>
</tr>
<tr>
<td>Macrophages (location specific</td>
<td>C1q, C1r/s, C2-C9, C1NH, fB, fD, fH, fI, P, CL-11</td>
<td>C1qR, CR1, CR3, CR4, C3aR, C5aR1/2</td>
</tr>
<tr>
<td>variation)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fibroblasts</td>
<td>C1q, C1r/s, C2-C9, C1NH, fB, fH</td>
<td>C1qR, CD46</td>
</tr>
<tr>
<td>Endothelial cells</td>
<td>C1s, C2, C3, C1NH, fB, fH, fI, CL-11, CL-12</td>
<td>C1qR, CR1, C5aR1/2, CD46</td>
</tr>
<tr>
<td>Epithelial cells (location</td>
<td>C1q, C1r/s, C2-C5, C1NH, fB, fH, Ficolin-1, Ficolin-3</td>
<td>C1qR, CR3, C5aR1/2, CD46</td>
</tr>
<tr>
<td>specific variation)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Keratinocytes</td>
<td>C3, C1NH, fB, fH</td>
<td>CR1, CR2, C5aR1/2</td>
</tr>
<tr>
<td>Adipocytes</td>
<td>C1q, C1r/s, C3, fB, fD, fH, fI, P</td>
<td>CR1, C5aR1/2</td>
</tr>
<tr>
<td>Astrocytes</td>
<td>C1q, C1r/s, C2-C9, C1NH, fB, fD, fH, I</td>
<td>C1qR, CR1, CR2, C3aR, C5aR1/2</td>
</tr>
<tr>
<td>Pancreatic islets</td>
<td>C1q, C1s, C3-C7, fB, fH, fI, C4BP</td>
<td></td>
</tr>
<tr>
<td>Retinal cells</td>
<td>C1q, C1r/s, C2-C5, fB, fH, fI, MBL, MASP1, MASP2</td>
<td>CR1, CD46</td>
</tr>
<tr>
<td>Chondrocytes</td>
<td>C1q, C1r/s, C2-C4, C1NH</td>
<td></td>
</tr>
<tr>
<td>Myocytes</td>
<td>C3-C5, fB, fH, fI</td>
<td>CD46</td>
</tr>
</tbody>
</table>
It is thus becoming increasingly evident that apart from being this ancient defense mechanism mainly operative in the extracellular milieu, complement from the intracellular domains can link its activation to a wide array of possibilities including cellular metabolic processes, intracellular defense reactions and further to diverse adaptive immune responses. These paradigm shifts in the field have provided new platforms for research and better understanding of molecular pathways underlying the wide reaching effects of complement functions in immunity and beyond.
2. AIMS OF THE STUDY

This thesis consists of two major projects and an additional project, all aimed at decoding the role of complement in the pathogenesis of eHUS with special emphasis on Shiga toxin 2a (Stx2a), the primary virulence factor of EHEC. The titles are followed by a condensed scientific background leading up to the aim of the projects.

2.1 Project I: Variable biological properties of two different preparations of Shiga toxin 2a, yielding new insights into eHUS pathogenesis

2.2 Project II: Shiga toxin 2a binding to complement components C3b and C5 and upregulation of their gene expression in human cell lines

2.3 Project III: Stx2a binding to MASP-1 and MASP-3 and the involvement of lectin pathway in eHUS pathogenesis.

The first project is a result of collaboration with Dr. Maurizio Brigotti, Department of Experimental, Diagnostic and Specialty Medicine, University of Bologna, Italy and the manuscript is currently under review. The second project has also been drafted into a manuscript as a first author contribution and is also currently under review. A part of the second project and the smaller third project has been carried out in Dr. Peter Garred’s laboratory of Molecular Medicine, Department of Clinical Immunology, Rigshospitalet, University of Copenhagen, Denmark.
2.1 Project I: Variable biological properties of two different preparations of Shiga toxin 2a, yielding new insights into eHUS pathogenesis

2.1.1 Scientific Background

Encoded as two immunologically distinct toxins, Stx1 and Stx2, the latter is most commonly associated with clinical isolates of EHEC [29]. Although Stx1a and Stx2a are clinically most relevant subtypes for humans [29], Stx2a is more potent than Stx1a [46] and is expressed more often than Stx1a [8]. A and B subunits of stx1 genes are only 57% and 60% similar in their nucleotide sequences to the respective subunits of stx2a [143]. Variants of stx2 show 63% to 99.7% nucleotide sequence identity in their A subunits and 57.4% to 95.2% nucleotide sequence identity in their B subunits with the corresponding subunits of classical stx2a [79, 143].

The B-pentamer subunit of the AB5 toxin, which is covalently bonded to the A subunit through a disulphide bond, binds to the globotriaosylceramide, GB3 receptor present on plasma membrane of target cells [144] triggering internalization of the toxin [29]. Some studies also report toxin internalization in GB3 negative cells through retrograde trafficking [145] and binding to kidney and brain endothelial cells via globotriaosylceramide (GB4) receptors [146]. Several reports have shown that human glomerular cell types including endothelial, mesangial and podocyte cells, as well as human tubular epithelial cells, express Stx receptors, mainly Gb3 [147].

During the transport the A subunit is cleaved by the protease furin resulting in two fragments, A1 and A2 (∼28 kDa and ∼4 kDa, respectively). The A1 fragment remains linked to the A2 fragment by a disulfide bond between C241 and C260 [148] and A2 interacts with the pentameric ring formed by the B subunits. The loop between the two cysteines contains the sequence (Arg-X-X-Arg), the consensus motif for cleavage by furin, which is also recognized by trypsin [148]. In the ER, the A1 subunit is released from the A2-B5 complex by reduction of the disulfide bond.
The A1 subunit is translocated from the ER into the cytosol or into the nucleus where it cleaves one adenine residue from the 28S RNA of the 60S ribosomal subunit or multiple adenines from DNA, hence inhibiting protein synthesis and inducing the formation of apurinic sites in chromatin [149-152].

Several in vitro studies have shown the cytotoxic effect of Stx2a on Vero cells and other cell lines, however, toxin sensitivity varies dramatically (showing up to 3-log differences in toxin concentration) [153-155]. These huge differences might be explained by the use of different purification methods, and by different experimental conditions (incubation time or cell passage). Beside the cytotoxic activity, other abilities of Stxs have been shown in vitro using purified preparations. Binding of Stxs to blood cells, like neutrophils [156, 157] or platelets [72, 73] and subsequent activation has been reported, however, these data have been discussed controversially in literature – including experimental data that could not confirm these findings [158, 159]. Furthermore, Stx has been demonstrated to activate complement in vitro and bind to fH [1], but one in vivo reports showed only limited effect of a complement blocking therapy [160].

Stx2a activates complement via the alternative pathway and also binds to fH [1] providing insights into the involvement of complement in the pathogenesis of eHUS. A key step in pathogenesis of eHUS is the interaction of Stxs with blood components before targeting the renal endothelial cells. Stx2a was reported to bind to neutrophils which was discovered at the Bologna laboratory [156] but preliminary data from the Innsbruck laboratory could not confirm the same previously. This discrepancy laid the idea a collaborative project between Dr. Maurizio Brigotti’s laboratory in Bologna and Dr. Reinhard Würzner’s laboratory in Innsbruck.

Most of the in vitro studies have been performed with Stx that has been purified from different strains and with different protocols, thus the toxins may differ in structure, activity, purity and
LPS content, which might explain part of the divergent results. The present study was thus aimed at performing a comparison of the binding of two sources of Stx2a (Stx2a purified in Innsbruck and Stx2a (BO) purified in Bologna) to fH, as well as of the cytotoxicity to Vero cells (gold standard in cytotoxicity assays towards Stxs), thus evaluating whether the differences in the purification procedure may have an influence on the biological properties of the toxin.

2.1.2 **Specific aims of this study**

- To analyze the binding capacity of Stx2a and Stx2a (BO) to fH by ELISA.
- To analyze the cytotoxic effects of Stx2a on Vero cells, in comparison the Stx2a (BO).

2.2 **Project II: Shiga toxin 2a binding to complement components C3b and C5 and upregulation of their gene expression in human cell lines**

### 2.2.1 **Scientific Background**

Although studies in the recent past have reported the involvement of complement, an important part of the innate immune system, in eHUS pathogenesis [1, 161-163], there remains a wide scope for substantial advancements in decoding the disease progression in blood and gut.

Our group has previously shown that the regulatory action of fH, which is promoted by fI, on the cell surface is inhibited by Stx2a, in turn activating the AP of the complement [1]. Furthermore, evidence of reduction in complement inhibitor CD59 on human renal tubular epithelial and glomerular endothelial cells upon stimulation with Stx2a [164] indicate that complement might be involved in the pathogenesis of eHUS.
Regardless of the recent affirmation of intracellular complement [165-168], the interaction of Shiga toxin with the intracellular complement is yet to be analyzed. It has been demonstrated that the C components such as C3, C4 and factor B are locally synthesized in the human intestine in vivo [169, 170]. In addition, some studies have shown synthesis of some of these complement proteins in extrahepatic sites by other cells including monocytes/macrophages, fibroblasts, endothelial [171, 172] and epithelial cells [173, 174]. With local complement production in a variety of non-immune cells, complement is naturally found intracellularly even without the presence of microbes or apoptotic bodies or are produced upon stimulation with cytokines or microbial factors. However, a landmark study in 2013 by Liszewski and co-workers showed that not only complement is present, it is also activated intracellularly [165]. Authors described that the tonic generation of the activated complement was crucial for cell homeostasis and cell survival in T-cells. Thus, local synthesis of C3 components [166, 168, 175] and C5 components [176, 177] argument for a plausible investigation of the interaction of Stx2a with the intracellular complement components in light of eHUS pathogenesis.

Human intestinal epithelium represents the point of release of Stx in the gut lumen upon bacterial lysis and through unknown mechanisms; the toxin translocates the gut-blood barrier in order to access the circulation system [141]. Accordingly, most commonly used human colon carcinoma cell lines (Caco-2, HT-29, and HCT-8) [90, 178], proximal tubular epithelial cell lines (HK-2) [154] and human microvascular endothelial cells [179], express Gb3 on the cell surface and are susceptible to Stx to a variable degrees. Previously, some studies have reported the synthesis of functional alternative pathway complement proteins including C3, fB and fH in proximal tubular epithelial cells HK-2 [166], emphasizing their potential role in kidney pathology.
The aim of this study was to affirm that complement is indeed involved in the development of EHEC-associated HUS. In particular, the aim was to investigate the role of complement, with special emphasis on the synthesis of intracellular complement proteins and regulators of the AP, in colon carcinoma epithelial HCT-8 cells, proximal tubular HK-2 cells and conditionally immortalized glomerulo endothelial CiGEnC cells upon stimulation with Stx2a.

2.2.2 Specific aims of this study

- To analyze the binding of Stx2a complement proteins C3b and C5 by ELISA.
- To perform the cytotoxicity assay in order to determine the time point for intracellular mRNA expression of complement.
- To assess the intracellular mRNA expression of the Alternative Pathway proteins and regulators in HK-2 cells by qPCR.
- To assess the intracellular mRNA expression of complement C3 and C5 in HCT-8 and CiGEnC cells by qPCR.
- To determine the production of C3 in cell lysis suspension or cell supernatant by ELISA.

2.3 Project III: Stx2a binding to rMASP-1 and rMASP-3 and the involvement of lectin pathway in eHUS pathogenesis.

2.3.1 Scientific Background

Stx2a has been known to bind to complement fH and it can be assumed that Stx2a activates complement, generates anaphylatoxins and TCC, and this activation proceeds predominantly via the AP [1]. The absent requirement of the classical pathway excludes the possibility that the presence of Abs in the serum that served as a source of complement may account for this
activation. In addition, a delayed cofactor activity of fH on the cell surface when bound to Stx2a suggested that complement activation by Stx2a may represent an important trigger for cell damage, and thus for the pathogenesis of EHEC-associated HUS, similar to that of aHUS [1].

The involvement of the LP in the pathogenesis of eHUS has not been studied previously. LP as described in 1.5.1.2, was earlier established to be fundamentally activated by MASP-2 by auto-activation and was thought to be the only active protease in the LP initiation. However recent studies show that MASP-1 activates MASP-2 and is essential for complement activation [180-182]. In addition, it has been lately proposed that MASP-1 is also critical for the AP activation in mouse model [183] and that MASP-1 activates MASP-3, which cleaved pro factor D and hence both MASP-1 and MASP-3 may be essential for the AP activation [184, 185] although these results have been challenged by Degn and coworkers. Another study reported that MBL-2 inhibition significantly protected against complement activation and renal injury induced by Stx2 in novel human MBL-2 expressing mice that lack murine Mbls. With contradictory yet probable link between LP and AP, further investigations are needed to decode the possible role of Stx2a and other EHEC virulence factors in the involvement of lectin pathway in eHUS pathogenesis.

The present study was aimed to investigate whether Stx2a binds to recombinant MASP-1 (rMASP-1) or to recombinant MASP-3 (rMASP-3), thus interfering with the advancement of complement attack.

2.3.2 Specific aims of this study

- To analyze the binding of Stx2a to Lectin Pathway complement proteases rMASP-1, rMASP-2, rMAP-1 and rMASP heavy chain (rMASP-HC1/3).
3. MATERIALS (summarized from all projects)

The lists of materials including preparation of all solutions are described below.

3.1 Chemical reagents

4-Nitrophenyl phosphate disodium salt hexahydrate (pNPP)……………… Sigma-Aldrich
Calcium chloride (CaCl$_2$)………………………………………………………… Merck chemicals
Crystal violet……………………………………………………………………… Sigma Aldrich
D-PBS without Ca and Mg………………………………………………………. PAA Laboratories
EGF (Epidermal growth factor)………………………………………………… Sigma Aldrich
Ethanol 70%……………………………………………………………………… Sigma Aldrich
Formalin solution………………………………………………………………… Sigma Aldrich
Fetal bovine serum……………………………………………………………… Sigma Aldrich
Gelatine……………………………………………………………………………. Merck chemicals
Glutamax……………………………………………………………………….. Invitrogen
Glycerol…………………………………………………………………………. Merck chemicals
Glycin……………………………………………………………………………. Bio-Rad
Hydrochloric acid……………………………………………………………… Sigma Aldrich
Hydrocortisone…………………………………………………………………. Sigma Aldrich
Insulin-transferrin sodium selenite (ITS)……………………………………… Sigma Aldrich
L-Glutamin 200mM…………………………………………………………… PAA Laboratories
Magnesium chloride (MgCl$_2$)………………………………………………… Merck chemicals
Penicillin/Streptomycin………………………………………………………… Invitrogen/PAA
Sodium hydrogen carbonate (NaHCO$_3$)……………………………………. Merck chemicals
Sodium carbonate (Na$_2$CO$_3$)…………………………………………………. Merck chemicals
Sodium chloride (NaCl)................................................................. Merck chemicals
Trypsin EDTA (10X).................................................................Sigma Aldrich
Tween 20.................................................................................Merck chemicals

3.2 Biological reagents

3.2.1 Antibodies

8B3, mouse anti-human (against the common HC of MASP-1/-3)..........1* (see p.53)
C3d, mouse anti-human.............................................................. Santa Cruz
C3c, rabbit anti-human.................................................................Dako
C5, mouse anti-human.............................................................. Santa Cruz
C5, rabbit anti-human.................................................................Dako
C7, mouse anti-human..............................................................Quidel
Factor H, sheep anti-human.........................................................The Binding Site
IgG, anti-mouse (whole molecule AP conjugated).............................Sigma Aldrich
IgG, anti-rabbit (whole molecule AP conjugated).............................Sigma Aldrich
IgG, anti-sheep (whole molecule AP conjugated)............................Sigma Aldrich
Stx2a-BB12, mouse anti-human.....................................................Toxin Technology

3.2.2 Cells, media and supplements

HK-2 cells.........................................................................................2* (see p.53)
DMEM/Hams F12 (Dulbecco's Modified Eagle Medium).................Gibco
CiGEnC cells..................................................................................3* (see p.53)

EGM-2 medium.................................................................................Lonza

Vero cells..........................................................................................ATCC-CCL-81

HCT-8 cells.......................................................................................ATCC-CCL-244-

RPMI 1640.........................................................................................Sigma-Aldrich

3.2.3 Purified proteins and cytokines

C3b, human complement............................................................Calbiochem

C5, human complement............................................................Calbiochem

C7, human complement............................................................Calbiochem

Factor H, human complement.....................................................Calbiochem

IFN-γ.................................................................Miltenyi Biotec

IL-6.................................................................Miltenyi Biotec

IL-1β.................................................................Miltenyi Biotec

LPS, bacterial component...........................................................In house

PGE₂......................................................................................Sigma-Aldrich

rMASP-1, human complement........................................1* (see p.53)

rMASP-3, human complement........................................1* (see p.53)

rMAP-1, human complement........................................1* (see p.53)

rMASP-HC-1/3, human complement.................................1* (see p.53)

Stx2a, bacterial component..................................................In house [186]

Stx2a (BO), bacterial component.................................4* (see p.53)

TNF-α.................................................................Miltenyi Biotec
3.2.4 Bacterial strains

EHEC (R82) ..........................................................5* (see p.53)

\textit{E. coli} C600 (933W) ................................................5* (see p.53)

3.2.5 Assays and Kit

C3 Prime Assay..........................................................Bio-Rad

C5 Prime Assay..........................................................Bio-Rad

Endosafe ®Endochrome-KTM assay.................................Charles River Lab.

GAPDH Prime Assay....................................................Bio-Rad

iScript™ Reverse Transcription Supermix for RT-qPCR ............Bio-Rad

MagAttract Direct mRNA M48 Kit....................................Qiagen

SsoFast™ EvaGreen® Supermixes..................................Bio-Rad

TaqMan Gene Expression Assays.....................................Applied Biosystems

Total RNA Kit, peqGOLD.............................................VWR International

Qubit™ RNA HS Assay Kit..........................................Invitrogen
3.2.6 Buffers and solutions

10X TBS (tris-buffered saline) (1l)

Tris base (formula weight 121.14) 24g
NaCl (formula weight 58.4g) 88g
Deionized water, dissolve in 900ml distilled water
Adjust pH to 7.6 with 12N HCl
Add distilled water to a final volume of 1l and store at 4°C

1X TBS (1l)

Mix 1 part of the 10X solution with 9 parts distilled water
Adjust pH to 7.6 again.

1X TBST (0.1% Tween 20) (1 l)

100ml of TBS 10X
900ml of distilled water
1ml Tween 20

1X TBST-Ca++ (1l)

Mix 1 part of the 10X solution with 9 parts distilled water
Add 2mM CaCl₂
Adjust pH to 7.6 again.
Coating buffer (1l)

**Solution 1:**

NaHCO₃ 12.42g  
Add 740ml deionized water

**Solution 2:**

Na₂CO₃ 5.5g  
Add 260ml deionized water  
Mix both solutions and store at 4°C

1% gelatin (100ml)

Gelatin 1g  
Coating buffer 100ml  
Dissolve gelatin in coating buffer at 37°C in water bath, store at -20°C

AP buffer (500ml)

Glycin (0.1M) 7.51g  
MgCl₂ (1mM) 0.2g  
ZnCl₂ (1mM) 0.13g  
Dissolve in 500ml of deionized water, adjust pH to 10.4 and store at 4°C

Substrate solution for ELISA

p-NPP (5mg/tablet) 1 tablet  
Add in 5ml AP buffer
3.2.7 Miscellaneous

Luria broth and agar ................................................................. Sigma-Aldrich
RNA later .............................................................................. Applied Biosystems
Igepal ...................................................................................... Sigma-Aldrich

3.3 Lab-wares and instruments

6-well Cell culture plate .............................................................. Sigma-Aldrich
96-well ELISA microplate (flat-bottom) ...................................... Sigma-Aldrich
ABI 2700 PCR machine: cDNA Rutine program ...................... Thermo Fiscer
Amicon filters ........................................................................ Sigma-Aldrich
C1000 Touch™ Thermal Cycler ............................................... Bio-Rad
Centrifuge ............................................................................ Hettich, Rotanta 460R
CFX96 Touch™ Real-Time PCR Detection System .................. Bio-Rad
ELISA reader (model 680 microtiter plate reader) ..................... Bio-Rad
Eppendorf tubes (0.5ml, 1.5 ml) .............................................. Greiner Bio-one
Falcon tubes (15ml, 50 ml) ...................................................... Greiner Bio-one
Freezer (-20°C) ..................................................................... Liebherr
Freezer (-80°C) ..................................................................... Snijders Scientific
Glassware ............................................................................. Schott Duran
Incubator ............................................................................. Heraeus
KingFisher™ mL Magnetic Purification System ....................... Thermo Scientific
Microcentrifuge, Mikro 200R ......................................................Hettich,
Microflow biological safety cabinet..........................................Politakis
Mini Trans-Blot System..............................................................Bio-Rad
Nanovue™ Plus Spectrophotometer..........................................GE Healthcare
Pipette (1000, 200, 100, 20, 10μl)............................................Biohit mLINE
Pipette (multichannel; 300, 100μl)...............................................Biohit mLINE
Pipette tips (1000, 200, 10μl)....................................................Greiner Bio-one
QuantStudio™ 12K Flex Real-Time PCR System.......................Applied Biosystems
Qubit® 2.0 Fluorometer...............................................................Invitrogen
T75 cell culture flask...............................................................Sigma-Aldrich
Vortex mixer.............................................................................Snijders
Water bath................................................................................Memmert

3.4 Softwares

CFX Manager Software version 2.1.............................................Bio-Rad
GraphPad Prism.........................................................................GraphPad Software
Microplate Manager.................................................................Sigma-Aldrich

1* Dr. Peter Garred, University of Copenhagen, Denmark
2* Dr. Michael Joannidis, Medical University Innsbruck, Austria
3* Dr. Simon Satchell, University of Bristol, England
4. Dr. Maurizio Brigotti, University of Bologna, Italy
5* Dr. Mohamed Karmali, Public Health Agency, Canada
4. METHODS

The methods for all the experiments performed in all the three projects are described conjointly in this section.

4.1 Toxin purification

EHEC (R82) and *E. coli* C600 (933W) was supplied by Dr. Mohamed Karmali (Public Health Agency of Canada’s Laboratory for Foodborne Zoonoses, Ottawa, Canada). Stx2a was purified in Innsbruck (Austria) by the following steps: culture, sonication, concentration on Amicon filters, dialysis, hydroxyapatite chromatography, dialysis, concentration on Amicon filters, chromatofocusing, ammonium sulfate precipitation, and dialysis [187]. For selected project (Project: I), another type of Stx2a designated as Stx2a (BO) was used which was isolated in Bologna (Italy) as described in [188]: culture, ammonium sulfate precipitation of proteins in cell-free broth, dialysis, receptor analog affinity chromatography on Galα1-4Galβ-O-spacer–BSA–Sepharose 4B (Glycorex, Lund, Sweden), concentration on Amicon filters, passage through ActiCleanEtox column (Sterogene Bioseparations, Carlsbad, CA) to remove trace endotoxin contaminants. The amount of contaminating LPS was determined by using the Endosafe®Endochrome-KTM assay [186]. The test method is based on the Chromogenic technique as described elsewhere (United States Pharmacopeial Convention, Bacterial Endotoxins Test).

4.2 Cleavage of Shiga toxins with trypsin

Elsewhere, trypsin was freshly prepared (1mg/ml in 0.1mM HCl) and diluted to 0.05mg/ml in TBS before use. The trypsin inhibitor phenylmethylsulfonyl fluoride (PMSF) was freshly prepared (1mg/ml in absolute ethanol) and diluted to 0.7μg/ml in water before use. Stx2a (BO)
(4μg) was incubated with 50ng of trypsin in 10μl TBS pH 7.5 for 1 h at 37°C. At the end of the incubation, 1μl of PMSF (corresponding to 0.7ng) was added and the sample was further incubated 10 min at 37°C. Trypsin-treated treated toxins were stored at -80°C. Elsewhere, toxins were analysed by SDS-PAGE in reducing and non-reducing conditions using a 16% (wt/vol) gel followed by staining with Coomassie blue.

4.3 Analysis of binding of Stx2a to fH by ELISA

In concentrations of 2μg/well, Stx2a, Stx2a (BO), LPS (5.17 EU, equivalent to the amount present in Stx2a), Stx2a (BO) plus LPS (5.17 EU), trypsin-treated Stx2a (BO) and BSA (2μg/well; serving as negative control) were immobilized in 100μl of coating buffer on wells of a microtiter plate overnight at 4°C. As positive control, fH alone was used. After blocking with 1% (w/v) gelatin at room temperature for 30 min, 2μg of purified fH or 100μl of TBST alone serving as negative control, were added to appropriate wells for 6 h at 37°C. After washing, bound fH was detected with a primary sheep anti-human fH Ab (1:1,000). The bound protein was then detected with a secondary alkaline phosphatase (AP)-conjugated anti-sheep antibody (1:1,000). The reactions were developed with 4-nitrophenyl phosphate disodium salt hexahydrate and the optical density of the reaction was then measured at 415nm versus 490nm.

4.4 Cell culture

Vero cells and HCT-8 cells were maintained in RPMI -1640 medium and supplemented with fetal bovine serum to a final concentration of 10% at 37°C under 5% CO₂ conditions. CiGEnC cells were cultured in EBM-2 medium using a growth factor kit, as described previously [189].
HK-2 cells were cultured in growth medium consisted of a mixture of equal parts of DMEM and Ham’s F-12 nutrient mix containing 5µg/ml insulin, 5µg/ml transferrin, 5ng/ml sodium selenite, 2mM glutamax, 10 ng/ml epithelial growth factor, 36ng/ml hydrocortisone, 100U/ml penicillin and 100µg/ml, as described previously [190].

4.5 Vero cell cytotoxicity assay

The cytotoxicity assay was performed as described previously [45]. 10⁴ Vero cells per well were seeded in 96-well plates and incubated at 37°C and 5% CO₂ for 24 h. Cells were then administered with serially diluted concentrations of Stx2a and Stx2a (BO) ranging from 10pg/ml to 10µg/ml (~150fM-150nM) in fresh medium, and medium only as control, for additional 48 h under the same conditions for comparative studies. Further, the cells were fixed with 100µl of 2% formalin for 3 min and stained with 100µl of 1% crystal violet for 1 h. After washing with distilled water, 100µl of 50% ethanol was added to the wells for 15 min at shaking conditions. The absorbance was measured at 550nm as an estimate of the density of cells that remained in the wells.

4.6 Analysis of binding of Stx2a to complement C3b, C5 and C7

To establish whether Stx2a binds to C3b, microtiter plates were coated with Stx2a (2µg/well) in 100µl coating buffer overnight at 4°C. After blocking with 1% (w/v) gelatin, each well was incubated with 0.5, 1 or 2µg of C3b in 100µl of TBST for 2 h at room temperature. Similarly, the binding of Stx2a to C5 or C7 was established by incubating the overnight Stx2a (2µg/well) coated microtiter with 0.5, 1 or 2µg of C5 or C7 in 100µl of TBST for 2 h at room temperature. After additional washing steps, bound C3b, C5 or C7 were detected with a primary rabbit anti-
C3c antibody (1:500), rabbit anti-C5 antibody (1:200) or mouse anti-C7 antibody (1:200), respectively, followed by a goat anti-rabbit AP-conjugated IgG (1:1,000) for C3b and C5, and goat anti-mouse AP-conjugated IgG (1:1,000) for C7, all diluted in TBST. The binding capacity was detected with 4-nitrophenyl phosphate disodium salt hexahydrate as the substrate. C3b, C5 or C7 alone were used as a positive control. BSA (0.5, 1 or 2µg/well) was coated as negative control in place of Stx2a. Photometric readouts were done at 415nm versus 490nm.

4.7 Cytotoxicity assay to exclude lysis during expression studies

The cytotoxicity assay was performed to determine the appropriate time point for the assessment of intracellular mRNA expression of the complement components, as described previously [45]. $10^4$ HCT-8 cells, CiGEnC or HK-2 cells were seeded per well in 96-well plates and incubated at 37°C and 5% CO₂ for 24 h. Cells were then administered with Stx2a concentrations of 10ng/ml, 100ng/ml, 1µg/ml and 10µg/ml in fresh medium, and medium only as control, for additional 9, 12, 18, 24, 48 and 72 h for each cell line under the same conditions for comparative studies. After washing with PBS, the cells were fixed with 100µl of 2% formalin for 3 min and stained with 100µl of 1% crystal violet for 1 h. After washing with distilled water, 100µl of 50% ethanol was added to the wells for 15 min at shaking conditions. The absorbance was measured at 550 nm as an estimate of the density of cells that remained in the wells.

4.8 Alternative Pathway intracellular complement profile analysis of HK-2 cells

HK-2 cells were seeded at 0.3 x 10⁶ per well seeding density in 6-well plates and incubated at 37°C and 5% CO₂ up to 80% confluency. Cells were then administered with 1ng/ml, 10ng/ml and 100ng/ml Stx2a in fresh medium and medium only as untreated control for 6 h; and co-incubated
with cytokines TNF-α (5ng/ml) and IFN-γ (10ng/ml) as positive control for 24 h [166]. Cellular supernatants were collected and stored at -80°C until analysed. Cells were trypsinized and stored in RNA later. mRNA was extracted using the MagAttract Direct mRNA M48Kit and processed in Thermo Scientific™ KingFisher™ mL Magnetic Purification System as per manufacturer’s instructions. The mRNA was quantified using Qubit™ RNA HS Assay Kit in a Qubit® 2.0 Fluorometer. The mRNA was normalized and reverse transcribed with the ABI 2700 PCR machine: cDNA Rutine program in 20µl reaction volume. Gene expression was assessed by quantitative real-time PCR with TaqMan probes specific for TOP2B (Hs00172259_m1), GAPDH (Hs02786624_g1), C3 (Hs00163811_m1), C5 (Hs01004342_m1), C6 (Hs01110040_m1), C7 (Hs00940408_m1), C8b (Hs00163867_m1), C9 (Hs01036216_g1), fH (Hs01009582_m1), fI (Hs01105402m1), fB (Hs00156060_m1), fD (Hs00157263_m1) and Properdin (Hs01106971_g1) obtained from Applied Biosystems. Quantitative real-time PCR was performed in triplicate loading 1ng of cDNA per reaction of volume of 10µl and carried out on QuantStudio™ 12K Flex Real-Time PCR System. Reaction conditions included incubation at 50°C for 2 min and 95°C for 10 min followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. PCR runs included no-template controls and no-reverse transcriptase controls. The target genes were normalized to housekeeping genes, TOP2B and GAPDH and results were expressed as fold changes from untreated cells using the ΔΔCt method.

**4.9 Intracellular C3 and C5 expression in HCT-8 and CiGEnC Cells**

HCT-8 and CiGEnC cells were seeded at 0.3 x 10^6 per well seeding density in 6-well plates and incubated at 37°C and 5% CO₂ up to 80% confluency. Cells were then administered with 100ng/ml, 1µg/ml and 10µg/ml Stx2a in fresh medium, medium only as untreated control and co-incubated with cytokine cocktail of IL-1β (10³U/ml), TNF-α (10ng/ml), IL-6 (10³U /ml) and
PGE$_2$ (1µg/ml) as positive control, for 6 h. Cellular supernatants were collected and stored at -80°C until analyzed. Total RNA was extracted using the Total RNA Kit, peqGOLD as per manufacturer’s instructions. The mRNA was quantified using NanoVue™ Plus Spectrophotometer. The mRNA was normalized and reverse transcribed using the iScript™ Reverse Transcription Supermix for RT-qPCR as per the manufacturer’s instructions. The mRNA expression of C3 was analyzed by real-time PCR using gene-specific primer/probe pairs. A GAPDH (human)-PCR using specific primer/probe pairs served as internal control to quantify the relative gene expression of target gene. The SsoFast™ EvaGreen® Supermix was used for target amplification and runs were performed on the CFX96 real-time detection system. Reaction conditions included incubation at 50°C for 5 min and 95°C for 1 min followed by 40 cycles at 95°C for 5 s and 60°C for 5 s. The results were analyzed by the ΔΔCt method using the gene expression software of the cycler.

4.10 Determination of C3 in cell supernatant and lysates treated with Stx2a

HCT-8 and CiGEnc cells were treated with Stx2a concentrations of 1µg/ml and 10µg/ml as in (4.9). Supernatants were measured for their C3 contents after treatment with 1% Igepal in TBS for 15 min. Mouse anti-C3d Ab (2µg/ml) was immobilized overnight at 4°C in 100µl of coating buffer onto wells of a microtiter plate. Following blocking with 1% (w/v) gelatin at room temperature for 30 min, 100µl of cell culture supernatant concentrated with amicon filter or cell lysate was added for 2 h at room temperature. 1µg of purified C3b in 100µl of TBST served as positive control and TBST alone as negative control. After washing, bound C3 was detected with a primary rabbit-anti-C3c Ab (1:500), followed by a goat anti-rabbit AP-conjugated IgG (1:1,000), all diluted in TBST. The binding capacity was detected with 4-nitro phenyl phosphate.
disodium salt hexahydrate as the substrate and photometric readouts were done at 415nm versus 490nm and compared to data obtained from C3 standard curve.

4.11 Analysis of binding of Stx2a to rMASPs by ELISA

Binding of Stx2a to rMASP-1, rMASP-3, rMAP-1 and rMASP heavy chain (rMASP-HC1/3) was investigated by ELISA. In concentrations of 1μg/well, Stx2a and BSA (1μg/well; serving as negative control) were immobilized in 100μl of coating buffer on wells of a microtiter plate overnight at 4°C. Additionally, positive controls were also set up by immobilizing rMASP-1, rMASP-3, rMAP-1 and rMASP-HC1/3 in concentrations of 1μg/well in 100μl of coating buffer. After blocking with 1% (w/v) gelatin at room temperature for 30 min, 1μg of purified rMASP-1, rMASP-3, rMAP-1 and rMASP-HC or 100μl of TBST-calcium buffer alone serving as negative control, were added to appropriate wells for 2 h at room temperature. After washing, bound proteins were detected with a primary mouse-8B3 Ab (2μg/ml). The bound proteins were then detected with a secondary alkaline phosphatase (AP)-conjugated anti-mouse antibody (1:1,000). The reactions were developed with 4-nitrophenyl phosphate disodium salt hexahydrate and the optical density of the reaction was then measured at 415nm versus 490nm.

4.12 Statistical analyses

The results were analyzed by the use of GraphPad Prism (version 7) software. Students’s t-test was performed to compare the paired means of the two measurement groups. P values of <0.05 were considered significant.
5. RESULTS

5.1 Purification and characterization of Stx2a

Different *E. coli* strains, EHEC (R82) or C600 (933W), were used to purify Stx2a in two different laboratories located in Innsbruck, Austria (Stx2a) and Bologna, Italy, (Stx2a (BO)) respectively, following two different purification methods. Both toxin preparations (final concentrations: Stx2a =1.6 mg/ml; Stx2a (BO) = 0.52 mg/ml) were divided in small aliquots and stored at -80°C. Elsewhere, sequencing analyses confirmed that the stx2a genes code for Stx2a protein and that the subunits A are identical for the two different toxins [186]. The B subunit showed one mismatch at position 36 in the alignment, which, however, did not change the protein sequence [186].

As measured by the Limulus assay elsewhere, the amount of LPS was higher in Stx2a (45EU/ml, 56EU/mg of protein) than in Stx2a (BO) (35EU/ml, 67.3EU/mg of protein) [186], and this was considered in the functional assays described below, i.e. addition of controls for contaminating LPS.

Since preliminary data from the Innsbruck laboratory could not confirm Stx2a binding to human neutrophils, as previously demonstrated in the Bologna laboratory [191], comparative analysis of the biological activity of the two toxin preparations was performed.

5.2 SDS-PAGE analysis of Stx2a

SDS-PAGE analysis performed at non-reducing conditions revealed the presence of two Coomassie-stained bands corresponding to A- and B-subunits in Stx2a (Fig. 4), whereas an additional band appeared at reducing conditions (Fig. 4) having the apparent molecular mass of the A1-fragment (27.6 kDa) and migrating faster than the A-subunit (31.7 kDa).
Figure 4: SDS-PAGE analysis of Stx2a. SDS-PAGE analysis of Stx2a (9μg) and Stx2a (BO) (9μg) performed in non-reducing and reducing conditions. Molecular mass markers were 10-50 kDa. After electrophoresis, gels were stained with Coomassie blue. The mobility (Rf, distance of protein migration/distance of dye migration) of calibration proteins were plotted vs log of their kDa. The Rf of A-subunit and A1-fragment of each preparation allowed calculation of the molecular masses reported under Results.

Elsewhere, densitometric analysis showed that Stx2a consists of 16% A chain and of 84% A1-fragment [186]. Since the A1-fragment was observed only in the presence of mercaptoethanol as reducing agent (Fig. 4), it was concluded that the A-subunit of Stx2a is cleaved in the loop between C241 and C260 and that the two resulting fragments A1 and A2 are linked by an intact disulfide bond. Stx2a (BO) run at reducing conditions (Fig. 4) showed a single 31.7 kDa band for the intact A-subunit. Treatment of Stx2a (BO) with trypsin [192], which mimics the single proteolytic cleavage of the Stx A-subunit occurring in cells, obtained 80% A1-fragment (27 kDa), hence reproducing the pattern observed in Stx2a (data not shown).
5.3 Stx2a and Stx2a (BO) differ in their ability to interact with complement factor H

Binding studies were performed with both toxin batches, since Stx2a was demonstrated to bind to fH as part of its activating effect on the complement system via the alternative pathway previously [1]. The results confirmed that Stx2a interacts with fH, and that its binding capacity was significantly higher in comparison to Stx2a (BO) and to all negative controls (p < 0.0001 for all) (Fig. 5).

![Figure 5: Stx2a binding to fH. Stx2a and trypsin-treated Stx2a (BO) bind to complement fH. Immobilized Stx2a or Stx2a (BO) or trypsin-treated Stx2a (BO) (2µg/well each) was incubated with fH (2µg). As controls trypsin + trypsin inhibitor (PMSF) or BSA were incubated with fH. Additional controls were performed by incubating the proteins with buffer (TBST) instead of fH. As positive control fH was used. The binding capacity of Stx2a and trypsin-cleaved Stx2a (BO) to fH was significantly higher compared to Stx2a (BO) and to all negative controls (p < .0001 for all).](image-url)
FH binding was also evaluated with LPS (amount present in Innsbruck preparation) added to Stx2a (BO) and LPS alone and was found to have no additional effect on the binding capacity whatsoever (Fig. 6). Thus, the binding patterns of the two toxin preparations to fH were opposite (Stx2a active, Stx2a (BO) inactive) to each other.

![Figure 6: LPS binding to fH. LPS has no impact on fH binding.](image)

**Figure 6: LPS binding to fH. LPS has no impact on fH binding.** Immobilized Stx2a or LPS (amount present in Stx2a) or Stx2a (BO) + LPS (amount present in Stx2a) (2µg/well each) was incubated with fH (2µg). As controls LPS (amount present in Stx2a) or BSA was incubated with fH. Additional controls were performed by incubating the proteins and LPS with buffer (TBST) instead of fH. As positive control fH was used. The fH binding capacity of Stx2a was significantly higher compared to Stx2a (BO) + LPS and LPS alone and to all negative controls (p < .0001 for all).

### 5.4 Nicked Stx2a (BO) behaves like Stx2a

Upon treatment by trypsin, Stx2a (BO) bound to fH with values similar to those obtained with the Innsbruck preparation (Fig. 5). Thus the trypsin treated Stx2a (BO) mimicked the nicked Stx2a suggesting that the fH binding property is an attribute of the toxin structure.
5.5 Vero cells are cytotoxic to both toxin types

Despite the difference in their structure, Stx2a and Stx 2a (BO) did not generate any statistical difference in their cytotoxic ability towards Vero cells and were almost identical (Fig. 7). In addition, it has been shown that both the toxins are fully active on GB3+ cells [186].

![Graph showing cytotoxicity profiles of Stx2a and Stx2a (BO) on Vero cells](image)

**Figure 7:** Cytotoxicity profiles of Stx2a and Stx2a (BO) on Vero cells are very similar. Vero cells were incubated for 48 h with a broad range (10fg/ml-10μg/ml) of Stx2a concentrations (150aM-150nM). Residual cell density was measured by crystal violet staining method and the percentage of living cells is shown on the y-axis.

5.6 Stx2a binds to complement C3b and C5

Binding of Stx2a to complement C3b or C5 or C7 was analyzed by ELISA. Stx2a was coated onto the solid phase of microtiter plates for this assay and C3b or C5 or C7 diluted in TBST were overlaid on top of Stx2a. Both C3b and C5 showed a dose dependent binding to Stx2a upon detection with anti-C3c and anti-C5 Ab respectively, whereas C7 did not seem to bind to Stx2a even at higher concentrations, upon detection with anti-C7 Ab, in comparison to C3b and C5 at
similar concentrations and behaved like the negative controls with BSA coated wells in place of Stx2a (p < 0.0001 for all) (Fig. 8).

![Graph showing Stx2a binding to C3b and C5.](image)

**Figure 8: Stx2a binding to C3b and C5.** C3b and C5 bind to Stx2a but C7 does not. Immobilized Stx2a (2 μg/well) was incubated with 0.5 μg or 2 μg of C3b or C5 or C7. As controls, BSA in place of Stx2a or TBST in case of complement proteins was incubated with same concentrations. As positive control, C3b or C5 or C7 was used alone. Stx2a significantly bound to C3b and C5 and the binding capacity was dose dependent and all negative controls (p < .0001 for all).

### 5.7 HCT-8, CiGEnC and HK-2 cell lines exhibit viability up to 9 h of Stx2a administration

Stx2a was administered to stimulate HCT-8, CiGEnC and HK-2 cells and Vero cells as reference cells with increasing concentrations of 10 ng/ml, 100 ng/ml, 1 μg/ml and 10 μg/ml of Stx2a in fresh medium and medium only as control for 9, 12, 18, 24, 48 and 72 h (data shown for 2 time points, 9 h and 48 h) (Fig. 9). All the cell lines were cultured in serum-free conditions to eliminate any serum-driven artefacts. At 9 h time point, Stx2a, at all given concentrations, did not cause any significant growth inhibitory effect on HCT-8, CiGEnC, HK-2 and Vero cell lines. The same holds true for 12 h and 18 h time point in case of HCT-8 cells whereas 48 h and 72 h time points showed gradual but significant decrease in cell viability with increasing Stx2a concentrations.
Figure 9: Cytotoxic activity of Stx2a. Stxa is administered in concentrations of 10ng/ml-10µg/ml and plotted against control (medium only) as percent living cells in CiGENC, HCT-8, HK-2 and Vero cells at 9 h (A1, B1, C1, D1) and 48 h (A2, B2, C2, D2) time points. All cell lines at all Stx2a concentrations are viable at 9 h time point.
In case of CiGEnC cells, a consistent tendency of decrease in cell viability with increase in Stx2a concentrations was observed with increasing time point, with a drop from 100% to 41% cell viability at 10ug/ml and 91% to 60% cell viability at 100ng/ml of Stx2a administration at 72 h. HK-2 cells exhibited 100% cell viability for all concentrations of Stx2a at 9 h time point while gradually decreased their cell viability up to 75% upon stimulation with the highest Stx2a concentration at 48 h time point (Fig. 9C1-C2), with cell viability further decreasing up to 55% at the 72 h and 10ug/ml Stx2a concentration. Vero cells being highly susceptible to Stx2a were chosen as the gold standard commonly used in Stx cytotoxicity assays and exhibited results similar to CiGEnC cells (Fig. 9D1-D2). This experiment was performed to determine a singular time point for further experiments, at which all cells are viable upon Stx2a administration and any activation in the cells is not a result of cell cytotoxicity.

5.8 Stx2a upregulates C3 and C5 mRNA expression in HK-2 cells

HK-2 cells contain the GB3 receptor susceptible to Stx2a and were used as a screen for selecting suitable concentrations of Stx2a for analysis of mRNA expression of AP specific complement components regulators such as fH, fI, fB, fD and P; AP complement protein C3 which forms the AP convertase; and terminal pathway complement components C5, C6, C7, C8 and C9 which assemble to form the MAC. Since HK-2 cells showed no cytotoxicity even at the highest administered Stx2a concentration up to 9 h at least, time point of 6 h was chosen to evaluate the effect on mRNA expression of complement components in order to exclude any possible complement expression due to cytotoxicity and cell death. mRNA expression was analyzed using real time PCR (RT-qPCR). RT-qPCR revealed a significant increase in C3 and C5 mRNA expression upon administration with Stx2a concentrations of 1ng/ml, 10ng/ml and 100ng/ml in comparison to untreated cells (Fig. 10), although the fold increase remained constant even with
increasing concentrations of Stxa. Cytokine stimulation was used as control and expressed C3, C5, fH, fB and fP upregulation. The relative normalized expression has been expressed as fold change.

![Image](https://via.placeholder.com/150)

**Figure 10: C3 and C5 mRNA upregulation in HK-2 cell lines.** mRNA was extracted from HK-2 cells and the mRNA expression of AP specific complement proteins and regulators was evaluated by real-time polymerase chain reaction (PCR) analyses. Data from the real-time PCR were normalized against TOP2B and GAPDH.

**5.9 Upregulation of C3 and C5 in HCT-8 and CiGENC cells at higher Stx2a concentrations**

Gut epithelial cells HCT-8 and glomerulo endothelial cells CiGENC were administered with increasing concentrations of Stx2a (1ng/ml-10µg/ml) for 6 h against untreated cells. Neither of the two cell lines showed any significant change in regulation of C3 and C5 mRNA at concentrations (1ng/ml-100ng/ml) subjected to HK-2 cell lines except for when HCT-8 cells were administered with 100ng/ml of Stx2a, there was significant upregulation of C5 mRNA. Thus, HCT-8 and CiGENC cells were treated with higher concentrations of Stx2a (1µg/ml and 10µg/ml) for the same time period. A significant change was observed with the high Stx2a concentrations of 10µg/ml yielding most significant increases in C3 and C5 mRNA expression (Fig. 11A).
Figure 11: C3 and C5 mRNA upregulation in HCT-8 and CiGENC cell lines. A. With increasing concentrations of Stx2a administered to HCT-8 cell lines for 6 h against untreated cells, all normalized against housekeeping gene GAPDH, a significant increase in C3 and C5 mRNA was observed. B. Results similar to HCT-8 cells were observed in CiGENC cells but the expression was more profound.

In case of CiGENC cells, profound expression of both C3 and C5 mRNA was observed with increasing concentrations of Stx2a (1µg/ml-10µg/ml) thus establishing the expression and upregulation of complement C3 and C5 when compared against untreated cells and normalized against housekeeping gene, GAPDH (Fig. 11B). The upregulation was more profound in CiGENC cells as compared to HCT-8 cells. As controls, cells were administered with cytokine cocktail (as
mentioned in 4.9) and showed significant upregulation of complement C3 and C5 mRNA in both the cell lines (data not shown).

5.10 Complement protein C3 obscure in HCT-8 and CiGENC cells

Cells were administered with Stx2a concentrations of 1µg/ml and 10µg/ml for 6 h and investigated for the presence of complement C3 in cell supernatant and lysates. When plotted against a standard curve, neither of the cell lines showed any detectable presence of C3 in the concentrated cell supernatant and lysate and both the administered concentrations.

5.11 Lectin pathway components MASP-1 and MASP-3 bind to Stx2a

Binding of Stx2a to recombinant LP serine proteases rMASP-1 and rMASP-3 was analyzed by ELISA. Stx2a was coated onto the solid phase of microtiter plates for this assay and rMASP-1 or rMASP-3 diluted in TBST-Ca++ buffer was overlaid on top of Stx2a.

rMAP-1, the inactive, alternate spicing gene product of MASP1 which also encodes for MASP-1 and MASP-3 was also overlaid on top of Stx2a. In addition, binding of Stx2a was also investigated with the common heavy chain of MASP-1 and MASP-3, designated as rMASP-HC1/3. rMAP-1 also constitutes of most of the heavy chain.

Both rMASP-1 and rMASP-3 bound to Stx2a upon detection with anti-8B3 antibody which detects the common heavy chain of MASP1 gene products (Fig. 12). rMASP-3 bound Stx2a more significantly to Stx2a in comparison to rMASP-1 whereas rMAP-1 and r rMASP-HC1/3 showed no binding to Stx2a and behaved like the negative controls where Stx2a was replaced with BSA (p < 0.0001 for all) (Fig. 12).
Figure 12: Stx2a binding to rMASP-1 and rMASP-3. Stx2a exhibited significant binding to rMASP-1 and even more profound binding to rMASP-3 whereas it showed no binding to the inactive r-MAP-1 and the common heavy chain r-MASP-HC1/3. BSA was coated in place of Stx2a as negative control and purified recombinant proteins alone were used as positive controls (p < 0.0001 for all).
6. DISCUSSION

6.1 Variable biological properties of two different preparations of Shiga toxin 2a, Stx2a and Stx2a (BO), yielding new insights into eHUS pathogenesis

The primary virulence factors in EHEC-associated HUS (eHUS) are the Shiga toxins. Amongst the Stx types, Stx2a is strongly associated with severe disease, including eHUS [1]. Thus several in vitro studies have been performed with purified Stx2a to elucidate the patho-mechanism of this most extreme outcome of EHEC infection. However, for Stx purification there is no standardized protocol available and thus different and often poorly detailed Stx purification methods employing different Stx-producing strains have been used.

Different characteristics of Stx2a have been described in the literature that might contribute to the pathogenesis of eHUS. These characteristics include cytotoxicity against various cells, often using Vero cells as model, protein synthesis inhibition, neutrophil binding, platelet binding and activation, formation of leukocyte/platelet aggregates, binding to different serum proteins, such as HuSAP or complement fH, and complement activation [1, 154, 162, 191, 193-197]. However, several of these Stx2a-induced features are discussed controversially. Either the Stx2a-induced effect was not comparable in magnitude or some of these effects could even not be reproduced by other groups [198, 199]. It seems unlikely that these variations are only due to modifications in the experimental set-up; rather differences in the Stx preparations are very likely to contribute. Thus, the aim of the present project was to compare two different Stx preparations with respect to published Stx2a-induced features.

The two EHEC strains (C600 and R82) used for the Stx preparations were shown to share the same DNA sequence for the stx gene, elsewhere, indicating that the purified Stx proteins also share the same protein sequence [186].
Upon comparing the abilities of the two Stx2a preparations with respect to complement fH binding, it was observed that Stx2a could bind to fH, as published several years before [1]. However, binding analyses of fH using Stx2a (BO) revealed no or only very minor binding to fH. Limulus assay performed elsewhere revealed differences in the LPS amount in Stx preparations: Stx2a yielded a many-fold higher LPS amount. As LPS contamination of the Stx preparations was shown to differ significantly, we examined whether this difference might corroborate and explain the differences shown for fH binding. It could be concluded that the difference in the amount of LPS present in both the toxins is not the reason for the difference in their ability to bind fH.

However, elsewhere, SDS PAGE analyses of the toxins revealed that in case of Stx2a, the A-subunit is cleaved in two fragments whose molecular masses correspond to A1 and A2 fragments. These two fragments of Stx2a are linked to each other by a disulfide bond, shown by the fact that only the mercaptoethanol-reduced gels resolved A1 and A2 fragments, whereas use of non-reducing sample buffer revealed only one band for the A-subunit. Cleavage of the A-subunit was found to happen early in the purification procedure of Stx2a at the sonication step (data not shown). As a matter of fact, this step is lacking in the purification method yielding Stx2a (BO) which has an uncleaved and intact A-subunit [186].

In further experiments Stx2a (BO) was treated with trypsin to mimic the same cleavage seen during the Stx2a purification. Trypsin-treatment of Stx2a (BO) restored fH binding activity at values similar to those obtained in the case of the nicked Stx2a. The fact that Stx2a and trypsin-treated Stx2a (BO) did bind to fH could at first suggest that the binding site for fH might be located in the nicked area of the A-subunit. However, the Stx2a B subunit has previously been suggested to contain the binding site for fH [200]. It is thus hypothesized that nicking of the A subunit might influence the quaternary structures of the whole toxin affecting A/B subunit
interactions, thereby explaining the different abilities of Stx2a preparations concerning the binding to fH.

In addition to fH binding, elsewhere, investigation of Stx2a-induced abilities revealed activation of platelets for both preparations and only slight differences in the enzymatic activity. Conversely, striking differences have been found when comparing Stx2a and Stx2a (BO): binding of Stx2a to neutrophils could be demonstrated for Stx2a (BO), but not for Stx2a and trypsin treated Stx2a (BO), suggesting that the binding site for neutrophils might be located in the cleaved area of the A-subunit, but not in the active site of Stx2a, as cytotoxic activity was unchanged and protein synthesis inhibition was even more pronounced in the case of Stx2a [186].

Elsewhere, formation of leukocyte-platelet aggregates in human blood treated with Stx2a also revealed remarkable differences: Stx2a (BO) induced a significantly higher percentage of neutrophil- and monocyte-platelet formation than Stx2a [186].

Nicking of the Stx2a A-subunit has been described during the cellular transport of Stx2a. The A1 fragment is released in the ER and subsequently conveys protein synthesis inhibition at the ribosome [201]. However, nicking of the A subunit might also occur in the intestinal mucus [202] or is likely induced by bacterial proteases released by lysed EHEC during infection. In this case, one can assume the presence of nicked, but possibly also native Stx2a in blood during the natural course of EHEC infections and in the transition from hemorrhagic colitis to eHUS.

There are very limited data showing presence of Stx2a in blood of EHEC-infected patients. Lopez and coworkers have detected free Stx2a in sera of three EHEC-infected patients during the prodromal intestinal phase before the onset of eHUS [203] and they have found very low amounts of Stx2a in sera of eight patients with overt HUS [204]. Arfilli and coworkers have demonstrated the presence of functional active Stx2a in sera of three EHEC-infected patients.
during hemorrhagic colitis [205]. However, none of these studies presented any data on the structure and/or conformation of Stxs in the blood of eHUS patients.

However, it seems worth to note that cytotoxic activity of both toxin preparations was comparable indicating that the receptor binding sites located in the B subunit [206] of Stx2a are not affected and that also the cooperation between B and A chains (binding to Gb3 receptor and intracellular enzymatic action, respectively) was operative for both toxin batches.

In conclusion, this study reveals more insights into (i) the effects of purification protocols on the structure and function of isolated Stx2a, and (ii) if it is not an artefact and if both the toxin forms exist in vivo, the variable biological properties could be the underlying key points of eHUS pathogenesis, such as, Stx2a binding to fH and cytotoxicity towards the gold standard, Vero cells. Different Stx purifications using different bacterial strains and different purification protocols lead to different abilities of the toxin. Thus, there is urgent need for a standardized protocol for Stx purification. On the other hand, on the basis of the structure/function relationship described here, an important goal would be the characterization of the structure of Stx2a circulating in EHEC-infected patients and its relation to the pathogenic process, i.e. development of eHUS or recovery after the intestinal prodromal phase. Furthermore, the understanding of the real structure and conformation of HUS-inducing Stx2a could allow the development of appropriate animal models aimed at studying the impact of the different toxin form (nicked or native) on eHUS pathogenesis. This may improve strategies for the prevention of the life-threatening transition from hemorrhagic colitis to eHUS in EHEC-infected patients, especially children.
6.2 Shiga toxin 2a binding to complement components C3b and C5 and upregulation of their gene expression in human cell lines

EHEC associated-HUS is an important cause of pediatric acute kidney injury causing significant morbidity and mortality. Stx2a has been demonstrated to bind to fH in the fluid phase and activate complement, in addition delaying its cofactor activity on Chinese hamster ovary cells, upon binding [1]. Specifically, Stx2a targets the membrane-binding portion of fH. Therefore, fH can still function in fluid phase but not on cell surfaces, which is identical to the effect produced by the fH loss of function mutations in aHUS. Further studies on the role of complement in eHUS rendered reduced expression of complement inhibitor CD59 on human renal tubule and glomerular endothelial cells [164]. These studies were corroborated by studies from Thurman and colleagues who have reported elevated levels of complement components Bb and soluble MAC (sC5b-9) in the serum of 17 eHUS patients [161], and Ståhl and coworkers who have provided evidence of platelet-leukocyte complexes and microparticles (released from cells) in the blood of eHUS patients [207], elucidating the role of complement in eHUS pathogenesis.

Project II of this thesis presents the most direct evidence of complement involvement in eHUS pathogenesis by showing that Stx2a binds to the two most central molecules of the complement cascades, C3b and C5 but not to C7. It should be noted that C7 was just a control which could also have been any other terminal complement component. It is possible that binding, in particular of C3b, in the fluid phase can trigger opsonization of Stx2a via the GB3 receptors on monocytes and macrophages. The toxin is not cytotoxic to these cell types and the cells respond to the toxin by upregulating the production of cytokines such as IL-1β and TNF-α [208]. Furthermore, Stx2a could also act as an antigen for C3b binding and activate the complement in the fluid phase. The AP is slowly activated spontaneously by hydrolysis of the internal C3
thioester bond and further triggered by contact with various proteins, lipids and carbohydrate structures on microorganisms and other foreign surfaces [121].

Immunoglobulins together with complement component depositions are frequently encountered in glomeruli of diseased kidneys. However, the question remains whether these complement proteins are synthesized hepatically and thus acquired from the circulation or are produced locally by intrinsic renal cells. This study here shows that increasing concentrations of Stx2a can upregulate expression of C3 and C5 mRNA in the HK-2 cell line. These GB3 positive cells were used for screening Stx2a concentrations that could stimulate complement upregulation and concentrations as low as 1ng/ml were able to upregulate C3 expression up to approximately 33% and C5 expression up to 78%, and a concentration of 100ng/ml was able to upregulate C3 expression up to approximately 91% and C5 expression up to 120%. Furthermore, CiGEnC cells were able to upregulate C3 and C5 mRNA expression at higher Stx2a concentrations of 1µg/ml and 10µg/ml, but not at lower concentrations.

There has been previous recognition of complement production in extrahepatic sites, and this local production may be involved in inflammatory responses [209]. As a matter of fact, the activation of these complement proteins can also occur in the cytoplasm prior to its secretion, in contrast to the well-established pathways of complement [209]. Local complement components with increased mRNA expression for several complement genes like C3, C4 and C2, were found in the nephritic kidneys of two murine models of immune-complex disease [210, 211] which spontaneously develop SLE-like disease. For fB, two mRNA transcripts, differing in size, were found in nephritic kidneys, suggesting a possible contribution of local complement from the infiltrating cells, in addition to intrinsic production [210, 211].
Recently, Satyam and coworkers demonstrated that intestinal injury occurs during ischemia prior to reperfusion and that locally produced C3 in the villus epithelium primarily contributes to this injury [212]. Also, Feucht et al. [213] demonstrated expression of C4 mRNA in human kidneys in amounts comparable to those of the liver. Early studies by Sacks et al. [214] in kidney biopsies from patients with immune complex glomerulonephritis, cell-mediated interstitial nephritis, or non-immune glomerular injury showed significant C3 expression in the first two groups of patients while this was not obvious in the latter group. In another study, C2 mRNA expression was detected in the biopsies of patients with cellular rejection and cyclosporin A nephrotoxicity [215].

However, it is difficult to ascertain the source of complement in these studies. To overcome this problem, Andrews and coworkers investigated donor-specific C3 mRNA in kidneys of patients with a renal graft pertaining to the fact that the patients were mismatched in their C3 allotypes allowing them to discriminate between the C3F and C3S allotypes [216]. Donor-specific C3 mRNA expression was observed in six of the nine case studies and by staining of frozen sections of renal cortex material, not only in tubular epithelial cells, but also glomerular cells were identified as producers of C3.

Shiga toxin has been known to cause organ injury in two ways; by direct cytotoxic activity on GB3 positive cells and by binding to fH [1], which binds to C3 of the AP and deactivates complement on self cells. While this explains the disposition of systemic complement in organ injury, several studies have also explained a possible relationship between local complement production and tissue injury. Studies in the passive Heymann nephritis model of membranous glomerulonephritis demonstrated a parallel relationship between an increase in renal epithelial expression of C4 and the development of proteinuria [217]. Moreover recent evidence shows that
C3 production by kidney epithelial cells regulates acute renal transplant rejection [218, 219]. Other studies suggested the progressive increase in synthesis of C2, C4, C3 and fB in nephritic kidneys with the development of renal injury [211, 213]. While these studies do not establish a definitive role for the contribution of locally produced complement, significant contribution by hepatocyte derived complement could not be excluded.

It has been reported by Hurley and group that Stxs cross polarized intestinal epithelial cells (IECs) via a transcellular route and remain biologically active and that this diaspora is enhanced significantly in the opposite direction by neutrophil transmigration across basolateral-to-apical side of polarized T84 IECs [220]. While the mechanism of displacement of Stxs across gut-blood barrier has been decoded to some extent, its implications upon the intracellular homeostasis still remain ambiguous. Upon stimulating a gut epithelial cell line, HCT-8, with increasing concentrations of Stx2a, upregulation of C3 and C5 mRNA expression was observed here at higher Stx2a concentrations of 1µg/ml and 10µg/ml, but not at lower concentrations. C3 gene expression was earlier found in intestinal epithelial Caco-2 cells producing C3 along with other complement components with intracellular cathepsins cleaving C3 to produce anaphylatoxin C3a [212]. The study also reported that addition of cathepsin inhibitors resulted in reduction of C3a generation and mice treated with cathepsins inhibitors or cathepsin B-deficient mice suffered limited intestinal injury during the ischemic phase of ischemia-reperfusion injury.

The possibility of intracellular AP C3 convertase formation cannot be entirely excluded and recent studies showing C3 and C5 cleavage by proteases of the cathepsin family could only connect dots for the possibility of new paths of complement activation [209]. Thus locally produced complement and its subsequent possible activation in the gut can account for previously unrecognized damage occurring during eHUS diarrhea. This also upholds with the renal damage
caused by complement activation by Stx2a during eHUS and aggravation by locally produced complement therefore cannot be ruled out completely.

In addition, locally produced complement can also contribute to previously unanticipated roles in tissue development and homeostasis, involving metabolic functions via crosstalk between complement and intracellular sensors and effector pathways. Advancements in this expanse have been produced by Kemper and coworkers elucidating the role of locally produced and intracellularly activated C3 components in T-cell homeostasis [165].

The absence of detection of complement proteins in cell lysates or in cell supernatant could account for complement being masked or for activating the resting cells resulting in inflammatory events. Studies have suggested mRNA production and secretion of pro-inflammatory molecules C5a and C3a by human colonic and proximal tubular cell lines, respectively. These cells are known to express C5a receptor (C5aR) and C3a receptor (C3aR) and can detect and respond, in turn activating the ERK pathways and cytokine upregulation [166, 175, 221].

However, to prove conclusively that local production of complement contributes to local homeostasis of regulation or remodeling, either during development or following injury, one has to await studies in complement deficient animals and/or transplantation of kidneys from complement-sufficient animals or individuals into complement-deficient counterparts. Further possibilities are to repopulate renal cells of complement-deficient animals by complement-sufficient cells. In addition, reintroduction of missing components under the control of tissue-specific promoters may help to establish a role of particular components in renal disease.

Systemic levels of complement are mainly determined by hepatic production and fulfil important roles in immune defense and immune complex processing. Because of the large size of
complement components, one can imagine that penetration of plasma complement into tissues is limited. Therefore it is possible that in many tissues locally produced complement may compensate for this relative lack of plasma complement. The local presence of various growth factors and cytokines may enhance local synthesis of complement and provide means for enhanced defense against local infections and/or modulating of injured tissue. At the same time, uncontrolled upregulation can also lead to dysregulations and can have serious unprecedented implications, such as, in case of eHUS. With this study, evidence of local complement production in gut and renal cell is provided as well as the involvement of complement in eHUS pathogenesis corroborated. This upregulation could act as a signal for pathogen invasion and opens new doors for further investigations into the pathology of EHEC leading to eHUS.

### 6.3 Stx2a binding to MASP-1 and MASP-3 and the involvement of lectin pathway in eHUS pathogenesis.

The conventional textbook views have described LP and AP as two individual pathways where the former is activated by recognition of PAMPs or DAMPs by the PRMs of the LP and the latter by spontaneous hydrolysis of C3 which is amplified upon antibody-independent binding of intrinsic or extrinsic danger signals. Contrary to this, new ideas have emerged suggesting the promiscuous nature of MASPs, the LP serine proteases, in their substrate binding specificities thereby involving in a multitude of previously unprecedented biological pathways.

Shiga toxin is actively involved in organ injury through activation of the complement via the AP, as described previously. For the first time, this project presents preliminary results of the interaction of Stx2a with recombinant MASP-1 and MASP-3 thus providing initial insights into the possible involvement of LP in eHUS. MASP-1 and MASP-3, both of which are encoded by
MASP1, differ in their serine protease domains [113] and bind to Stx2a differentially, with MASP-3 being more profound in binding to Stx2a than MASP-1. It can be postulated that the toxin binds to these proteases at domains barring the common heavy chain as Stx2a showed no binding to rMASP-HC. To support this theory, Stx2a also did not bind to the inactive, non-enzymatic rMAP-1, which is a product of differential splicing of the MASP1 gene and lacks the serine protease domain completely along with the CCP part of the heavy chain.

It is unknown whether this binding further activates or impedes the LP. Preliminary experiments could not hint on the role of this binding, but certainly opens avenues for further investigations.

Recent breakthrough reports by Takahashi and coworkers in 2010 stated that MASP-1 is an essential protease of both the lectin and alternative complement pathways [183]. The group showed that the serum of MASP1/3−/− deficient mouse model has inactive AP with inactive pro-factor D (pro-fD) in circulation [183]. Furthermore, they showed that MASP-1 converted pro-fD into the active form in vitro. This article embarked sparks of conflict when soon after Degn et al. published in 2012 that a patient harboring defective MASP gene resulting in deficiency of both MASP-1 and MASP-3 had a perfectly normal functioning of the AP and rather a nonfunctional LP, which could be restored by MASP-1 [222]. This also implied that although MASP-2 is able to auto-activate under artificial conditions, MASP-1 is crucial for LP activation under physiological conditions [222]. Finally, reports by Dobo et al. in 2016 suggested that in normal resting blood unperturbed by ongoing coagulation or complement activation, fD is present predominantly in its active form, suggesting that resting blood contains at least one pro-fD activating proteinase that is not a direct initiator of coagulation or complement activation [223]. They further showed that all three MASPs were able to activate pro-fD in vitro whereas in resting blood neither MASP-1 nor MASP-2 was able to activate pro-fD. By evolving a specific MASP-3
inhibitor, they could unambiguously prove that activated MASP-3 is the exclusive activator of pro-fD in resting blood, which demonstrated a fundamental link between LP and AP [223].

With evidence of a definitive link between AP and LP, and with the previous knowledge of interactions of Stx2a with various components of AP viz. fH, C3b and C5, and serine proteases of LP viz. MASP-1 and MASP-2 in vitro, there remains a huge scope for investigating the involvement of LP in eHUS and further explore the influence of Stx2a over the LP-AP link. The preliminary results of this project from this thesis can pan out into a probable collaborative project between LP specializing lab of Dr. Peter Garred in Copenhagen, Denmark and AP specializing lab of Dr. Würzner in Innsbruck, Austria.
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Transplantation


8. APPENDIX

8.1 Publications


- Chatterjee S, B-O B, Justesen L, Posch W, Kenno S, Garred P, Orth-Höller D, Würzner R. Shiga toxin 2a binds to complement components C3b and C5 and upregulates their gene expression in human cell lines. Manuscript ready for submission. 2019
8.2 Posters

- **Chatterjee S, Posch W, Kenno S, Orth-Höller D, Würzner R**

  *Upregulation of complement C3 gene expression in human cell lines by shiga toxin 2a and binding of the toxin to C3*

  VTEC, Abstract book, 2018 (Florence, Italy)

  CIIT Science Day, Abstract book, 2018 (Innsbruck, Austria)

- **Chatterjee S, Posch W, Kenno S, Orth-Höller D, Würzner R**

  *Shiga toxin 2a upregulates C3 expression in gut and renal cells and also binds to C3*

  Abstract book Page 29,

  DGHM, 2018, (Bochum, Germany)

- **Chatterjee S, Brigotti M, Striegel I, Orth-Höller D, Würzner R**

  *Variable biological properties of two different preparations of Shiga toxins yielding new insights into eHUS pathogenesis*


  EMCHD, 2017, (Copenhagen, Denmark) [224]

- **Chatterjee S, Rosbjerg A, Striegel I, Huber S, Garred P, Orth-Höller D, Würzner R**

  *Shiga toxin 2a binds to MASP-3 – Involvement of Lectin Pathway in eHUS pathogenesis?*

  HUS & related disorders, Abstract book, A2, 2016 (Innsbruck-Igls, Austria)
8.3 Curriculum Vitae

Sneha Chatterjee
Doctoral Programme "HOROS – Host Response in Opportunistic infections"
Division of Hygiene & Medical Microbiology
Medical University of Innsbruck
Shöpfstrasse 41/2
A-6020 Innsbruck
Sneha.Chatterjee@i-med.ac.at, snehachatterjee0@gmail.com

Education:

2015-2018 Ph.D. Dissertation
Doctoral Programme: HOROS
Medical University of Innsbruck, Austria

2013-2015 M.Sc. Applied Microbiology
School of Bio-Sciences and Technology
Vellore Institute of Technology, VIT University, India
M.Sc. Dissertation
Division of Microbiology and Cell Biology,
Indian Institute of Science, India

2010-2013 B.Sc. (H) Microbiology
Department of Microbiology
University of Delhi, India

Further Education: 1. Stay abroad programme (Ph.D) in Lab. of Molecular Medicine,
Righospitalet, Copenhagen, Denmark (Apr 2018-Aug 2018).
2. Workshops and conferences:

VTEC 2018 (Florence, Italy)
EMCHD 2017 (Copenhagen, Denmark)
HUS 2017 (Innsbruck Austria)
Bio-summit meet 2014 (Vellore, India)

3. International talks:

Infection and Immunity, Joint Doctoral Retreat; HOROS Innsbruck & Univ. Copenhagen (2016)

Publications:

