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# ***Staphylococcus aureus* $\alpha$ -toxin – natural fragments and effects on intestinal epithelial cells**

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**Karolinska  
Institutet**

Stockholm, June 2011

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**Front cover:** Top view of heptameric *S. aureus*  $\alpha$ -toxin missing residues 1-71 (i.e.  $\alpha$ -toxin fragment 26 kD).

Published by Karolinska Institutet. Printed by *Larserics Digital Print AB*

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ISBN 978-91-7457-404-3

## ABSTRACT

*Staphylococcus aureus* is a human pathogen, commonly found in healthy humans as a part of normal flora.  $\alpha$ -toxin is thought to be largely responsible for the pathogenesis of the bacterium. This cytotoxin has been shown to irreversibly damage the membrane of a great variety of cells including erythrocytes, endothelial cells, and mouse adrenocortical cells.  $\alpha$ -toxin is secreted as a water soluble monomer that binds to mammalian cell membranes, oligomerizes and forms a heptameric transmembrane pore. Pore formation leads to osmotic shock and cell death.

One of the aims of the present work was to understand the biological relevance of four naturally occurring  $\alpha$ -toxin fragments isolated from *S. aureus* culture medium. All four fragments bound to and formed transmembrane channels in egg-phosphatidyl glycerol vesicles. Oligomer formation on the lipid membrane was a prerequisite of channel formation. Interestingly, alleviated hemolytic activity was partially recovered by acidification of the medium. We have demonstrated that some toxin fragments can be proteolytically generated from intact staphylococcal  $\alpha$ -toxin by *S. aureus* extracellular co-expressed proteases. All isolated fragments induced intoxication of mouse adrenocortical Y1 cells *in vitro*. Only one fragment, missing the first eight N-terminal amino acids, induced irreversible intoxication of Y1 cells in the same manner as the intact toxin.

After we established that  $\alpha$ -toxin fragments indeed were biologically active, we demonstrated that  $\alpha$ -toxin treatment induced loss of junctional proteins (ZO-1, ZO-3, E-cadherin, and occludin) in human intestinal Caco-2 cells. Surprisingly, when  $\alpha$ -toxin was applied from the basolateral side of the model epithelium the trans-epithelial resistance (TER) decreased significantly in a dose- and time-dependent manner; while no significant changes in TER were induced by application from the apical side. To investigate the influence of  $\alpha$ -toxin on calcium homeostasis of Caco-2 cells, we measured  $[Ca^{2+}]_i$  in calcium (1 mM) containing and calcium free buffer.  $[Ca^{2+}]_i$  was significantly increased about 10 min after the addition of  $\alpha$ -toxin, whereas no significant signal increase was observed in calcium free buffer. Our result shows a positive correlation between an increase in intracellular  $[Ca^{2+}]$  and epithelial barrier function and suggests that  $\alpha$ -toxin treatment might be involved in maintaining TER against external stimuli. Our finding gives a possible explanation of how bacteria disseminates in the blood-stream of sepsis patients: in *S. aureus*-associated sepsis, the membrane damaging  $\alpha$ -toxin may circulate in the blood and affect the intestinal barrier, resulting in translocation of enteric bacteria. This might lead to the spread of endotoxin (LPS) in the blood and further aggravate sepsis syndrome.

In order to prove this hypothesis we performed animal experiments in order to determine whether bacterial translocation across the intestinal epithelium can be induced by intravenous application of  $\alpha$ -toxin. The present work indicates a strong correlation between intestinal hyperpermeability and bacterial translocation and suggests that  $\alpha$ -toxin may thus aggravate the septic condition. Demonstrating  $\alpha$ -toxin involvement in serious inflammatory disease syndrome, caused by a vicious circle of *E. coli* endotoxemia and bacterial translocation, will be a challenge for the future.

## List of Publications

- I. Young-Keun Kwak, Martin Högbom, Patricia Colque-Navarro, Roland Möllby, and Beatrix Vécsey-Semjen. Biological Relevance of Natural  $\alpha$ -Toxin Fragments from *Staphylococcus aureus*. J. Membr. Biol. (2010) 233:93–103.
- II. Beatrix Vécsey-Semjen, Young-Keun Kwak, Martin Högbom, and Roland Möllby. Channel-Forming Abilities of Spontaneously Occurring  $\alpha$ -Toxin Fragments from *Staphylococcus aureus*. J. Membr. Biol. (2010) 234:171–181.
- III. Young-Keun Kwak, Elena Vikström, Karl-Eric Magnusson, Beatrix Vécsey-Semjen, Patricia Colque-Navarro, and Roland Möllby. The *Staphylococcus aureus*  $\alpha$ -toxin perturbs the barrier function in Caco-2 epithelial cell monolayers by altering junctional integrity. Resubmitted to *Infection and Immunity* 2011 Mar.
- IV. Young-Keun Kwak, Beatrix Vécsey-Semjen, Patricia Colque-Navarro, and Roland Möllby. *Staphylococcus aureus*  $\alpha$ -toxin promotes translocation of enteric bacteria *in vitro* and *in vivo*. Manuscript.

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## LIST OF ABBREVIATIONS

[Ca <sup>2+</sup> ] <sub>i</sub>	Cytosolic calcium concentration
Caco-2	Human colon carcinoma cell line
ER	Endoplasmic reticulum
EPG	Egg-phosphatidyl glycerol
HRBC	Human red blood cell
HU	Hemolytic unit
MODS	Multiple organ dysfunction syndrome
RRBC	Rabbit red blood cells
TBS	Tris buffer saline
TER	Transepithelial electrical resistance
TSST-1	Toxic shock syndrome toxin-1
ZO-1	Zonula occluden-1

# 1 Introduction

## 1.1 *Staphylococcus aureus*

### 1.1.1 General

*Staphylococcus aureus* (staphylé, “bunch of grapes”; coccus, “grain” or “berry”; aureus, “golden”: yellow grapelike cocci)) is the most virulent and the best known member of the *Staphylococcus* genus. It is a facultative anaerobe Gram-positive coccus. Other than *S. aureus*, the genus comprises several clinically important species such as *S. epidermidis*, *S. lugdunensis*, and *S. saprophyticus*. Major staphylococcal diseases range from mild skin infection (e.g. impetigo, cellulitis, scalded skin syndrome) to serious systemic infection (e.g. bacteremia or sepsis, pneumonia, and endocarditis) which may further lead to heart failure and toxic shock syndrome contributed by various bacterial pathogenic strategies.

### 1.1.2 Virulence factors

A number of virulence factors associated with the pathogenic strategies of *S. aureus* have been identified (Table 1). Classified into cell structural components and extracellular enzymes, and toxins, they express diverse biological functions. Expression of each virulence factor occurs in a coordinated manner. Several factors involved in the regulation of the virulence factors are related to culture medium and growth rate. For example, their microbial surface components that recognize adhesive matrix molecules (MSCRAMM) are generally expressed in exponential growth phase, whereas extracellular virulence factors such as toxins and enzymes are synthesized after post-exponential growth phase. This might be due to bacterial preferential virulence factors required for different stages of infection and the importance of regulatory genes in virulence as was demonstrated by Chung et al 1994 [1, 2].

#### 1.1.2.1 Extracellular enzymes

The major enzymes produced by *S. aureus* comprise metalloprotease, serine protease, nuclease, and lipase. Most *S. aureus* strains produce hyaluronidase and  $\beta$ -lactamase. Several major gene regulators such as SspA (serine protease), Aur (metalloprotease), and SspB (cysteine protease) are characterized.

A serine protease, V8 protease (*S. aureus* serine glutamylendopeptidase) is encoded by SspA gene and synthesized as an inactive preproenzyme which is further processed by proteolytic cleavage yielding an inactive proenzyme [3, 4]. The inactive proenzyme can

be further activated by *S. aureus* metalloprotease, aureolysin [5]. V8 protease has narrow substrate specificity by specifically cleaving the carboxy-terminal side of dicarboxylic amino acids. Therefore, it was considered as a digestion enzyme providing bacteria with low molecular nutrients. Additionally, V8 protease impairs host defense properties by cleaving human immunoglobulin heavy chains, as demonstrated in vitro [6], and degrades the human antimicrobial peptide LL-37 [7].

Aureolysin is a zinc-dependent extracellular metalloprotease which consists of 301 amino acids [8]. Aureolysin is synthesized as a preproenzyme and the *aur* genes have been shown to be widely distributed among *S. aureus* strains. The protein expression is up-regulated by *agr* and repressed by *sarA*. It cleaves peptide bonds on N-terminal side of residues with low substrate specificity and zinc is required for activity. It has been shown that aureolysin activates an inactive precursor of a *S. aureus* protease, V8 protease [9].

The enzyme cleaves a complement factor C3, weakening the host immune response [10]. It also cleaves cathelicidin LL-37, a human bactericidal peptide, thus enabling *S. aureus* to evade the innate immune system [7]. Aureolysin is also significantly involved in the fibrinolytic system of *S. aureus* by degrading plasminogen activator inhibitor for immune evasion [11].

#### 1.1.2.2 Extracellular toxins

The functions of extracellular toxins produced by *S. aureus* include degradation, hemolysis and apoptosis of host cells and disturbance of the host immune defense system.

Two toxins,  $\alpha$ -hemolysin and  $\beta$ -hemolysin, have been shown to have key roles in *S. aureus* pathogenicity.

##### $\alpha$ -hemolysin ( $\alpha$ -toxin)

A water-soluble 33kD polypeptide protein,  $\alpha$ -toxin (encoded by the *hla* gene) contains 293 amino acids. It is synthesized by most *S. aureus* strains during the exponential growth phase, and toxin expression is coordinately controlled by the accessory gene regulator (*agr*), staphylococcal accessory regulator (*sar*), and *S. aureus* exoprotein expression regulator (*sae*) [12, 13].  $\alpha$ -toxin monomers bind to the lipid bilayer of eukaryotic cell membranes, form membrane bound oligomers, and after conformational changes form a heptameric transmembrane channel with an inner diameter of 1-2 nm. Channel formation leads to chemiosmosis, the release of small molecular weight



cytoplasmic contents, and cell lysis. The structure of the active heptameric transmembrane channel had been determined in detergent micelles by Song et al., [14] but the three-dimensional structure of the monomeric  $\alpha$ -toxin is deduced from the heptameric structure in lack of crystallographic data.

The open heptameric transmembrane channel consists of three domains: the cap, rim, and stem domains, formed a shape like a mushroom. The hydrophilic cap domain is composed of  $\beta$ -sandwich structures held together by the seven amino latches from seven protomers. The rim domain forms a three-strand  $\beta$ -sheet under the cap domain. An aromatic amino acid rich crevice is formed between the cap and rim domains involved in interactions with phospholipid head groups of target cell membranes [14, 15]. The stem domain is composed of a 14-strand anti-parallel  $\beta$ -barrel, forming the effective transmembrane channel.

The steps leading to channel formation might be 1) binding of protomers to susceptible cell membranes [16], 2) forming oligomers, and 3) insertion of the anti-parallel  $\beta$ -strand into the membrane, forming the transmembrane stem of the channel [17].

Conformational changes have been shown to accompany heptamerisation and channel formation [18-20].

Target cells of  $\alpha$ -toxin include rabbit erythrocytes, human platelets, erythrocytes, monocytes, lymphocytes, and endothelial cells. Interestingly, different cells show a wide range of sensitivity to  $\alpha$ -toxin attack. For example,  $\alpha$ -toxin is 400 times more reactive against rabbit erythrocytes than to human erythrocytes [21, 22].

The diameter of the final  $\alpha$ -toxin channel depends on the type of target cells and the concentration of applied toxin. For example, larger sized channels, through which  $\text{Ca}^{2+}$  and nucleotides could permeate, were formed by higher concentrations of  $\alpha$ -toxin application but only monovalent ions could permeate through smaller sized channels formed when lower concentrations of  $\alpha$ -toxin were applied [23, 24].

### $\beta$ -hemolysin

Identified at first by Glenn and Stevens in 1935 [25],  $\beta$ -hemolysin was demonstrated to be highly hemolytic to sheep erythrocytes but not to rabbit erythrocytes, and its toxicity was enhanced by incubation under 10 °C after treatment at 37 °C (i.e. hot-cold lysis).

**Table 1. Virulence factors of *Staphylococcus aureus***

Virulence factors	Function/Effect
<b>Cell structural components</b>	
Capsule	Inhibit chemotaxis and phagocytosis
Peptidoglycan	Prevent osmotic lysis; Cause inflammatory response [26]
Teichoic acid	Bacterial adherence [27]
Protein A	Prevent antibody-mediated clearance
<b>Extracellular enzymes</b>	
Nuclease	
Lipase	Lipid hydrolysis
Metalloprotease (Aureolysin)	Host immune evasion
Thiolprotease	
Hyaluronidase	Damage connective tissue
Alkaline/acid phosphatase	
$\beta$ -lactamase	Antibiotic resistance
Serineprotease (V8 protease)	Host immune evasion
<b>Extracellular toxins</b>	
Hemolysin ( $\alpha$ -, $\beta$ -, $\gamma$ -, and $\delta$ -)	Cytolysis and hemolysis
Exfoliative toxin (A and B)	Cause scalded skin syndrome by desmosomal cadherin cleavage (for review, [28])
Enterotoxins (A, B, C, D, and E)	Food poisoning and toxic shock syndrome [29]
Toxic shock syndrome toxin-1	Superantigens, endothelium permeability [30]
Succinic oxidase factor	
<b>Secreted proteins</b>	
Coagulase	Avoid phagocytosis by forming fibrin coating
Staphylokinase	Facilitate bacterial penetration by lysing fibrin clot [31]

\*Table adapted from [32] (for reviews [33]).

A 39-kD polypeptide protein,  $\beta$ -toxin (encoded by the *hly* gene) contains 330 amino acids and is produced by a number of *S. aureus* strains in exponential growth phase like other exotoxins.

$\beta$ -hemolysin activity is highly species-dependent. For example, it is highly sensitive on sheep, cow, and goat erythrocytes but not on murine and canine erythrocytes. The reason might be due to its dependency on membrane sphingomyelin contents which are hydrolyzed to phosphorylcholine and ceramide by  $\beta$ -toxin [34].

Hot-cold lysis with  $\beta$ -toxin can be explained in a two-stage process which includes: 1) hydrolysis of sphingomyelin which is generally located on the outer leaflet membrane

by  $\beta$ -toxin at 37 °C and, 2) physical disruption of the membrane on cooling due to the phase separation between phosphorylcholine and ceramide [35].

#### Other *S. aureus* toxins

Enterotoxins and Toxic shock syndrome toxin 1 (TSST-1) have an effect on attachment and permeability of the endothelial barrier [30].

## **1.2 *Staphylococcus aureus* diseases**

### **1.2.1 General**

*S. aureus* is the most virulent species in the *Staphylococcus* genus. *S. aureus* was the most common cause of nosocomial cases of pneumonia, surgical-wound infection, and was the second most common cause of bloodstream infection between 1990 and 1992. The overall incidence of staphylococcal infections during the last decade significantly increased in the United States [36, 37].

*S. aureus* disease is caused through two different infection mechanisms. In the non-toxin-mediated infection mechanism, *S. aureus* directly invades and destroys the target tissue causing conditions such as bacteremia (presence of bacteria in the blood), endocarditis, pneumonia, osteomyelitis, and septic arthritis [9]. In the toxin-mediated infection mechanisms, toxins produced by the bacteria cause conditions such as staphylococcal scalded skin syndrome, staphylococcal food poisoning, and toxic shock syndrome [32].

**Bacteremia:** Most *S. aureus* bacteremia are hospital-acquired [38]. With its abilities to adhere to and colonize target tissue, to invade the bloodstream and to evade host immune responses, *S. aureus* can efficiently infect humans [39]. The infection begins with adherence and colonization of *S. aureus* on the nares and damaged skin using several virulence factors such as clumping factor, various binding proteins to host matrix proteins (e.g. fibronectin, fibrinogen, elastin, and collagen) and coagulase. Once the organism succeeds in colonization, further staphylococcal invasion is facilitated by various exo-toxins (e.g. exfoliative toxin, hemolysins), exo-enzymes (e.g. metalloprotease, hyaluronidase) [6]. Once *S. aureus* enters into the host, it can evade and/or disturb the host's immune defense by secreting protein A, leukotoxins, and superantigens (e.g. enterotoxins and TSST-1) [40]. The overall mortality rate attributed

to *S. aureus* infections has been estimated to range between 11–43% in the past 15 years [41].

Sepsis: sepsis is defined as a systemic inflammatory response to infection. Severe sepsis is accompanied by organ dysfunction, perfusion abnormalities, and/or hypotension [42].

Interestingly, not all patients with sepsis may have bacteremia [43] and not all bacteremic patients may have systemic inflammatory responses [44]. Sepsis is often initiated at the infected foci and recruitment of host immune cells to this site leads to an inflammatory cytokine cascade [45]. *S. aureus* is one of most common Gram-positive bacteria found in patients with sepsis [46, 47]. Several virulence factors of *S. aureus* may be involved in the inflammatory cascade including bacterial cell wall components such as peptidoglycan, teichoic acid, capsule, and extracellular proteins such as toxic shock syndrome toxin-1, and hemolysins [48].

Peptidoglycan isolated from *S. aureus* has been shown to cause systemic inflammation in rats, showing an increased value of liver injury marker (e.g. alanine aminotransferase), renal failure marker (e.g. creatinine), tumor necrosis factor- $\alpha$ , and IL-6, and IL-10 [49]. Together with protein A, *S. aureus* peptidoglycan has been shown to induce spontaneous aggregation of human platelets, suggesting that it might be one of the causes of disseminated intravascular coagulation (DIC) in staphylococcal sepsis [45]. Injection of peptidoglycan from *S. aureus* has also been shown to induce DIC, leukopenia, and thrombocytopenia in animal models [50, 51].

As a superantigen, TSST-1 non-specifically stimulates the proliferation of CD4 T cells through its interaction with Major histocompatibility complex class II (MHC II) and T-cell antigen receptor (TCR) resulting in an excess monocyte stimulation and secretion of large amount of cytokines such as TNF- $\alpha$ , IL-1, which may in turn cause an inflammatory cascade [52-54]. *S. aureus*  $\alpha$ -hemolysin also has been reported to stimulate monocytes leading to the secretion of IL-1 and TNF- $\alpha$  [55].

## **1.2.2 Defense barriers**

### *1.2.2.1 Endothelial barrier*

Endothelial cells form cell linings in blood vessels and the endothelium separates blood from tissue. Endothelial cells are elongated with sizes between 25–50  $\mu\text{m}$  in length and 10–15  $\mu\text{m}$  in width. Cells bind to each other with adherence junctions (vascular

endothelial (VE) cadherin  $\alpha$ - and  $\beta$ -catenin), and tight junctions (e.g. zona occludins (ZO-1 and ZO-2) and occludin) similar to those formed by epithelial cells [56]. Rather than being an inert barrier, the endothelial barrier allows the exchange of small molecules such as gases, nutrients, and waste products, as well as letting signal molecules or substances cross the barrier for communication with tissue. On the other hand, high-molecular-weight proteins and blood cells are unable to cross the barrier and remain in the circulatory system. Increased endothelial permeability can be observed in acute inflammatory reactions caused by bacteremia and endotoxemia, leading to fluid/plasma extravasation [57, 58].

Bacterial endo- and exo-toxins have been shown to affect the endothelial barrier function either by inducing uncontrolled release of vasoactive mediators (e.g. NO, PGI<sub>2</sub>, and PAF) or direct breakdown of the endothelial barrier [59].

*S. aureus*  $\alpha$ -toxin has been shown to form calcium-permissive pores and cause the release of PGI<sub>2</sub>, NO, and PAF from endothelial cells. Furthermore, the excessive release of vasoactive mediators is induced by cytoplasmic calcium influx through the channels formed by *S. aureus*  $\alpha$ -toxin [60, 61].

It has also been reported that *S. aureus*  $\alpha$ -toxin directly affects the junctional proteins leading to increased endothelial permeability which may in turn promote the inflammatory cascade [62, 63]. In the porcine pulmonary endothelium, the toxin increases endothelial permeability by forming intercellular gaps [64].

#### 1.2.2.2 Epithelial barrier

Epithelial cells form the linings, or the epithelium, of the internal or external surfaces of different organs such as the intestines, lungs, kidneys, and liver. As the first line of host defense, the epithelium protects the organs from endogenous bacteria, bacterial products, and other injuries. Similar to endothelial cells, epithelial cells are physically bound to each other with tight junction proteins (e.g. ZO-1, ZO-3, and occludin), and adherence junction proteins (e.g. E-cadherin and  $\beta$ -catenin). Tight junction proteins are mainly involved in physical barrier functions and are located in the apical side of the epithelium while adherence junctions are involved in signaling functions and the mechanical strength of the junctional complex [65, 66]. Its selective-permeability property enables the epithelial barrier to transport various ions and necessary molecules without uncontrolled passages of antigens or indigenous bacteria in the intestine [67]. Increased intestinal epithelial permeability can be observed in many different

inflammatory diseases including multiple trauma [68], burn injury [69], hemorrhagic shock [70], sepsis [71] and multiple organ dysfunction syndrome (MODS) [72].

Several bacterial toxins have been shown to induce intestinal epithelial hyperpermeability. For example, *E. coli* endotoxin induced translocation of enteric bacteria, even in genetically endotoxin-resistant mice [73]. *Clostridium difficile* toxins induce epithelial hyperpermeability by disrupting intracellular F-actin which is connected to junctional proteins [74] while *Vibrio cholerae* toxin disrupts tight junctions [75]. *S. aureus*  $\alpha$ -toxin has been reported to induce hyperpermeabilization in isolated rat intestine [76] but its pathophysiological mechanism remains as yet unclear.

### **1.3 Bacterial translocation**

#### **1.3.1 General**

Bacterial translocation is the passage of viable and non-viable bacteria and/or their metabolic products which reside in the lumen of the intestine, into the sterile extra intestinal areas via movement through the intestinal epithelial barrier [77]. The bacteria translocating from the intestinal tract pass through the epithelial mucosa and lamina propria in this process and might reach the mesenteric lymph nodes and further sites such as the liver or spleen, [78, 79].

#### **1.3.2 Influencing factors**

Analysis of animal model studies revealed three major conditions under which bacterial translocation may occur [80]. These are:

- 1) Bacterial overgrowth in intestine
- 2) Impaired host immune defenses
- 3) Impaired intestinal barrier function

#### **1.3.3 Clinical implications**

Multiple organ dysfunction syndrome (MODS) is defined as “the presence of altered organ function in an acutely ill patient such that homeostasis cannot be maintained without intervention”. The syndrome can be subdivided into primary MODS, which is a direct result of a well-defined insults (e.g. pulmonary contusion, renal failure), and

secondary MODS, which is characterized by the consequence of a host systemic inflammatory response against the insult [81]. MODS is one of the most common causes leading to death in the intensive care unit [82]. It has been reported that the increased intestinal permeability on admission to the intensive care unit (ICU) is significantly associated with the development of multiple organ dysfunction syndrome [72].

## 2 Aims of the study

The overall aim of this thesis is to investigate the role of *S. aureus*  $\alpha$ -toxin and toxin fragments in the pathogenesis of *S. aureus*. Two major studies were undertaken to achieve this aim.

### 1. Study on the role of $\alpha$ -toxin fragments in *S. aureus* pathogenesis (Paper I and II)

- ▶ To determine the origin of naturally occurring  $\alpha$ -toxin fragments.
- ▶ To investigate the relationships between protein structure and biological function of the  $\alpha$ -toxin fragments and other possible factors involved.

### 2. Study on the role of *S. aureus* $\alpha$ -toxin in pathogenesis (Paper III and IV)

- ▶ To determine the effect of  $\alpha$ -toxin on the permeability of the intestinal epithelium.
- ▶ To investigate the effect of  $\alpha$ -toxin on enteric bacterial translocation in mice



### 3 Materials and Methods

#### 3.1 Hemolysis assay

Rabbit erythrocytes were washed three times by spinning down in PBS at 3000 rpm for 3 min. A part of the erythrocytes were taken from the sediments and suspended in TBS (pH 7.5) at 1% concentration.

A 100 µl volume of test sample was serially diluted with the same amount of TBS in a 96-well plates and 100 µl of 1% rabbit erythrocyte suspension was added. After incubation of the plates at 37 °C for 30 min, the absorbance of the erythrocytes was measured by spectrophotometer at 620 nm.

In order to obtain precise value of hemolytic unit, a two-point interpolation method was used for the calculation as follows [83]:

$$\text{Titer (HU/ml)} = 2^X$$

$$X = n + ((A(50) - A(n)) / (A(n+1) - A(n)))$$

Where..

HU = hemolytic unit

n = the last dilution number that resulted in lysis of less than 50%

A(50) = 50% lysis reference absorbance value

A(n) = absorbance value of the dilution number n

A(n+1) = absorbance value of the dilution number n+1

#### 3.2 Cell culture and transepithelial electrical resistance (TER)

A two compartment cell culture model was used to grow Caco-2 cells in monolayers with tight junctions (Fig. 3.1). Briefly, Caco-2 cells (passage 80–90), grown in Dulbecco's minimum essential medium (DMEM) (GIBCO/BRL) with 20% fetal bovine serum and 1% penicillin/streptomycin, were seeded at a density of  $10^5$  cells/cm<sup>2</sup> into permeable culture inserts (3415, pore size 3 µm, Transwell, Corning, NY) in 24-well plates. The cells were cultivated at 37°C in 5% CO<sub>2</sub> until they formed a monolayer with a transepithelial electrical resistance (TER) of 500–700 Ω·cm<sup>2</sup>. It took approximately 10–15 days to reach a confluent layer. Before the start each experiment, cells were incubated in antibiotic-free media overnight. The TER across the Caco-2 cell monolayers was measured with an epithelial volttohmmeter (World Precision Instruments, Sarasota, FL). The TER value was calculated as follows [84]:

$$\text{TER } (\Omega \cdot \text{cm}^2) = (\text{Monolayer resistance } (\Omega) - \text{Blank resistance } (\Omega)) \times \text{Insert membrane growth area } (0.33 \text{ cm}^2)$$

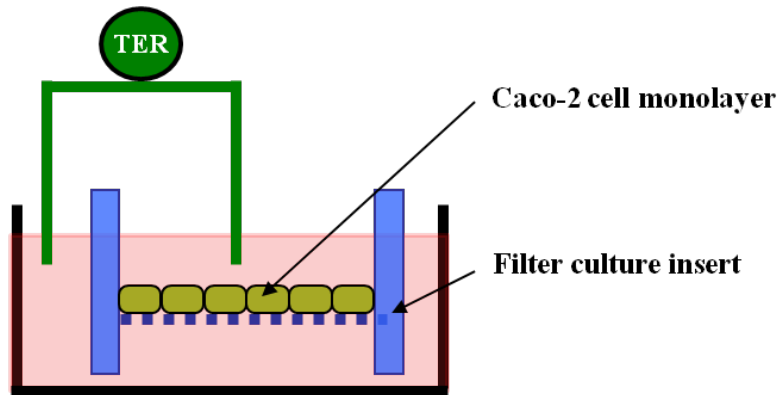


Figure 3.1 The measurement of transepithelial electrical resistance (TER) in the two-compartment cell culture model system with Caco-2 cells.

### 3.3 Bacterial translocation model with *Escherichia coli*

As per the two-compartment cell culture model described above, a Caco-2 cell monolayer grown on polycarbonate cell culture inserts (12 mm diameter, 8  $\mu\text{m}$  pore size, Millipore, Carrigtwohill, Ireland) was washed twice in PBS and incubated for 2 h in antibiotic-free media. *S. aureus*  $\alpha$ -toxin was applied basolaterally (i.e. in the outer well) at a concentration of 0.4  $\mu\text{g}/\text{ml}$ , 5 min before bacterial infection.

*E. coli* was cultivated at 37°C overnight and washed once in PBS by centrifugation. The bacteria were resuspended in PBS at a density of  $10^9$  CFU/ml.

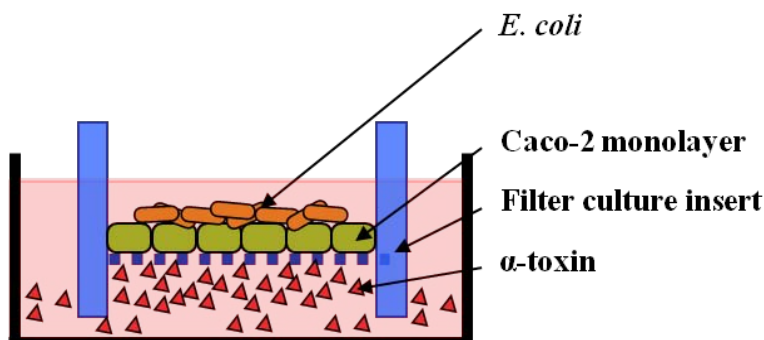


Figure 3.2 The two compartment translocation assay model used to investigate the effect of *S. aureus*  $\alpha$ -toxin on Caco-2 cell monolayer integrity following *E. coli* infection.

The Caco-2 cell monolayer ( $\text{TER} = 500\text{--}600 \Omega\cdot\text{cm}^2$ ) was thereafter infected with approximately  $2\times 10^8$  CFU/ml of *E. coli* for 5 h (See Figure 3.2). The infected Caco-2 cell monolayers were incubated in 5%  $\text{CO}_2$  at  $37^\circ\text{C}$  and TER was measured upon each sampling. Samples (200 $\mu\text{l}$ ) were drawn from the basolateral compartments and serially diluted in PBS. A 100 $\mu\text{l}$  of each dilution was then plated onto MacConkey agar plates. The plates were incubated at  $37^\circ\text{C}$  for 24 h, and the number of translocated *E. coli* (CFU) was counted.

## 4 Results and Discussion

One of the major objectives in this thesis is to investigate the possible role of various *S. aureus*  $\alpha$ -toxin fragments by biochemical and biological characterization in the structural and functional view of aspects.

### 4.1 Staphylococcal $\alpha$ -toxin fragments (Paper I and II)

After finding  $\alpha$ -toxin fragmented during the cultivation of strain *S. aureus* Wood 46 strain in our group, we got interested in the generation and possible biological relevance of these naturally occurring toxin fragments. A hypothesis that the generation of the naturally occurring toxin fragments might be due to the proteolytic cleavage with co-expressed staphylococcal proteases was supported by further experimental studies.

#### Purification and N-terminal sequence analysis (Paper I)

Five naturally occurring  $\alpha$ -toxin fragments were successfully purified from *S. aureus* culture supernatant with the sizes of 32.5 kD, 31 kD, 26 kD, and 8 kD. N-terminal amino acid analysis of the toxin fragments revealed that most fragments were cleaved at amino acid residues either in their N-terminal amino latch or glycine-rich regions which are highly protease-resistant in their heptameric structure [85].

It has been shown that the intact  $\alpha$ -toxin could be cleaved by other enzymes such as trypsin, pronase, and proteinase K at the same site in the glycine-rich region (which becomes the central loop in the heptameric structure) as was observed in the isolated toxin fragments [86, 87].

There are a number of studies showing the biological relevance of the N-terminal amino latch. For example, the N-terminal region of  $\alpha$ -toxin has been shown to be critical to the formation of transmembrane pores in rabbit erythrocyte membranes [88] and the loss of N-terminal amino latch causes rapid inhibition of oligomerization [85, 89, 90].

Interestingly, our isolated toxin fragments showed a considerable ability for oligomerization and pore-formation in further experiments.

#### Biochemical characterization (Paper I and II)

Most isolated  $\alpha$ -toxin fragments formed oligomers in membranes of egg-phosphatidyl glycerol (EPG) vesicles at different pH levels. Toxin fragments of sizes 32 kD and 31 kD formed oligomers at all pH ranges from pH 3.5–7.4 with relatively lower efficiency

than the intact  $\alpha$ -toxin, and the 26-kD fragment formed three different sizes of oligomers, i.e. multimers. However, no oligomers were formed by the 32.5-kD fragment at pH 4.5 (Paper I), whereas it did release chloride from EPG vesicles with 38% efficiency at pH 4.0. (Paper II), revealing that pH is an important factor for  $\alpha$ -toxin activity.

The ability to form transmembrane channels was further investigated by monitoring the chloride efflux from egg-phosphatidyl glycerol (EPG) vesicles. All the toxin fragments caused a release of chloride from the EPG vesicles in a pH-dependent manner, as was observed in intact  $\alpha$ -toxin-treated EPG vesicles. For example,  $\alpha$ -toxin-treated EPG vesicles released chloride with 91% efficiency at pH 4.5, treatment with the 32.5-kD fragment caused the released of chloride with 38% efficiency at pH 4.0, the 31-kD fragment caused the released of chloride with 53% efficiency at pH 4.75, and vesicles treated with the 26-kD fragment released chloride with 82% efficiency at pH 4.5.

Further, the correlation between oligomer formation and chloride efflux by the toxin fragments was investigated by using the 32.5-kD fragment. A positive correlation was observed and pH was revealed to be a significant influencing factor.

These results reveal the ability of  $\alpha$ -toxin fragments to form oligomers and transmembrane channels. Channel formation is significantly influenced by specific amino acid residues in the N-terminal amino latch as well as pH conditions, which might be critically important for structure rearrangement and oligomerization.

In order to confirm that the reason for toxin fragment generation might be due to the actions of staphylococcal extracellular enzymes, we attempted to reproduce the toxin fragments by proteolysis of intact  $\alpha$ -toxin with staphylococcal V8 protease and aureolysin. Approximately 8 different protein fragments were produced, and the data from N-terminal sequence analysis revealed that the proteolytically-generated 26-kD fragment was identical to the naturally occurring 26-kD fragment, showing that the proteolytic cleavage site is between E-71 and G-72.

### **Biological activities (Paper I and II)**

C-terminal intact  $\alpha$ -toxin fragments (32-kD, 31-kD and 26-kD) showed hemolytic activity on rabbit red blood cells (RRBC) exhibiting a single hemolytic peak at pH 5.5 which coincided with a minor hemolytic peak of the intact  $\alpha$ -toxin. In addition, the 32-kD toxin fragment showed 86% hemolytic activity on human red blood cells (HRBC)

at pH 5.5 and the activity reached a maximum value at pH 5.0, the same pH value as for intact  $\alpha$ -toxin. However, none of the other fragments showed any hemolytic activity on HRBC.

These results enabled better understanding of how *S. aureus*  $\alpha$ -toxin fragments affect eukaryotic cells. Based on the present findings, we believe their mechanisms of action not only depend on the protein structure but also on pH levels. The results revealed that fragments might contain specific amino acid residues for binding to either RRBC or HRBC which may have different receptors or membrane lipid compositions. Additionally, the loss of activity due to missing amino acid residues could be recovered by acidification of the medium.

The binding of intact  $\alpha$ -toxin and some of the  $\alpha$ -toxin fragments (i.e. the 32-kD, 31-kD, and 26-kD fragments) to the membrane of rabbit red blood cells was proved by autoradiography of  $H^3$ -labeled  $\alpha$ -toxin fragments bound to RRBC membranes.

In contrast to intact  $\alpha$ -toxin, variable sizes of oligomers (i.e. multimers) were formed by the toxin fragments, e.g. the 32-kD fragment formed hexamers, heptamers, and octamers after 20 h incubation, the 31-kD fragment formed heptamers after 20 h incubation, and the 26-kD fragment formed decamers and dodecamers after 45 min incubation.

Since the amino acid residues 9–12 are critical for multimer formation by interacting with an adjacent monomer in the cap domain in the early stage of multimerization [88, 89], multimers might not readily be formed by  $\alpha$ -toxin fragments in the same conditions.

Cytotoxic effects of the toxin fragments were then determined on mouse adrenocortical Y1 cells. Toxin fragments caused different morphological changes compared to  $\alpha$ -toxin i.e. treatment with the 31-kD and 26-kD fragments caused swelling of FM Y1 cells, whereas intact  $\alpha$ -toxin caused cell shrinkage and detachment from the culture plate as a consequence of intoxication. The results of the cell viability assay revealed that 87% of the cells died upon 6 h incubation with 0.01  $\mu$ g intact  $\alpha$ -toxin and a similar toxicity was achieved by 10  $\mu$ g of the 32-kD fragment for 24 h incubation. Following 24 h incubation with 20  $\mu$ g of the 26-kD fragment, 19% of cells died. The cytotoxicity in FM Y1 cells by intact  $\alpha$ -toxin and the 32-kD fragment, both of which contain critical amino acid residues 9–12 for multimer formation [88], was irreversible.

Furthermore, the results from the co-incubation tests with the fragments and  $\alpha$ -toxin revealed that the 31-kD fragment inhibited the cytotoxic effect of  $\alpha$ -toxin by occupying the same binding-sites on FM Y1 cells.

Results from tests of  $\alpha$ -toxin on (5-<sup>3</sup>H) uridine-labeled FM Y1 cells revealed that the 32-kD fragment had similar pore-forming kinetics as that of the intact  $\alpha$ -toxin, exhibiting 43.5% nucleotide release in the same time frame. However, the 31-kD and 26-kD fragments induced very low level of nucleotide release, exhibiting 16.6 % and 13.9 %, respectively. The 32.5-kD fragment did not induce any nucleotide release at all (0.36%).

In summary, 1) the naturally occurring  $\alpha$ -toxin fragments cleaved at the N-terminal region were biologically active, and 2) their oligomerization and transmembrane channel formation in biological membranes after conformational changes were triggered by protonation. 3) These findings significantly support the hypothesis that *S. aureus* escapes host immune defenses by secreting modified cytolytic toxins that may be activated in the low pH conditions prevailing inside phagosomes.

#### **4.2 Staphylococcal $\alpha$ -toxin on human epithelial cells (Paper III)**

During a serious *S. aureus* infection, the bacteria and exotoxins spread in the blood stream and further damage the endothelial barrier, resulting in increased vascular permeability [62, 91]. However, there are only a few studies demonstration the same effect on the epithelial barrier, which is important as a first line of defense.

For instance, an important function of the intestinal epithelial barrier is to keep a number of enteric bacteria and their products in the intestinal lumen. An increase in intestinal epithelial permeability may enable bacterial translocation through the epithelium and into the blood which may lead to a serious septic condition.

We hypothesized that intestinal epithelia can be damaged by the  $\alpha$ -toxin present in the serum on the basolateral side, causing bacterial translocation that may further aggravate the septic condition.

The other major objective of this thesis is to investigate the effect *S. aureus*  $\alpha$ -toxin on intestinal epithelium permeability to support the hypothesis.

### **Effect on the integrity of Caco-2 cell monolayer**

After about 1 h of incubation with  $\alpha$ -toxin, the TER of the Caco-2 cell monolayer began to decrease and reached a significantly lower level after 3 h. The decrease in TER of the Caco-2 cell monolayer that was induced by staphylococcal  $\alpha$ -toxin was dose- and time- dependent, up to 0.8  $\mu$ g/ml  $\alpha$ -toxin.

The apparent expression levels of various junctional proteins of the Caco-2 cell monolayer, such as ZO-1, ZO-3, occludin, E-cadherin, and  $\beta$ -catenin, were determined to investigate the involvement of junctional proteins in the TER decrease. Our results revealed that the levels of 3 tight junction proteins (ZO-1, ZO-3, and occludin) and 1 adherence junction protein (E-cadherin) decreased significantly at different rates and degrees by  $\alpha$ -toxin treatment. The cellular level of occludin decreased most dramatically to 61% within 3 h. Interestingly, no change in  $\beta$ -catenin level was observed. Similar patterns of decrease in the different junctional proteins in the inter-cellular space were shown by immunofluorescence and confocal imaging. The results from confocal imaging and image analysis revealed that the  $\beta$ -catenin was not degraded but relocalized from the intercellular area into the cytoplasm, similar to cancer progression.

In order to prove the hypothesis that septic condition might be aggravated by enteric bacterial translocation through a hyper-permeabilized intestinal epithelial barrier due to the action of  $\alpha$ -toxin in the blood stream, we set up an experimental model. Caco-2 cells were grown on cell culture inserts until they formed monolayer, and  $\alpha$ -toxin was added to the basolateral compartment of the two-compartment cell culture model and the TER of Caco-2 cell monolayer was monitored. The TER decreased significantly after 8 hours incubation with basolaterally applied  $\alpha$ -toxin in a dose- and time-dependent manner, whereas no significant changes in TER were induced by apical application of  $\alpha$ -toxin. The results revealed that a significant decrease in TER was specifically due to the action of basolaterally applied  $\alpha$ -toxin. Additionally, the apparent expression levels of ZO-3, occludin,  $\beta$ -catenin, and E-cadherin were significantly decreased by basolateral application of  $\alpha$ -toxin to Caco-2 cell monolayer, e.g. the cellular levels of ZO-3 and E-cadherin decreased by 81% and 97% after 3 h incubation, respectively.

### **Pathophysiological effect on Caco-2 cells**

The cytosolic calcium concentration ( $[Ca^{2+}]_i$ ) of  $\alpha$ -toxin-treated Caco-2 cells was monitored to investigate the influence of  $\alpha$ -toxin on calcium homeostasis of Caco-2 cells.  $[Ca^{2+}]_i$  of Caco-2 cells in calcium (1 mM)-containing buffer was significantly



increased about 10 min after the addition of 0.8 µg/ml α-toxin, whereas no significant signal increases were observed in the Caco-2 cells in calcium-free buffer, or after the pretreatment with either an intracellular calcium chelator (25 µM BAPTA/AM), or with an inhibitor of intracellular Ca<sup>2+</sup> store replenishment (0.5 µM thapsigargin). The results revealed that the α-toxin-induced increase in cytosolic free Ca<sup>2+</sup> was not due to release from intracellular Ca<sup>2+</sup> stores, like calciosomes or ER, but rather due to Ca<sup>2+</sup> influx from the extracellular environment.

Furthermore, in order to investigate the involvement of [Ca<sup>2+</sup>]<sub>i</sub> in the TER drop that was observed among Caco-2 cell monolayers treated with α-toxin for 1 h, an intracellular calcium chelator (25 µM BAPTA/AM) was applied to the Caco-2 cell monolayer after the α-toxin treatment, and the TER of the cell monolayer was monitored over 8 hours.

With intracellular calcium chelation, the extent of the TER drop was increased compared to control cells. This result shows a positive correlation between an increase in intracellular [Ca<sup>2+</sup>] and epithelial barrier integrity, and this finding suggests that the intracellular [Ca<sup>2+</sup>] increased by α-toxin treatment is might be involved in maintaining TER against external stimuli.

In conclusion, we have demonstrated that the basolateral application of α-toxin increased the permeability of Caco-2 cell monolayers which were used as a model of the intestinal epithelium, by: 1) decreasing the cellular level of tight/adherence junction proteins, which regulate the paracellular permeability of the epithelium, 2) inducing cytoplasmic calcium influx from the external side instead of internal calcium storages, and 3) decreasing the TER of cell monolayers. Further, the relationship between cytoplasmic calcium influx and TER was shown.

### **4.3 Staphylococcal α-toxin on bacterial translocation in mice (Paper IV)**

Based on the results obtained in Paper III, we next tested the effect of α-toxin on the translocation of intestinal bacteria.

#### **Effect on bacterial translocation in an *in vitro* model**

The level of translocation across Caco-2 monolayers of an *E. coli* strain previously shown to be non-translocating in rats (i.e. *E. coli* 46-4) was determined after the basolateral addition of α-toxin. Following 3 h of toxin treatment, a significant number

of *E. coli* were found in the basolateral compartment of the translocation model used. A slight decrease in TER occurred during the same period. However, in the absence of  $\alpha$ -toxin (control), no significant *E. coli* translocation or TER decrease was observed.

It was shown that the increased epithelial permeability led to bacterial translocation. The reason for the increased TER in the *E. coli* challenged model might be due to a cellular reaction to bacterial structural components.

### **Effect on enteric bacterial translocation in an *in vivo* mouse model**

A sublethal dose of  $\alpha$ -toxin, which might give enough time for the translocation of enteric bacteria, in mice was determined to be 1.8  $\mu\text{g}$   $\alpha$ -toxin per mouse.

In the treated group ( $n = 13$ ), mild discomfort was observed 3 h after intravenous (i.v.) challenge with 1.8  $\mu\text{g}$   $\alpha$ -toxin in the tail vein. The symptoms were aggravated thereafter. Intraperitoneal hemorrhage ( $n = 4$ ) was observed at the dissection and two mice additionally showed pancreatic cysts. A significantly higher number of bacteria were found in the spleen of mice which were subjected to  $\alpha$ -toxin injection ( $P = 0.045$ , Mann-Whitney test) as compared to control mice.

A significantly higher number of bacteria were also found in the Peyer's patches of toxin-challenged mice compared to controls ( $P = 0.032$ , Mann-Whitney test).

The results thus showed that intravenously administered *S. aureus*  $\alpha$ -toxin may promote translocation of intestinal bacteria. Similar to our results, *E. coli* endotoxin (i.p.) has been shown to promote the translocation of enteric bacteria in a previous study [73].

Possibly, it can be speculated that even minor damage to the intestinal epithelial barrier by *S. aureus*  $\alpha$ -toxin might cause a leakage of *E. coli* endotoxin, and once endotoxin has entered into the peritoneal cavity, it facilitates further bacterial translocation by disrupting intestinal epithelial barrier, thereby establishing a vicious circle of *E. coli* endotoxemia and bacterial translocation which may thus cause massive inflammatory disease syndrome [92].

In this study, a possible virulence role of *S. aureus*  $\alpha$ -toxin has been demonstrated in relation to the intestinal hyperpermeability and bacterial translocation, which can be a major cause of sepsis and MODS.

Several conclusions can be drawn from these experimental studies (Paper III and IV):

- 1) Basolaterally applied *S. aureus*  $\alpha$ -toxin increased the permeability of the Caco-2 cell culture model of the human intestinal epithelium, as evidenced by decreased cellular levels of junctional proteins, decreased TER of Caco-2 cell monolayers, and increased cytoplasmic calcium concentration, demonstrating its positive relation to TER (Paper III).
- 2) Basolaterally applied *S. aureus*  $\alpha$ -toxin induced the bacterial translocation in an *in vitro* model (Caco-2), revealing that the increased epithelial permeability led to bacterial translocation.
- 3) Intravenously injected *S. aureus*  $\alpha$ -toxin promoted intestinal bacterial translocation in mice, evidenced by the significantly higher number of *E. coli* found in the Peyer's patches ( $P = 0.032$ , Mann-Whitney test) and spleen ( $P = 0.045$ , Mann-Whitney test).

## 5 Thesis summary

*Staphylococcus aureus* is commonly found in healthy humans, as part of the normal flora, but the bacteria is also known to cause severe infections if an opportunity arises. Deep infection in hospitalized patients with *S. aureus*, leading to sepsis, causes serious problems in the hospital and community settings.

*S. aureus* is a well-armed pathogen with a large number of virulence factors such as peptidoglycan (structural component), cytotoxins (extracellular toxin), and hyaluronidase (extracellular enzyme). These virulent bacteria also produce different kinds of cytotoxins to enable their spread into the blood stream of patients and evasion of immune defenses in the condition of sepsis.

One of the membrane damaging cytotoxins, *S. aureus*  $\alpha$ -toxin (33 kD;  $\alpha$ -hemolysin) is produced in the exponential growth phase together with extracellular proteases (e.g. aureolysin, V8 protease) either for housekeeping or pathogenic strategy.

In this study, we found several fragmented forms of *S. aureus*  $\alpha$ -toxin in the culture supernatant and we investigated their structure, biological function, and origin. In order to achieve the objectives of our study, we used various methods such as limited proteolysis to create toxin fragments artificially from intact  $\alpha$ -toxin, N-terminal sequencing to determine the amino acids residues of the isolated fragments, hemolysis test and nucleotide release test to determine their relevant biological activities in nucleated cells.

We found that most biologically active toxin fragments were cleaved at their N-terminal latch and their activity were highly due to the number of missing residues and dependent on pH conditions. Interestingly, alleviated hemolytic activity was partially recovered by protonation.

Besides that, we demonstrated that some toxin fragments can be proteolytically generated from intact staphylococcal  $\alpha$ -toxin by simultaneously expressed extracellular *S. aureus* proteases.

In *S. aureus*-associated sepsis, the membrane damaging  $\alpha$ -toxin may circulate in the blood and affect the intestinal barrier, resulting in translocation of enteric bacteria. This might lead to the spread of endotoxin (LPS) in the blood and consequently further aggravate the sepsis syndrome. In order to test this hypothesis, we investigated the

effect of  $\alpha$ -toxin on the permeability of human intestinal Caco-2 cell monolayers cultured on cell culture membrane inserts. Several molecular biology methods (Western blot, qRT-PCR, Immunofluorescence and Confocal imaging) were used to investigate the fate of junctional proteins, which are critical in regulating the permeability of the intestinal epithelium. In addition to that, cytoplasmic calcium changes in response to  $\alpha$ -toxin treatment were investigated to clarify the possible pathophysiological relevance of calcium influx. We found that basolaterally-applied  $\alpha$ -toxin significantly increased the permeability of Caco-2 cell monolayers by decreasing the amount of different junctional proteins (ZO-1, ZO-3, E-cadherin, and occludin). Additionally, the cytoplasmic calcium concentration was increased in response to  $\alpha$ -toxin treatment.

With this in mind, we performed animal experiments in order to determine whether bacterial translocation across the intestinal epithelium can be induced by circulating  $\alpha$ -toxin in the blood stream. A strong correlation between intestinal hyperpermeability and bacterial translocation suggests that  $\alpha$ -toxin may thus aggravate the septic condition with serious inflammatory disease syndrome caused by a vicious circle of *E. coli* endotoxemia and bacterial translocation.

## 6 Acknowledgements

I would like to express my sincere gratitude to all the people who supported me to complete this study and I wish to express my appreciation particularly to:

Professor **Roland Möllby**, my main supervisor, for his scientific supervision, kind guidance and care, and encouragement. I appreciate very much your careful and generous concern on me to work in the group with many nice people!

Dr. **Beatrix Vécsey-Semjén**, my co-supervisor, for her enthusiasm and countless efforts for me to finish this work and valuable advices and supervisions. Thank you very much!

Dr. **Patricia Colque-Navarro**, for her scientific support and special care for me and huge amount of  $\alpha$ -toxin that I have used during the study.

Associate Professor **Inger Kühn**, for her kind support with MALGA project.

Professor **Jan-Ingmar Flock** and Dr. **Margareta Flock** for all help.

Professor **Karl-Eric Magnusson**, for his acceptance for me to work on cell biology in Linköping University and consistent support.

Dr. **Elena Vikström**, for her kind and enormous help and support with the experiment with Caco-2 cells. Thank you very much!

Professor **Elisabeth Norin**, for her scientific discussion and help for planning animal experiment.

**Anna-Karin Persson**, for her kind and excellent technical assistant for animal experiment.

Professor **Man-Gi Cho** and Dr. **Chang-Hwa Jeong**, for valuable advice and support during my study.

Dr. **Sung Yun Kang** for his friendship and kind help with qRT-PCR.

Dr. **Nubia L. Ramos**, for her friendship and proofreading the manuscript. Thank you very much!

Thank you Dr. **Pascal Dammeyer** for your effort to bring me here in Stockholm and keep a nice friendship for long time.

Thank you Dr. **Abdul Kader** for the friendship and showing me your hospitality all the time.

Thank you **Stefano Sammiceli** for the friendship and spontaneous sub-project!

I thank you all my friends in Stockholm and **Pan-Gun, Eun-Sun, Philipp**, and **Hae-Sun** for your friendship. It was so nice to mingle with you!

Lastly, I would like to express my sincere gratitude to **my parents** and **sister** who have been waiting for this moment for long time on my behalf in all the time. Thank you very much!

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