LIPOOLIGOSACCHARIDE AND CYTOLETHAL DISTENDING TOXIN OF *HAEMOPHILUS DUCREYI* AND ANTIBODY RESPONSES

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GÖTEBORG 2009

Cover image: Internalization of Alexa Fluor 488 labeled HdCDT in HeLa cells after 48h, visualized by confocal microscopy.

TILL

FREDRIK

TOVE & NORA

MY MOMMA ALWAYS SAID, "LIFE WAS LIKE A BOX OF CHOCOLATES. YOU NEVER KNOW WHAT YOU'RE GONNA GET."

-FORREST GUMP

LIPOOLIGOSACCHARIDE AND CYTOLETHAL DISTENDING TOXIN OF *HAEMOPHILUS DUCREYI* AND ANTIBODY RESPONSES

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ABSTRACT

The Gram-negative bacterium *Haemophilus ducreyi* causes chancroid, a sexually transmitted infection characterized by persistent ulcers on genitals. The disease is prevalent in developing countries and facilitates transmission and acquisition of HIV.

This thesis focuses on two bacterial virulence factors, lipooligosaccharide (HdLOS) and cytolethal distending toxin (HdCDT). The carbohydrate part of HdLOS is short and sialylated. HdCDT, an AB_2 toxin composed of three protein subunits, induces DNA double strand breaks, cause cell cycle arrest and death of target cells. Protective immunity against *H. ducreyi* is not well understood.

The general aim was to investigate the role of HdLOS and HdCDT in antibody responses, specifically to: 1) evaluate the function and viability of human monocyte-derived dendritic cells (DC), macrophages (MQ) and $CD4^+$ T-cells after interaction with *H. ducreyi* bacteria, HdLOS and HdCDT, *in vitro*; 2) define immunogenic and adjuvant properties of HdLOS; 3) evaluate the impact of HdCDT on the serum antibody responses and 4) define a procedure for the generation of high antibody levels to HdCDT in the genital tract, using a mouse model.

Bacteria and HdLOS stimulated an inflammatory cytokine response in the DCs and MQs, and activated cells induced $CD4^+$ T-cells to proliferate and secrete INF-γ. HdCDT caused apoptosis of DCs, inhibited the secretion of cytokines and intoxication resulted in failure of $CD4^+$ T-cells activation, *in vitro*.

Purified HdLOS is an immunogenic T-cell independent antigen. The majority of HdLOS antibodies was specific for the inner core and did not neutralize endotoxin activity. HdLOS possessed adjuvant properties and significantly increased the antibody response to proteins tested.

Active HdCDT is weakly immunogenic and induced low levels of specific antibodies. HdCDT did not down-regulate the antibody response to *H. ducreyi* antigens, despite the toxic activity on mouse immune cells, *in vitro*.

High levels of specific HdCDT antibodies in serum and genital tissue, including neutralizing antibodies, were induced by parenteral immunization of mice with formaldehyde detoxified HdCDT alone or in combination with aluminum salts or lipid A based adjuvants. The HdCDT toxoid can be evaluated as useful component of a vaccine against *H. ducreyi* and other CDT producing bacteria.

Keywords: *Haemophilus ducreyi*, lipooligosaccharide, cytolethal distending toxin
ISBN 978-91-628-7916-7 Gothenburg, 2009

ORIGINAL PAPERS

This thesis is based on the following papers, which are referred to in the text by the given Roman numerals (I-III):

I. **Xu T, Lundqvist A, Ahmed HJ, Ericsson K, Yang Y, Lagergård T**
Interactions of *Haemophilus ducreyi* and purified cytolethal distending toxin with human monocyte-derived dendritic cells, macrophages and $CD4^+$ T cells
Microbes and Infection 6 (2004) 1171-1181

II. **Lundqvist A, Kubler-Kielb J, Teneberg S, Ahlman K, Lagergård T**
Immunogenic and adjuvant properties of *Haemophilus ducreyi* lipooligosaccharides
Microbes and Infection 11 (2009) 352-360

III. **Lundqvist A, Fernandez-Rodrigez J K, Ahlman K and Lagergård T**
Haemophilus ducreyi cytolethal distending toxin and antibody responses; induction of specific antibodies in the genital tract
Submitted manuscript

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TABLE OF CONTENTS

ABSTRACT 5

ORIGINAL PAPERS 7

ABBREVIATIONS 10

INTRODUCTION 11

1. *HAEMOPHILUS DUCREYI* BACTERIUM 11

2. CHANCROID 12

2.1. EPIDEMIOLOGY OF CHANCROID 12

2.2. CLINICAL MANIFESTATIONS, DIAGNOSIS AND TREATMENT 13

2.3. HISTOPATHOLOGICAL FEATURES 13

3. *HAEMOPHILUS DUCREYI* MODELS FOR THE STUDY OF PATHOGENESIS 14

4. *HAEMOPHILUS DUCREYI* PATHOGENESIS AND VIRULENCE FACTORS 16

5. HOST RESPONSE TO *HAEMOPHILUS DUCREYI* 18

6. LPS AND *HAEMOPHILUS DUCREYI* LIPOOLIGOSACCHARIDE 19

6.1. STRUCTURE OF HDLOS 20

6.2. HDLOS ROLE IN PATHOGENESIS AND IMMUNITY 21

7. *HAEMOPHILUS DUCREYI* CYTOLETHAL DISTENDING TOXIN AND THE CDTS 22

7.1. GENETICS OF HDCDT 23

7.2. *H. DUCREYI* CDT SUBUNITS AND HOLOTOXIN 23

7.3. CDT ACTIVITY *IN VITRO* 25

7.4. CDT ACTIVITY *IN VIVO* 28

7.5. ANTIBODY RESPONSE TO CDT 29

AIMS OF THIS STUDY 31

MATERIALS AND METHODS 33

RESULTS AND COMMENTS 43

1. THE FUNCTION AND VIABILITY OF HUMAN MONOCYTE-DERIVED-DENDRITIC CELLS, MACROPHAGES AND $CD4^+$ T-CELLS TO *H. DUCREYI* BACTERIA, HDLOS AND HDCDT, *IN VITRO* (PAPER I). 43

1.1. PHAGOCYTOSIS OF *H. DUCREYI* BY DCS AND MQS 43

1.2. Pro-inflammatory cytokine production by DC and MQs after induction by *H. ducreyi* bacteria, HdLOS and HdCDT 44
1.3. HdCDT action on DC viability and apoptosis 46
1.4. Activation of T-cells by DCs and MQs pulsed with *H. ducreyi*, HdLOS and HdCDT 46
1.4.1. Induction of $CD4^+$ T-cell proliferation 47
1.4.2. Cytokine secretion by $CD4^+$ T-cells 49
2. Immunogenic and adjuvant properties of HdLOS (paper II and III). 50
2.1. Immunogenicity of purified HdLOS 50
2.2. Specificity of antibodies induced by HdLOS 53
2.3. Anti-inflammatory activity of anti-HdLOS antibodies 53
2.4. Adjuvant activity of HdLOS 54
3. Evaluation of HdCDT impact on the serum antibody responses using a mouse model (paper III). 56
3.1. Mouse model 56
3.2. HdCDT influence on antibody response to HSP, HdLOS and BSA 56
3.3. Antibody response to HdCDT 57
4. Generation of antibodies to HdCDT in genital tract using a mouse mice model (paper III). 59
General discussion 61
Conclusions 67
Swedish summary - Populärvetenskaplig sammanfattning på svenska 69
Acknowledgments - Tackord 70
References 72

ABBREVIATIONS

AaCDT	*Aggregatibacter actinomycetemcomitans* cytolethal distending toxin
APC	Antigen presenting cell
CDT	Cytolethal distending toxin
cfu	Colony forming unit
DC	Dendritic cell
DTH	Delayed type hypersensitivity
FCS	Fetal calf serum
GUD	Genital ulcer disease
HdCDT	*Haemophilus ducreyi* cytolethal distending toxin
HdLOS	*Haemophilus ducreyi* lipooligosaccharide
HSP	Heat shock protein
HSV	Herpes simplex virus
LAL	Limulus amoebocyte lysate assay
LOS	Lipooligosaccharide
LPS	Lipopolysaccharide
MQ	Macrophage
MTT	Dimethylthiazol diphenyl tetrazolium bromide
NLS	Nuclear localization factor
NPP	*p*-nitrophenyl phosphatase
OMP	Outer membrane protein
PBMC	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
PI	Propidium iodide
PMN	Polymorphonuclear leukocytes
STI	Sexually transmitted infection
TLR4	Toll-like receptor 4

INTRODUCTION

The Gram-negative bacterium *Haemophilus ducreyi* is a strict human pathogen and the etiologic agent of the sexually transmitted infection (STI) chancroid (ulcer molle, soft chancre) (Fig 1). Chancroid is characterized by soft, painful, slow healing ulcers on the external genitalia which may be accompanied by regional lymphadenitis and bubo formation. The disease occurs worldwide with highest prevalence in the developing countries of Africa, Asia and Latin America. In these countries *H. ducreyi* is considered to be one of the major causes of genital ulcer disease and it facilitates the transmission and acquisition of HIV.

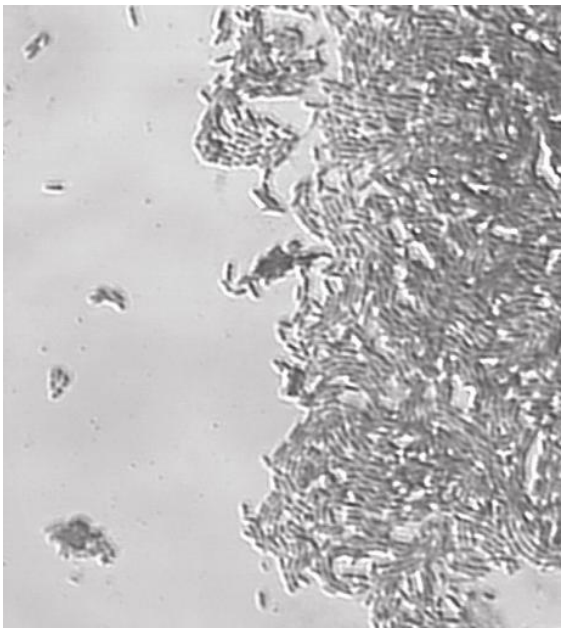

Figure 1 *Haemophilus ducreyi*. The picture was kindly provided by Teresa Lagergård

1. *HAEMOPHILUS DUCREYI* BACTERIUM

The bacterium *Haemophilus ducreyi* was first described in 1889 by Auguste Ducreyi [1]. It is a strictly human, fastidious Gram-negative coccobacillus that grows slowly with requirements of selective, enriched media and a microaerophilic environment for growth. For optimal growth a humidified and carbon dioxide enriched atmosphere at a temperature of 33° is applied. When grown on solid agar based media, bacterial colonies are characterized as small, non-mucoid, yellow-grey and semi-opaque. They are intercellular adhered and can be pushed intact across the agar surface. In liquid media the bacteria are arranged in chains and "schools of fish" [2-3].

The classification of *H. ducreyi* among the *Haemophilus* species was based on the specific growth requirements, biochemical properties and antigenic relatedness to other species in the group [3], however rRNA analyses have shown that a more correct classification would be in the *Actinobacillus* cluster of the *Pasteruellaceae* [4-5]. The genome of *H. ducreyi* 35000HP (HP refers to human passaged) was completely sequenced in 2001 and it is composed of a single $1.7x10^6$ bp chromosome that encodes for 1783 predicted genes.

2. CHANCROID

2.1. EPIDEMIOLOGY OF CHANCROID

Cases of chancroid occur worldwide with highest prevalence in the developing countries of Africa, Asia and Latin America. In these countries *H. ducreyi* was considered to be the major cause of genital ulcer disease for long time [6-7]. In a report from 1997, UNAIDS and the World Health Organization (WHO) estimated that the annual global incidence of chancroid was 6 million cases [8]. However, the incidences of chancroid are declining and recent studies performed on ulcer specimens from genital ulcer disease (GUD) patients in Tanzania and South Africa report decreased detection of *H. ducreyi* (detected in 1.2 - 5% of ulcers), on the contrary the prevalence of Herpes simplex virus have increased (detected in 83 - 85% of ulcers) [9-10]. The disease is reported to be more prevalent in men than in women, with ratios ranging from 3:1 in endemic areas to 25:1 in outbreak situations [3]. This difference in susceptibility between sexes is also seen in experimental infections in both humans and macaques [11-12]. One important cofactor in the spread of chancroid is prostitution and the prostitutes are considered as a reservoir of the disease in epidemic outbreak situations [13-14].

Genital ulceration caused by *H. ducreyi* disrupts the mucosal integrity and thus provides a portal of entry for HIV explaining the strong correlation between genital ulcer diseases (GUD) and the acquisition and transmission of HIV [15-17]. Furthermore, the presence and activation of HIV- susceptible cells in the genital tract increased as a part of the cellular immune response to *H. ducreyi*. The cutaneouse infiltrate of $CD4^+$ T-cells and macrophages that Spinola *et al*. reported of [18] are the primary targets for HIV and the increased CCR-5 receptor expression on the macrophages further increase the susceptibility of these cells to HIV [17]. *H. ducreyi* specific T-cell stimulating antigens might increase the viral replication in these cells [19]. The transmission of HIV is facilitated by viral shedding in ulcer exudates and from bleeding ulcers. Inappropriate handling of chancroid diagnostics and therapeutic procedure e.g. bubo aspiration, can also contribute to the spread of HIV.

2.2. CLINICAL MANIFESTATIONS, DIAGNOSIS AND TREATMENT

Infection with *H. ducreyi* is initiated by the entry of bacteria through micro abrasions in the epidermis. After an incubation period of 4-7 days post infection the chancre begins as a tender erythematous papules that transform to eroded pustules in 24-48 h, after another 2-3 days the pustule ruptures and a painful shallow ulcer is formed [7, 13]. The ulcer is described as soft, having ragged undermined edges, no indurations and purulent exudates covering the base of the ulcer with little inflammation of the surrounding skin [20]. The type and the severity of the ulcers vary, in men they typically occur on the prepuce and frenulum and in women on the vulva, cervix and in the perianal area [7]. Unilateral inguinal lymphadenopathy is another typical characteristic of chancroid, it occurs in 50% of cases, and the affected lymph nodes may develop into buboes that rupture to form inguinal ulcers. However, these symptoms seem to be less common in women [3]. Without efficient treatment the infection is persistent and it can take several months before the infection is resolved and the ulcers are healed.

The "golden standard" for the diagnosis of chancroid has been based on physical examination, laboratory culture of ulcer and bubo pus specimens, and also by the exclusion of other genital ulcer diseases, such as; syphilis, herpes simplex virus and lymphogranuloma venereum [7]. In order to improve the diagnostic of chancroid several attempts were made to develop PCR-based diagnostic techniques [21-24] and the multiplex PCR (M-PCR) assay that simultaneously detects *H. ducreyi, Treponema pallidum* and herpes simplex virus (HSV) type 1 and 2 from ulcer specimens is the most sensitive [25]. Still laboratory culture is the main diagnostic tool for detection of *H. ducreyi*, since this is the technique that is available in most clinical microbiology laboratories.

H. ducreyi have in the recent decades acquired antibiotic resistance by plasmid- and chromosomally- mediated mechanisms and nowadays is the treatment recommend for chancroid by WHO and the Centre for Disease Control; Azitromycin, Ceftriaxone, Ciprofloxacine or Erythromycin, in addition aspiration of buboes is recommended to avoid complications of spontaneous rupture [7].

2.3. HISTOPATHOLOGICAL FEATURES

Biopsies from chancroid proven ulcers have been used for the study of the histological features of natural occurring chancroid. The ulcers have been described as three discrete zones; the superficial zone, the edge and the base of the ulcer, consists of necrotic tissue, fibrin, neutrophils and many Gram-negative coccobacilli; the middle zone, dominated by endothelial cells, shows oedema and newly formed blood vessels perpendicular to the surface of the ulcer; the deep layer

manifesting a dense infiltrate of neutrophils, plasma cells, T-lymphocytes and leukocytes with fibroblastic proliferation [20, 26-28]. Biopsies from the pustule stage in experimental human infection showed similar histological features as the ones taken from ulcers in natural occurring infection [18, 29]. In this stage of the infection, the majority of the infiltrated cells was reported to be; T-cells (60-80% were $CD4^+$ and 20-40% were $CD8^+$) and mononuclear cells (mainly macrophages), even though a smaller portion of the infiltrated cells were B-cells and NK cells there were no plasma cells detected at this early stage of infection.

3. *HAEMOPHILUS DUCREYI* MODELS FOR THE STUDY OF PATHOGENESIS

Even though *H. ducreyi* is solely a human pathogen researchers have tried to establish models to be able to study the virulence and pathogenesis of the bacteria and both *in vitro* and *in vivo* models have been developed [12, 30-37]. Established cell lines as well as cell cultures of either human or animal origin have been used for the *in vitro* studies. The models mainly used for *in vivo* studies are;

1) Rabbits were for many years used in the evaluation of the virulence of *H. ducreyi* strains, by the use of this model, a strain was defined as virulent when intradermal inoculation of approximately 10^8 cfu bacteria generated a typical lesion, characterized by necrosis and eschar formation [30-31]. If no cutaneous lesion formed the strain was defined as avirulent. In 1991, Purcell *et al.* reported of a temperature dependency of the rabbit model [32]. Inoculation of 10^5 cfu *H. ducreyi* bacteria, unable to produce lesions in rabbit skin when the animals were housed at normal temperature, produced lesions when the rabbits were housed at a reduced temperature (15-17°C). Some histopathologic features are shared such as the presence of dermal perivascular lymphocytic and plasma cell infiltrates, but the main disadvantage of the rabbit model is that the lesion development do not resemble the human chancroid [32].

Wising *et al.* used the temperature dependent rabbit model for the investigation of toxicity and immunogenicity of purified *H. ducreyi* cytolethal distending toxin (HdCDT) and they also showed that HdCDT aggravated the dermal lesions caused by *H. ducreyi* [38-39].

2) The primate model developed by Totten *et al.* consists of adult pigtailed macaques (*Macaca nemestrina*) of both sexes inoculated with 10^7-10^8 cfu of *H. ducreyi* [12]. The males were inoculated on the foreskin and the females on the vaginal labia, all of the male macaques developed ulcers that closely resembled those of natural occurring chancroid, while none of the female macaques developed ulcers. Both the rabbit and the macaque model were reported to be dependent on the viability of the inoculated bacteria for formation of ulcers.

3) Mice were used in an attempt to develop a model that presented ulcers that more resembled natural occurring chancroid [33]. After intradermal or subcutaneous inoculation of 10^7 cfu *H. ducreyi* the animals developed both pustular nodules and severe ulceration that resembled a human infection. Further evaluation of the model showed that the same type of ulcers was formed when mice were inoculated with either heat killed *H. ducreyi* or 10^8 cfu of viable or heat killed *Neisseria gonorrhoeae* and also by inoculation of purified lipooligosaccharide (LOS) from any of the bacteria [34]. According to these data ulcers were not produced specifically by *H. ducreyi.*

When Campagnari *et al*. evaluated the role of LOS in experimental dermal lesions caused by *H. ducreyi* in mice and rabbits they concluded that *H. ducreyi* LOS might play an important role in the pathogenesis of chancroid and that the rabbit model could be useful for the study of *H. ducreyi* LOS at a cellular level [40]. Furthermore, Lagergård evaluated the capacity of *H. ducreyi* strains that were cytotoxin positive or negative, bacterial sonicates and purified LOS from the same strains for the ability to induce dermonecrotic lesions and ulcers in mice and rabbits [35]. The results showed that the same type of lesions was caused by the different bacterial strains, bacterial sonicate and LOS. Furthermore ulcer formation caused by bacterial sonicates and LOS was found to correlate with the endotoxic activity in the preparation, consequently Lagergård concluded that LOS could play a role in the ulceration caused by *H. ducreyi* in animals.

4) In the swine model juvenile pigs are injected with approximately $4x10^3 – 4x10^4$ cfu bacteria on the dorsal side of the ear with a Multi-Test Applicator (allergy testing device) [36-37]. The skin of the juvenile pig closely resembles the human skin both structurally and physiologically and the lesions that are developed in pigs have histological resemblance to natural human chancroid. A great advantage of this model is the possibility of multiple injection sites, which provides a valuable tool for the evaluation of different as well as genetically modified strains in the same animal. Furthermore, pigs have been reported to mount a partially protective humoral immune response to *H. ducreyi*, which might suggest this model as valuable for the evaluation of vaccine candidates [37].

5) The human model for experimental infection with *H. ducreyi* was developed by Spinola *et al.* in order to study the pathogenesis of *H. ducreyi* and the host inflammatory response to the organism [41]. The model is standardized and approximately 10^1 to 10^3 cfu of bacteria are delivered to the epidermis and dermis of the upper arm using a allergy testing device [42]. After inoculation, papules are formed within 24 hours that either evolve into pustules after another 2-5 days or resolve spontaneously. For safety reason three clinical end points are set up; 1) disease

at all sites resolved, 2) development of a pustule that is either painful or > 4 mm in diameter, or 3) 14 days has passed after inoculation. All volunteers are treated with one oral dose of ciprofloxacine when they reach either of the end points. Since infection is not allowed to proceed more than 14 days, this model only represents the early stages in chancroid. For the use of this model, the difference between the structure of the skin on the arm and on the external genitalia is important to keep in mind.

This model is extensively used in the search for putative virulence factors, occasionally in combination with the swine model. Many of the performed virulence studies are isogenic mutant-parent comparison trails and the mutated bacteria is classified as; 1) attenuated when it is unable to cause pustule formation even at doses 10-fold greater than the parent dose that resulted in pustule formation, 2) partially attenuated when the mutant is able to cause pustule formation at doses 2- or 3-fold greater than the parent dose that resulted in pustule formation, or 3) virulent when the mutant is able to cause pustule formation at a dose equivalent to the parent dose that resulted in pustule formation [43].

Despite all attempts there is still no usable model for the study of chronic *H. ducreyi* infection, which can be used for the evaluation of *H. ducreyi* cytotoxic activity on the development and persistence of ulcers. Neither is there any suitable model for the study of protection against infection.

4. *HAEMOPHILUS DUCREYI* PATHOGENESIS AND VIRULENCE FACTORS

H. ducreyi has developed several mechanisms for the initiation and establishment of infection. In the initial stage of infection bacteria are found co-localized with collagen, fibrin, PMNs and MQs, although bacteria are surrounded by phagocytes they remain extracellular throughout experimental human infection and resist killing by the phagocytes [44]. Furthermore, several reports have suggested the ability of *H. ducreyi* to evade complement mediated serum killing [45-47]. The cytotoxic virulence factors haemolysin, cytolethal distending toxin (CDT) and LOS are of great importance as they probably are involved in the establishment of persistent ulcers and the evasion of host immune responses[48]. LOS and CDT will be described in detail in a later section of this thesis (page 19-29).

Some of the virulence factors reported to be involved in the pathogenesis of *H. ducreyi* is listed in Table1.

Table 1. *Haemophilus ducreyi* virulence factors

VIRULENCE FACTORS INVOLVED IN ADHERENCE		REF
FgbA	Fibrinogen binder	[49]
FtpA	Fine tangled pili	[50]
GroEL	Heat shock protein	[51]
NcaA	Necessary for collagen adherence	[52]
TadA	Tight adherence protein	[53]
VIRULENCE FACTORS INVOLVED IN THE RESISTANCE TO PHAGOCYTOSIS		
LspA1, LspA2	Large supernatant proteins	[54]
SOD	Superoxide dismutase	[55]
VIRULENCE FACTORS INVOLVED IN SERUM RESISTANCE		
DltA	Ducreyi lectin A	[47]
DsrA	Ducreyi serum resistance A	[45]
MOMP	Major outer membrane protein	[56]
LOS	Lipooligosaccharide	[35]
VIRULENCE FACTORS INVOLVED IN CYTOTOXICITY		
LOS	Lipooligosaccharide	[35]
hhdA; hhdB	Genes encoding haemolysin	[48]
HdCDT	*H. ducreyi* cytolethal distending toxin	[57]
VIRULENCE FACTORS INVOLVED IN DISEASE PROGRESSION		
HgbA	Hemoglobin-binding OMP	[58]
PAL	Peptidoglucan associated lipoprotein	[59]

5. HOST RESPONSE TO *HAEMOPHILUS DUCREYI*

The human disease chancroid is caused by a natural *H. ducreyi* infection. Re-infection occurs frequently and the duration of the disease is often several months as a consequence of improperly treatment. Though, it must be mentioned that spontaneous resolution of the disease occasionally occurs. The course of disease is affected by the type of immune response that is generated during natural infection. Although chancroid is a sexually transmitted infection, not much is known about the immune response that is generated locally in the genital tract.

Infection is initiated when *H. ducreyi* enters the physical barrier that an intact skin constitute, this can occur via superficial abrasions in the genital skin or mucosa caused by sexual intercourse [3]. The first cells recruited to the site of infection are important in the innate immune response. In both natural chancroid and in the experimental human model there the lesions infiltrated with both PMNs and macrophages and the primary function of these cells are to phagocytose microbes, kill and eliminate them [60]. *H. ducreyi* is reported to be relatively resistant to phagocytic killing, *in vitro* [61-62]. In the human model, bacteria are reported to be closely related to, but not internalized by macrophages or neutrophils [44]. Furthermore, *H. ducreyi* were only able to produce lesions in granulocyte- and monocyte depleted mice, while no lesions were developed in normal or severe combined immune deficiency (SCID) mice [61]. Thus, indicating the importance of phagocytes in the defense against the bacteria in the mice model.

The activation of macrophages, natural killer cells (NK), antigen-specific $CD8^+$ T-cells and the release of cytokines/chemokines in response to antigens are important factors of the cell mediated immunity. In the experimental human model, $CD4^+$ $CD45RO^+$ T-cells and macrophages were reported to be the major cellular infiltrate, but $CD8^+$ T-cells and few B-cells were present in dermis. Furthermore, the expression of MHC class II (HLA-DR) in keratinocytes, dendritic cells and mononuclear cells was increased in pustules [18, 29]. These results suggest antigen presentation and cell mediated immune response in the early stages of infection.

T-cell mediated immune responses can be divided into two types, Th1 and Th2. The prior is characterized by the secretion of (interferon) IFN-γ, TNF-α, and IL-2, and by activation of macrophages, while Th2 type response is characterized by the lack of IFN-γ and the presence of IL-4, IL-5, IL-13 and the production of antibodies [63]. The type of cytokines that are expressed in natural occurring chancroid is not clear. Palmer *et al*. reported of the presence of IFN-γ in all lesions, whereas the expression of IL-2, IL-4, and IL-5 varied [29]. Humans are reported to

mount what appears to be a delayed type hypersensitivity (DTH) reaction in response to chancroid and experimental *H. ducreyi* infection [27, 29, 41, 64]. DTH reaction is a cell mediated response of the Th1 type, in which activation of macrophages and inflammation may cause tissue injury [65]. This type of response does not confer protection against subsequent re-infection, neither is it effective at clearing chancroid infections, as lesions can persist for weeks or months, and ulcer resolution is often incomplete in the absence of antibiotic therapy [66].

The majority of *H. ducreyi* bacteria present in chancroid lesions are reported to be extracellular [44]. The humoral immune response is of great importance in the defense against extracellular bacteria and their toxins. There are reports of serum antibodies specific to the heat shock protein (GroEL), LOS and HdCDT in chancroid patients [67-69]. The prevalence of antibodies to HdCDT and the individual components was significantly higher in both chancroid and periodontitis patients as compared with Tanzanian blood donors [70]. Furthermore, antibodies to *H. ducreyi* antigens are not detected in sera from experimentally infected humans [18]. Even though both cell mediated and humoral immune responses are induced by natural *H. ducreyi* infection the patients are not protected against subsequent infection.

Humphreys *et al*. used microarrays to profile the gene expression in infected and wounded skin of experimental *H. ducreyi* infected humans, and reported that DCs from volunteers that continuously resolved the experimental infection responded differently to *H. ducreyi* [71]. They suggested the non resolvers to promote a dysregulated T-cell response which contributed to the phagocytic failure, while the DC s from volunteers that resolved the infection probably promoted a Th1 response that facilitated bacterial clearance.

In the temperature-dependent rabbit model, the animals were shown to be protected against experimental challenge f a single experimental infection with *H. ducreyi*, immunization with cell wall components, a pilus preparation or purified hemolysin [72-75]. Pigs were not protected from subsequent infection by a single exposure to *H. ducreyi*, but after three inoculations pigs developed a modest, but significant level of protection against bacteria [36]. Protection was defined as a reduction in disease severity as indicated by reduced recovery of viable bacteria. In addition, transfer of *H. ducreyi* immune serum protected naïve juvenile pigs against challenge with bacteria, thereby proving pigs to mount an effective humoral immune response to *H. ducreyi* after multiple exposure to the organism [37].

6. LPS AND *HAEMOPHILUS DUCREYI* LIPOOLIGOSACCHARIDE

Lipopolysaccharide (LPS), a major component in the outer membrane of the Gram-negative bacteria, is considered to be an important virulence factor and is essential for the viability of

bacteria. LPS and LOS are reported to be involved in the pathogenesis of disease by activation of human immune cells and subsequent production of pro-inflammatory cytokines and chemokines [76-77].

LPS can be described as a complex glycolipid which can be divided structurally into three parts; 1) lipid A, the hydrophobic domain that is attached in the outer membrane and responsible for the biological activity, 2) the core, a non repeating oligosaccharide covalently attached to lipid A, which can be further divided into the inner core consisting of heptose and Kdo (3-deoxy-D-*manno*-2-octulosonic acid), and the outer core with structural diversity and an attachment site for the O-antigen, and 3) the O-antigenic side chain, a distal repetitive polysaccharide [78].

Lipid A is the moiety of LPS reported to be responsible for the biological activities referred to as "endotoxic" effects [76, 79-80]. An endotoxic effect is initiated by the binding of free LPS to LPS-binding protein and CD14, and thereafter recognition of complex by Toll-like receptor 4 (TLR4), subsequently leading to activation of intracellular signaling pathways resulting in the release of pro-inflammatory mediators (reviewed in [81]). The outcome of endotoxic activity varies, some important effects caused by endotoxin are lethal toxicity, pyrogenicity, induction of leukocytosis, platelets aggregation, complement activation, mitogenic activity of B-cells, adjuvant and immunomodulating activity, etc. [80]. Intradermal injection of lipid A in mice resulted in induction of vascular permeability followed by hemorrhagic necrosis in the dermis [82].

The LPS produced by *H. ducreyi* lacks the repeating O-antigens, thus an LOS, and contains a variable and branching core oligosaccharide [83-84].

6.1. Structure of HdLOS

The structure of *H. ducreyi* LOS (HdLOS) produced by different bacterial strains have been characterized in several studies [83, 85-91]. The carbohydrate chain in the LOS produced by different *H. ducreyi* strains were reported to vary in length from 5 to 11 monosaccharides, about 10% to 50% of the LOS molecules were sialylated and the majority of *H. ducreyi* strains expressed the nonasaccharide form of HdLOS [88]. HdLOS contains a major glycoform with immunochemical identity to paragloboside, a glycoshingolipid precursor of human blood antigens and molecular mimicry of host structures by HdLOS is a suggested mechanism for *H. ducreyi* to evade the natural host defense [86]. In addition, the terminal portion of the HdLOS is reported to be involved in the adherence to and the invasion of human keratinocytes by *H. ducreyi* [92].

The structure of HdLOS produced by different *H. ducreyi* strains have been determined, in 1995 and 1997, the structure of LOS from *H. ducreyi* strain 4438 and 7470 were published by Ahmed *et al*. (Fig 2) [85, 88-89].

A)

α-NeuAc-(2→3)-β-Gal-(1→4)-**β-GlcNAc-(1→3)-β-Gal-(1→4)-α-Hep-(1→6)-**β-Glc-α-(1→4)-α-Hep-(1→5)-α-KDO4P-Lipid A
|
α-Hep-(1→2)-α-Hep-(1→3)┘

B)

α-NeuAc-(2→3)-β-Gal-(1→4)-β-Glc-α-(1→4)-α-Hep-(1→5)-α-KDO4P-Lipid A
|
α-Hep-(1→2)-α-Hep-(1→3)┘

Figure 2. Structure of *H. ducreyi* LOS purified from (A) strain 7470 and (B) strain 4438; strain 4438 misses internal trisaccharide (in bold).

Post and Gibson defined two classes of *H. ducreyi* strains, based on the protein profiles and LOS structures from various strains, which generally are well conserved, class 1 strains predominantly express a nonasaccharide, whereas class 2 strains synthesise a pentasaccharide, the latter terminating in N-acetylglucosamine and does not act as acceptor for sialic acid [91].

6.2. HDLOS ROLE IN PATHOGENESIS AND IMMUNITY

The literature describes the biological activity of endotoxin well and as mentioned before LOS have been reported to be involved in the pathogenesis of disease by the activation of human immune cells and the subsequent production of pro-inflammatory cytokines and chemokines [76, 78, 80]. Nevertheless, the role of HdLOS in the pathogenesis of chancroid is still not well evaluated.

Intradermal injection of LOS was reported to cause inflammation and tissue destruction in rabbits and mice [34-35, 40]. The expression of glycoforms with sugar moieties that extend beyond the heptose trisaccharide core was not required for the formation of pustules by *H. ducreyi* in human subjects subcutaneously injected with different *H. ducreyi* LOS mutants [93]. Nevertheless the role of HdLOS in the pathogenesis of chancroid is still not well evaluated.

HdLOS specific antibodies were detected in sera from chancroid patients, as well as in sera form blood donors [94-95]. Alfa *et al*. described the differences between the levels of serum anti-HdLOS antibodies in sera from endemic chancroid areas in Uganda and Kenya, and Canada

where chancroid is not endemic. They reported HdLOS to stimulate a specific immune response to *H. ducreyi* and postulated that ELISA could be useful as serological tool [94]. In an outbreak of chancroid in the United States, LOS EIA was used for the detection of serum anti- HdLOS IgG antibodies and the result of the IgG LOS EIA performed on initial sera from GUD patients was compared with multiplex (M)-PCR result of genital lesion specimens [95]. The LOS EIA was found to have a low sensitivity, only 48% of the patients that were M-PCR positive for chancroid were positive in the LOS EIA. In addition, no increase in seropositivity was detected in follow up sera. The authors concluded that the LOS EIA might be a useful epidemiologic tool in areas where PCR cannot be applied and that the use of serological assays for diagnosis of chancroid was not applicable, based on the fact that it takes several weeks of ulcerative symptoms before antibodies are developed [95].

Odumeru *et al*. suggest the different composition of *H. ducreyi* LOS to be involved in the susceptibility to complement-mediated serum bactericidal activity [96]. Later, Frisk *et al*. reported antibodies specific to LOS to be incapable of enhancing the killing of bacteria [97]. However, the specificity of anti-HdLOS antibodies as well as their contribution to immunity needs further evaluation.

7. *HAEMOPHILUS DUCREYI* CYTOLETHAL DISTENDING TOXIN AND THE CDTS

H. ducreyi produces a cytolethal distending toxin (HdCDT), first described in 1992 by Purvén and Lagergård [57]. The HdCDT belongs to the CDT family of recently discovered bacterial protein toxins.

The first report of this novel type of toxin activity was presented in 1987 by Johnson and Lior [98]. They observed that culture supernatant from pathogenic strains of *Escerichia coli* caused distension of cells cultured *in vitro,* that after 3-5 days resulted in cell death. Later the authors report that both *Shigella* spp and *Campylobacter* spp possessed the same type of toxic activity and named the putative toxin, "cytolethal distending toxin" [99-100]. In 1994, the toxin was sequenced from a pathogenic *E. coli* strain for the first time and it was found that the CDT was encoded by three chromosomally linked genes [101].

Throughout the years, several CDT producing Gram-negative bacteria other than *H. ducreyi* have been identified and sequenced through the years, e.g. *E. coli*, *Shigella* spp., *Campylobacter* spp., *A. actinomycetemcomitans*, several *Helicobacter* spp., *Salmonella paratyphi* (only *cdtb)* and *Salmonella enterica* serovar Thypi (only *cdtb*) [100, 102-113]. So far, no Gram-positive bacteria have been found to produce CDT. The CDT-nomenclature proposed

by Cortes-Bratti *et al.* that specifies the different CDTs by indicating the producing bacterium before CDT, e.g. HdCDT for *H. ducreyi* CDT, will be employed in this thesis[114].

7.1. GENETICS OF HDCDT

The HdCDT similarly to other CDT is encoded by closely located nearly, or slightly overlapping open reading frames (ORFs), *cdtA*, *cdtB*, and *cdtC*, with three known promoters located upstream from the first ORF, with a rho-independent transcription terminator located 3' from the third ORF [115] Transcript analyses of the *H. ducreyi cdt* gene cluster indicated the three genes to be transcribed in a single transcript [115]. The *cdt* gene cluster is chromosomally located in all species, except for one *E. coli* strain where it is located on a Vir plasmid [104]. Furthermore is it possible that the *cdt* genes have disseminated among *H. ducreyi* and other Gram-negative bacteria, since it is known that DNA close to the *cdt* genes have been acquired by horizontal transfer [104, 107, 115]. Nucleotide sequence analysis of *H. ducreyi* chromosomal DNA both upstream and downstream from the *cdt* gene cluster reveal the presence of ORFs with homology to proteins involved in transposition [115]. Moreover, sequences upstream the *cdt* gene cluster of *A. actinomycetemcomitans* were found to be related to a bacteriophage attachment site [107]. Likewise, sequences near the genes encoding EcCDT-III on the Vir plasmid were found to be phage and insertion-sequence remnants [104]. In the genomes of *S. enterica* serovar Thypi and *S. paratyphi* have only sequences that encodes for the *cdtB* gene been found and no homologues are detected that encode for *cdtA* and *cdtC* [112] However have *S. Typhi* been found to encode for two genes, pltA and pltB, in the same pathogenicity islet as cdtB, with homology to components of the pertussis toxin, including its ADP-ribosyl transferase subunit [116].

7.2. *H. DUCREYI* CDT SUBUNITS AND HOLOTOXIN

The *cdtA*, *cdtB* and *cdtC* genes in *H. ducreyi* were found to encode for proteins with predicted molecular weights of 24.7, 31.5, and 20.6 kDa, respectively, the molecular weight of the mature form of these proteins were found to be 23, 29, and 19 kDa, respectively [122, 125]. The amino acid identity for CDTs from different species varies from 19 to 97% [117]. When the HdCDT subunits were compared with other CDTs, the CdtB was found to be the most conserved one of the proteins and displayed higher homology (47-50%), than CdtA (25-33%) and CdtC (21-25%), [117]. The highest homology were reported between the HdCDT and *A. Actinomycetemcomitans* CDT (AaCDT), ranging from 92 to 97% [107, 117].

In 2002, the three HdCDT subunits as well as the HdCDT holotoxin were successfully purified by Wising *et al.* [38].

The CdtB produced by *E. coli* and *C. jejuni* were described to possess a deoxyribonuclease I (DNase I)-like enzymatic activity [118-119]. A position-specific homology between the CdtB subunit from EcCDT-II and mammalian DNase I was reported by Elwell and Dreyfus [118]. The homology pattern was found at specific residues involved in enzyme catalysis (Glu86, His154, Asp229, His261), DNA binding (Arg123, Asn194), and metal ion binding (Glu62, Asp192, Asp260).Furthermore, the CdtB contained a pentapeptide sequence (aa 259-263: Ser-Asp-His-Tyr-Pro) found in all DNase I enzymes. The DNase activity, as well as the cytotoxicity, was abolished by point mutations of conserved residues and the catalytic DNase activity appeared to be essential for the cytotoxic activity of CDT [118].

Frisk *et al*. constructed recombinant plasmids that allowed the expression of each HdCDT gene individually or in different combination in *E. coli* and *Vibrio cholera* and found that it was it the CdtB component that possessed the DNase activity [120]. Moreover, have Shenker *et al.* reported of a phosphatidylinositol (PI)-3,4,5-triphosphate phosphatase activity of AaCdtB, when conserved amino acids critical to catalysis in the phosphatidylinositol 5-phosphatase family of enzymes were mutated, the activity was abolished [121]. In addition, these mutations caused a decrease in the ability to induce G2 arrest.

From the beginning the roles of HdCdtA and HdCdtC in the generation of cytotoxicity were not defined. However, several studies have demonstrated that all three subunits must be expressed for the generation of cytotoxicity by *H. ducreyi* and other CDT producing bacteria [102, 106, 115, 120].These findings were confirmed when crystal structure of HdCDT was determined in 2004 by Nesic *et al*.[122]. The crystal structure of the holotoxin revealed that HdCDT is a tripartite complex consisting of an enzyme of the DNase-I family, CdtB, bound to two lectin-type structures, homologous to the B-chain repeats of the plant toxin ricin, CdtA and CdtC. The subunits form a complex with three independent molecular interfaces characterized by globular and non-globular interactions (Fig 3) [122]. The authors suggested, based on the close interplay of CdtA and CdtC that they probably work together for a related function, which is likely to be the receptor binding of the complex. This knowledge supports the hypothesis that HdCDT is an AB_2 toxin, with CdtA-CdtC as the binding subunit (B_2) and CdtB as the active subunit (A).

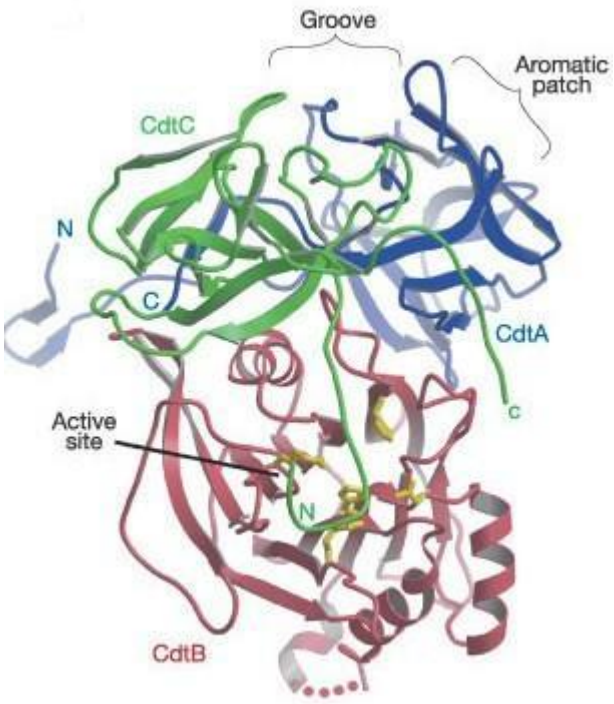

Figure 3. Crystal structure of HdCDT, shown as a ribbon cartoon tracing the three polypeptide chains. N, amino terminus; C, carboxy terminus.
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7.3. CDT ACTIVITY *IN VITRO*

In 1992, the effects of a cytotoxin produced by *H. ducreyi* were reported by Purvén and Lagergård [57]. Epithelial cells were observed to be enlarged, elongated and rounded as a response to *H. ducreyi* cytotoxin intoxication. The human epithelial cell lines HEp-2, HeLa, and A549 were reported to be more sensitive to the cytotoxic activity than human and animal fibroblasts, even though morphological changes were observed in these cells [57].

The most apparent morphological effect of CDT is the cell distension which leads to a three-five fold increase of the cell size. Together with cellular distension the actin cytoskeleton is strongly promoted; this effect can be detected as the appearance of actin stress fibers [123-124]. After continued incubation with cytotoxin, cells round up, show membrane blebbing in some cases, and then deteriorate completely (Fig 4). In contrast to fibroblasts and epithelial cells, T-cells, B-cells and dendritic cells do not distend, but rather become apoptotic and fragmented after CDT treatment [125-127]. Nowadays almost all known CDT are reported to cause this type of distension on cultured cells, *in vitro* [112].

A B

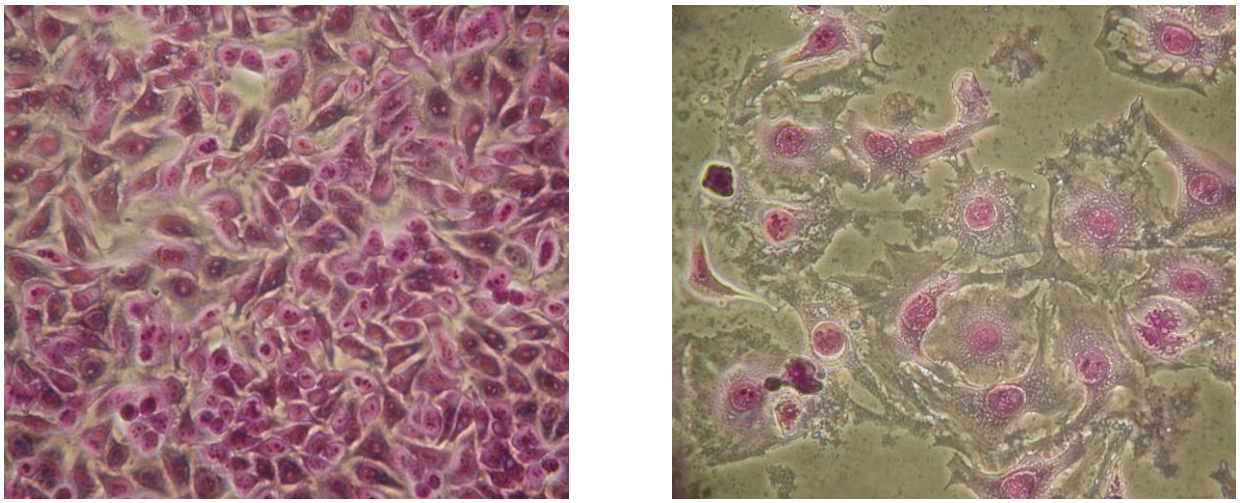

Figure 4. Morphological changes caused by HdCDT intoxication of human HeLa cells.
A) Cells incubated with PBS. B) Cells treated with HdCDT (10 ng/ml) for 48h.
Above pictures were kindly provided by Teresa Lagergård.

The initial step in CDT intoxication is the binding of the toxin to the cell, and similar to other intracellular toxin the internalization is an important step, still unfortunately no receptor has been identified for HdCDT. Thus, for AaCDT is the ganglioside GM3 reported to be a possible CDT receptor [115]. The binding of AaCDT to its cell surface receptor is suggested to be mediated predominantly by the CdtA subunit [128]. Expression of recombinant HdCdtA and HdCdtC in *E. coli* resulted in the formation of a non-covalent CdtA-CdtC complex, capable of binding to HeLa cells, addition of purified CdtB caused cell death within 72 h [129]. HeLa cells pretreated with the CdtA-CdtC complex were prevented from cell death by addition of *H. ducreyi* culture supernatant containing HdCDT holotoxin, which completely killed untreated cells. Guerra *et al*. reported the binding of HdCDT to the plasma membrane of sensitive cells to be dependent on cholesterol [130]. This was confirmed for AaCDT when Boesze-Battaglia *et al*. reported that cholesterol-rich cytoplasmic membrane lipid rafts were involved in the delivery of AaCdtB into target cells [131]. By depletion, inhibition and genetically manipulation studies HdCDT were shown to enter HEp-2/HeLa cells by endocytosis via clathrin-coated pits [132]. The cellular intoxication was inhibited if the fusion of early endosomes with downstream compartments was blocked or if agents that disrupted the Golgi complex were used. The authors concluded that the toxin, after uptake via clathrin-coated pits, required transport in vesicles at least to the Golgi apparatus before it could be activeated [132]. Another study confirmed these results by reporting that the toxin were internalized via the Golgi complex and thereafter

retrogradely transported to the endoplasmic reticulum (ER) [130]. In an assay using transient expression of a series of truncated AaCdtB-green fluorescent protein (GFP), a 76-amino acid sequence (residues 48-124) was reported to constitute an atypical nuclear localization signal (NLS) [133]. Microinjected His-tagged AaCdtB-GFP entered the nucleus within 3-4 hours. It was also demonstrated that a holotoxin containing an 11-amino acid truncation in the identified NLS of AaCdtB were unable to intoxicate the cells. This observation suggested that the identified NLS may be functional for nuclear localization of the toxin also when cells are naturally intoxicated [133]. In 2004, two NLS sequences, designated NLS1 and NLS2 were identified in the carboxyterminal region of EcCdtB-II [134]. Most recently it was found that HdCdtB was heat stable and resisted degradation, it was suggested that the toxin subunit did not unfold before exiting the ER and might move directly from the ER lumen to the nucleoplasm [135]. This though HdCdtB does not contain any known conventional NLS.

The outcome of HdCDT intoxication varies depending on the cell type affected. Several cell types are reported to be sensitive to the toxin e.g. HEp-2, HeLa, Jurkat T-cells, THP-1, HaCat, Vero and Don Fibroblasts [115, 124, 136]. Intoxication of HeLa cells with HdCDT caused DNA double strand breaks, similar to ionizing radiation (IR) [137]. The toxin is believed to act as a genotoxin by causing DNA lesions, and consequently induce cellular responses that result in cell cycle arrest and cell death through apoptosis/necrosis. HdCDT intoxication of; epithelial cells and normal keratinocytes causes cell cycle arrest exclusively in G2, normal fibroblasts show cell cycle arrest in both G1 and G2 phase of the cell cycle while B-cells are reported to undergo apoptosis [126]. Moreover, HdCDT is reported to inhibit proliferation of normal human T-cells and B-cells, and to affect normal human endothelial cells (HUVEC) [138-139]. The two rabbit cell lines; SIRC (fibroblast-like cells from cornea) and RK13 (epithelial-like cells from kidney) were affected both in the metabolic activity and arrested in the G2 phase of the cell cycle by HdCDT intoxication [38]. Wising *et al*. described that HdCDT induce different levels of apoptosis and necrosis in a dose- and time dependent manner in different types of cells. Early and late apoptosis were induced in more than 90% of T-cells and monocytes, but only in 26-32% of the epithelial cells, kerationcytes and fibroblasts [140]. The authors concluded that HdCDT effectively eliminate the cells that are involved in immune responses by inducing apoptosis. Additionally, cells that are of importance in the healing of chancroid ulcers were driven into apoptosis or necrosis.

7.4. CDT ACTIVITY *IN VIVO*

The knowledge of how HdCDT contribute to the pathogenesis of *H. ducreyi* in natural hosts or in experimental animal models is relatively limited. However since there is a high prevalence of *cdt* in *H. ducreyi*, *C. jejuni*, and *A. actinomycetemcomitans* CDT might be an important virulence factor in infections caused by these pathogens. There are, however, only few studies reported on the actual importance of CDT *in vivo*.

The *in vivo* activities of CDT in *H. ducreyi* infection were studied using isogenic mutants to the different CDT components. An isogenic *H. ducreyi cdtC* mutant was reported to be as virulent as the wild-type strain in the temperature-dependent rabbit model of chancroid, despite the fact that it was not cytotoxic to HeLa cells and keratinocytes [141]. In addition isogenic *H. ducreyi cdtA* and *cdtB* mutants were presented to be as virulent as the wild-type strain with regard to lesion production in the same rabbit model [142]. Furthermore, the expression of CDT was not required for pustule formation by *H. ducreyi* in the experimental human model [143]. Wising *et al*. used the temperature dependent rabbit model and reported of HdCDT induced inflammation in rabbit skin when injected alone and HdCDT contributed to the local aggravation and retardation of the healing of skin when it was injected together with a non-toxin producing strain of *H. ducreyi* [39].

For *C. jejuni* the CDT was reported to be essential for a persistent infection of the gastrointestinal tract. One *C. jejuni cdtB* mutant was able to enteric colonization of SCID mice, but the invasiveness was impaired compared to wild-type bacteria, implying that CDT might have a role in the pathogenesis (invasion) of *C. jejuni* [144]. Moreover, an investigation of a *C. jejuni cdtB* mutant in wild-type and nuclear factor (NF)-κB deficient mice suggested that CDT may have a pro-inflammatory activity *in vivo*, as well as a potential role in the ability of *C. jejuni* to escape immune surveillance [145]. The *cdtB* mutant was less efficient than wild-type *cdtB* in colonizing the wild-type mice but not in NF-κB deficient mice. Despite 100% colonization of the NF-κB deficient mice the *cdtB* mutant produced less gastritis than the wild-type bacterium [145]. In 2008, Jain *et al*. showed that CDT producing *C. jejuni* strains adhered to and invaded epithelial cells more efficiently than CDT deficient *C. jejuni* strains by the use of the suckling mice model. They concluded that CDT is responsible for intestinal pathology and the colon is the major target.[146].

H. hepaticus is an enterohepatic pathogen of mice that colonize the lower gastrointestinal tract and the liver, and HhCDT is reported to contribute to an increased mucosal inflammation or liver disease in susceptible mouse strains [147]. Young *et al*. provided evidence for a potential pro-inflammatory activity of CDT when they described that strains of *H. hepaticus*

which expressed a functional CDT caused severe colitis in a murine model of inflammatory bowel disease, using IL-10 deficient mice [148]. Recently, *H. hepaticus* CDT were shown to play an important role in promoting the progression of infectious hepatitis to pre-malignant, dysplastic lesions, *in vivo* [149].

An *E. coli* strain that expressed the *cdt* genes from *S. dysenteriae* was reported to be able to induce watery diarrhea in a suckling mouse model and the toxin caused a certain damage in the descending colon of these mice [150].

7.5. ANTIBODY RESPONSE TO CDT

Very little is known about the HdCDT specific antibody response in humans. Purvén *et al*. detected cytotoxin neutralizing antibodies in the majority of sera from patients with culture-proven chancroid [69]. CDT neutralizing antibodies were also detected in sera from patients infected with *C. jejuni* [151].When the prevalence of antibodies to HdCDT and the individual components were studied, the antibody levels were found to be significantly higher in both chancroid and periodontitis patients as compared with Tanzanian blood donors [70]. Moreover, antibodies to CdtC were less frequently detected than CdtA and CdtB antibodies; however they correlated well with the neutralizing capacity of the sera. These data suggest that the level of anti-HdCDT antibodies and their neutralizing capacity may be insufficient for induction of protective immunity [70]. Earlier this year Xynogala *et al*. detected antibodies to AaCDT with neutralizing capacity in human subjects infected with *A. actinomycetemcomitans*, thus demonstrating that AaCDT is produced during natural infection of humans [152]. Furthermore, 20 of 23 human subjects with localized aggressive periodontitis were found to be unable of mounting a significant anti-CDT response and the authors suggested that this may in part explain their relative susceptibility to the disease.

Nevertheless, experimental infection with toxin producing *H. ducreyi* or immunization with bacterial sonicate, purified HdCDT or HdCDT components in the temperature dependent rabbit model resulted in the development of toxin neutralizing antibodies, although levels were low [120]. Moreover, Lagergård *et al*. immunized mice with formaldehyde detoxified HdCDT and high levels of toxin neutralizing antibodies were generated [153].

AIMS OF THIS STUDY

THE GENERAL AIM OF THIS THESIS WAS TO INVESTIGATE THE ROLE OF *HAEMOPHILUS DUCREYI* LIPOOLIGOSACCHARIDE (HDLOS) AND *HAEMOPHILUS DUCREYI* CYTOLETHAL DISTENDING TOXIN (HDCDT) IN ANTIBODY RESPONSES.

THE SPECIFIC AIMS WERE:

1. TO EVALUATE THE FUNCTION AND VIABILITY OF HUMAN MONOCYTE-DERIVED-DENDRITIC CELLS, MACROPHAGES AND $CD4^+$ T-CELLS AFTER INTERACTIONS WITH *H. DUCREYI* BACTERIA, HDLOS AND HDCDT, *IN VITRO* (PAPER I).

2. TO DEFINE IMMUNOGENIC AND ADJUVANT PROPERTIES OF HDLOS (PAPER II AND III).

3. TO EVALUATE THE IMPACT OF HDCDT ON THE SERUM ANTIBODY RESPONSES USING A MOUSE MODEL (PAPER III).

4. TO DEFINE A PROCEDURE FOR THE GENERATION OF HIGH ANTIBODY LEVELS TO HDCDT IN THE GENITAL TRACT USING A MOUSE MODEL (PAPER III).

MATERIALS AND METHODS

REAGENTS (I AND II)

Recombinant human GM-CSF (rhGM-CSF; 1.665×10^6 U/ml, 150 μg) and IL-4 (rhIL-4; 2.9×10^4 U/μg, 25 μg) were purchased from R&D Systems (Abingdon, UK).

Phytohemagglutinin (PHA) and trypan blue were obtained from Sigma Chemical Co. (St. Louis, MO, USA). [^{3}H]-thymidine was purchased from Amersham (Buckinghamshire, UK).

The oligosaccharide derivatives conjugated to human serum albumin (HSA), lacto-N-neotetraose-APD-HSA conjugate (Gal-β1-4GlcNAc-β1-3Gal-β1-4(Glc)-APD-HSA) and monosialyl-lacto-N-neotetraose-APD-HSA conjugate (αNeu5Ac2-3Gal-β1-4GlcNAc-β1-3Gal-β1-4(Glc)-APD-HSA), and oligosaccharides Lacto-N-neotetraose, LNnT (Galβ1-4GlcNAcβ1-3Galβ1-4Glc) and 3'sialyllactose (αNeu5Ac2-3Galβ1-4Glc) were obtained from IsoSep (Tullinge, Sweden). Lipid A-Kdo-Kdo (di[3-deoxy-D-manno-octulosonyl]-lipid A, ammonium salt) purified from heptose-deficient *E. coli* mutant WBB06 was obtained from Avanti Polar Lipids (Alabaster, AL).

Flourescein isothiocyanate (FITC) was purchased from Sigma-Aldrich Inc. (St Louis, USA).

BACTERIAL STRAINS, CULTURE CONDITIONS AND LABELING (I, II AND III)

H. ducreyi strains used in this thesis were CCUG 4438 [Institute Pasteur Collection (CIP) 542], CCUG 7470 [Institute Pasteur Collection (CIP) 76118] obtained from the Culture Collection, University of Gothenburg (CCUG) and ATCC 35000 obtained from the American Type Culture Collection.

Bacteria were cultivated on chocolate agar plates Grand Lux (GL) (Department of Bacteriology, University of Gothenburg, Sweden) or in brain heart infusion (BHI) broth supplemented with 1% hemin/histidine (BHI-hemin; Sigma), 0.04% L-histidine (Fluka Chemie AG, Buch, Switzerland), 10% calf serum (FCS), 1% IsoVitale X, and 3 μg/ml vancomycin (Department of Bacteriology, University of Gothenburg, Sweden) as described previously [35]. Plates and liquid cultures were incubated in an oxygen-depleted, CO_2-enriched humidified atmosphere at 33°C for 15-18 h in an anaerobic jar with anaerocult C (Merck, Darmstadt, Germany), bacteria cultivated in liquid medium were rotated at 100 rpm.

Non-capsulated *H. influenzae* CCUG 7566 was cultivated on GL plates at 37 °C for 24 h in a CO2-enriched atmosphere.

Bacteria were labeled with FITC as described previously [61]. Briefly, bacteria grown in liquid medium for 15-16 were washed with PBS twice and killed with gentamycin treatment, followed by centrifugation and resuspension in 2 ml PBS containing 1mg/ml of FITC. After

incubation at 4°C for 1h bacteria were washed and the labeled bacteria were blocked with a balanced saline solution containing 0.1% gelatine (GH-BSS) and stored at -20°, until used.

CELLS AND CULTURE CONDITIONS

All cell cultures were maintained at 37°C in a humidified atmosphere with 5% CO_2, as described [140].

CELL LINES (I, II AND III)

Human: Epithelial cell line HeLa (ATCC® CCL2™) was cultured in Earle Minimum Essential Medium supplemented with 8% FCS, 0,02 mg/ml gentamicin, 2 mM L-glutamine. The keratinocyte cell line HaCaT (from Dr. N. E. Fusenig, Heidelberg, Germany) and normal foetus fibroblasts (Department of Virology, University of Gothenburg, Sweden) were cultured in Eagle's medium with 10% FCS, 1% glucose, 1% Na-pyruvate, 1% L-glutamine, and 1% penicillin-streptomycin (PEST). The primary human umbilical vein endothelial cells (HUVEC; Cascade Biologics Inc., Portland, OR, USA) were cultured in medium M200 (Cascade Biologics).

Mouse: B-cell line X16 (ATCC® TIB-209™), T-cell hybridoma 3DO54.8, dendritic cell line FSDC [154], macrophage cell line P388D1 (ATCC® CCL-46™) and lung epithelial cell line MLE12 (ATCC® CRL-2110™) were cultured in Iscove´s Modified Dulbecco´s Medium (GIBCO™) supplemented with 10% foetal calf serum (FCS) and 0,02 mg/ml gentamycin. The intestinal epithelial cell line m-IC_{c12} was maintained as described [155].

PBMC (II)

Peripheral blood mononuclear cells (PBMC) were separated from heparinized blood samples from volunteer donors by density gradient centrifugation on Ficoll-Paque™ Plus (Amersham Biosciences AB, Sweden) and cultivated in RPMI 1640 with 5% heat-inactivated (at 56 C for 30 min) human AB serum, as described [61]. Viability, assayed by the trypan blue exclusion test, was >95%.

GENERATION OF MONOCYTE DERIVED DCS AND MQS (I)

Monocytes were purified from PBMCs by two different methods. In the first method CD14 MicroBeads (Miltenyi Biotech, Germany) were employed according to the manufacturer's instructions. The second method utilized cell adherence to cell culture plates, which were incubated 37 C for 2h at in 5% CO_2, then washed twice with PBS to remove the non-adherent cells.

DCs were generated by cultivation of the monocytes for 5 days in RPMI supplemented with 5% heat-inactivated human AB serum, 0.3 mg/ml L-glutamine, 0.1 mg/ml gentamycin, 800 U/ml GM-CSF and 1000 U/ml IL-4 [56]. For the generation of MQs was the RPMI medium supplemented with 5% FCS and the cells were cultivated for 7 days [156-157]. Surface markers on the DC and MQ populations were analyzed by flow cytometry after staining with antibodies against CD11c and CD14 [156]. The percentages of DCs expressing $CD11c^+$ were 79–87% and 95% for DCs that were derived from adherent and CD14 MicroBead purified monocytes, respectively. The percentage of $CD14^+$ MQs was 89–94%. The DCs and MQs used in the experiments were from populations in which ≥79% of the cells were $CD11c^+$ or $CD14^+$. Cell viability was determined at the start of each experiment by the trypan blue exclusion test and was > 90%. There were no significant differences in purity, expression of surface marker or cytokine production between the DCs generated from monocytes purified by different methods. A higher number of DCs were generated from adherent monocytes after maturation, thus was this method applied for the production of DCs.

$CD4^+$ T-CELLS (I)

$CD4^+$ T-cells were isolated from heparinized blood samples from volunteer donors using $CD4^+$ cell isolation kit (Dynal Biotech ASA (Oslo, Norway).

ANTIGEN PREPARATIONS

All protein determinations in this thesis were done using the Bio-Rad colorimetric assay with bovine serum albumin as standard.

HDCDT PURIFICATION, CHARACTERIZATION AND LABELING (I AND III)

The HdCDT complex was purified from *E. coli*, HdCdtA, HdCdtB, and HdCdtC recombinants as described previously [38]. Briefly, the HdCDT complex was reconstituted by immobilizing Glutathione S-transferase (GST)-HdCdtB fusion protein on a 5-ml GST HI-trap column followed by the addition of pre-mixed sonicate from *E. coli* (pAF-tac1) HdCdtA and HdCdtC recombinants. The HdCDT toxin complex was cleaved from the column by the addition of thrombin, which cleaves of the complex leaving the GST moiety on the column. The purified HdCDT preparation contained all the HdCdtA, HdCdtB, and HdCdtC subunits, as detected by SDS-PAGE followed by Coomassie blue staining and immunoblotting. The cytotoxic activities of the HdCDT preparations ranged from 10^6 to 10^8 cytopathic units per ml (CPU/ml), with 1 CPU being defined as the dilution of the HdCDT preparation that caused 50% inhibition of HeLa cell growth compared to a PBS-treated control [39]. The endotoxin content, as quantified by the Limulus amoebocyte lysate assay (LAL, Sigma), was <0.05 μg/1 mg protein [158].

HdCDT was labeled using Alexa Fluor 488 protein labeling kit according to manufactures instructions (Molecular Probes Europe BV, Leiden, The Netherlands). The cytotoxic activity after the labeling procedure was decreased by 10%.

The toxicity of HdCDT was neutralized by the incubation of HdCDT at concentrations ranging from 3-30 ng/ml with rabbit anti-HdCDT serum at 37° for 60 min [38].

HdCDT TOXOID PREPARATION (III)

Formaldehyde treatment of HdCDT was carried out as described [38]. The purified HdCDT preparation was diluted to 0.5 mg protein/ml in phosphate-buffered saline that contained 25mM lysine, and formaldehyde solution (formalin) was slowly added with stirring at the room temperature to a final concentration of 0.75 %. The mixture was incubated for 12 h at room temperature followed by 24 h at 37 °C and then 24 h at 4 °C, and was then extensively dialyzed against sterile PBS at 3–8 °C. The toxoid preparation was completely detoxified as estimated by cytotoxicity assay. The SDS-PAGE followed by Coomassie blue staining and immunoblotting showed similar pattern as described [153]. The final protein concentration was 225 µg/ml.

H. DUCREYI LIPOOLIGOSACCHARIDE PREPARATIONS (I, II AND III)

HdLOS7470 was hydrolyzed by using acetic acid or anhydrous hydrazine. In the first method, LOS was treated with 1% acetic acid at 100°C for 60 min, ultra centrifuged (35000 rpm for 5 h), and the carbohydrate-containing supernatant was fractionated on a Bio-Gel P-4 column (1.0 × 100 cm) in 0.05 M pyridine acetate buffer, to obtain an oligosaccharide (OS) preparation. In the second method, LOS was treated with anhydrous hydrazine (10 mg/ml) at 37°C for 30 min, to remove *O*-linked fatty acids, then precipitated with acetone on ice, cooled to -20°C, centrifuged, washed twice with cold acetone, re-suspended in water and freeze-dried (LOS-H).

H. DUCREYI HEAT SHOCK PROTEIN (HSP) PREPARATION (III)

HSP GroEL 58.5- kDa was purified from *H. ducreyi* strain 7470 as described previously with minor modifications [159]. Briefly, bacteria were re-suspended in PBS, vortexed with glass beads and centrifuged at 9300 x *g* for 20 min. The supernatant was applied to an ion-exchange column (Mono Q; Amersham Biosciences), the 58.5-kDA protein was eluted with 0.2 M NaCl and applied to a Sephadex 200 column (Amersham Bioscience). Fractions were pooled and concentrated using a Vivaspin column, with a molecular weight cut-off of 10,000 kDa (Vivascience, Hannover, Germany). At all stages, the HSP-containing fractions were monitored by dot-blotting or by SDS-PAGE and immunoblotting with the anti-HSP monoclonal antibody B11 (kindly provided by Dr C.A. Ison). The final protein concentration was 250 µg/ml.

CELL METHODS

PHAGOCYTOSIS ASSAYS (II)

Phagocytosis of FITC-labeled gentamycin-killed *H. ducreyi* by DCs and MQs was done using Phagotest according to the manufacturer's instructions (Orpegen Pharma, Heidelberg, Germany) and measured by flow cytometry (FACS; Becton Dickinson, San Jose, CA) as described previously[61]. Briefly, 100 µl of DCs or MQs (8×10^5 cells) were mixed with 20 µl (1×10^9 CFU/ml) of FITC-labeled bacterial suspension; at a ratio of approximately 1:25and incubated at 37°C for 60 min. The FITC-labeled *Escherichia coli* provided with the assay kit were used as control and a non-capsulated strain of *H. influenzae* was used for comparison. Samples were analyzed by flow cytometry with blue–green excitation at 488 nm using an argon ion laser and FACSCalibur CellQuest software. The cell populations were analyzed by means of forward and side light scatter. A live gate was set in the red fluorescence FL2 histogram, and a marker was set in the histograms, i.e. the fluorescence intensity (FL1) of the control sample in order to exclude background cell auto-fluorescence. The percentages of cells having ingested bacteria as well as their mean values for the FL1 channel were recorded. The mean and standard deviation (mean ± S.D.) of three independent experiments were calculated. A cell population that was exposed to labeled bacteria at 0°C was used as the negative control, and this value was subtracted from the test results, to exclude the background.

CYTOKINE ASSAYS (II)

The culture supernatants from APCs, keratinocytes, endothelial and epithelial cells and fibroblasts were collected after 24 h of incubation with the bacteria or bacterial antigens, and assayed for IL-1β, IL-6, IL-8, TNF-α, and IL-12. The culture supernatants from DCs that were stimulated first with *H. ducreyi* for 24 h and then treated with different doses of HdCDT and reverse were tested after 48 h for IL-12 production.

The culture supernatants from antigen-pulsed APCs that were co-cultured with $CD4^+$ T-cells were collected after 48 h, and the levels of IL-4, IL-13 and IFN-γ were measured. ELISAs with specific capture and detection antibodies were used to measure the cytokine levels. The levels of IL-1β, IL-6, IL-8 and TNF-α were determined by ELISA (R&D Systems). For IL-4, IL-12, IL-13 and IFN-γ, Opt EIA kits (BD Pharmingen, Heidelberg, Germany) were used. All of the cytokines were analyzed in three separate experiments.

HDCDT INDUCTION OF DC APOPTOSIS (II)

DC apoptosis was measured after treatment with 0.5 µg/ml of HdCDT for 24, 48 and 72 h. Apoptosis was estimated by two-color flow cytometry using annexin V-FITC (PharMingen, San

Diego, CA) and propidium iodide (PI, Sigma), as described [160]. Annexin V binds to phosphatidylserine that is translocated during apoptosis from the inner to the outer leaflet of the plasma membrane. Live cells with intact membranes are distinguished by their ability to exclude PI, which readily penetrates dead or damaged cells. Readings at 488 nm excitation and 525 nm (PI: 625 nm) emission wavelengths were recorded using the FACStar flow cytometer (Becton Dickinson Biosciences, San Jose, CA), and the subsequent analysis was performed using the CellQuest Pro software (Becton Dickinson). Dual analysis was introduced using a quadrant dot plot, in which 'necrotic cells' were identified as single PI-positive, 'early apoptotic cells' were annexin V-FITC-positive only, and cells in 'late apoptosis' were recognized as double-positive for annexin V-FITC and PI. Cells that stained negative for both annexin V-FITC and PI were classified as live cells. The number of cells in each category was expressed as a percentage of the total number of stained cells counted.

CYTOTOXIC ANALYSES (III)

Time and dose dependent effects of treatment with HdCDT on the cell lines; HeLa, FSDC, P388D1, MLE12, m-IC_{c12}, X16 and 3DO54.8 with HdCDT were examined by microscopy as described [115, 120, 159]. Briefly, 100µl of cells were seeded in 96-well plates at a density of $4x10^4$ cells /ml (adherent cell lines HeLa, FSDC, P388D1, MLE12 and m-IC_{c12} were allowed to adhere over night), and HdCDT (0.1, 1, 10, 50, 100, 200 and 1000 ng) was added to the cells. Further, the cytotoxic effect was measured with MTT assay at the time points 24, 48 and 72 h.

MTT ASSAY (III)

The colorimetric MTT assay is based on the cleavage of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) into water insoluble crystals of formazan by metabolically active cells as described previously [140]. 50µg of MTT was added to the HdCDT treated cells, described above, which were further incubated at 37º for 4h. The formazan crystals were dissolved by the addition of 100µl acidic isopropanol (0.1 N HCl) (MERCK KgaA, Darmstadt, Germany) and the absorbance was measured on BioTek Power Wave xs at the wavelengths 570 nm and 630 nm. The difference in absorbance at the two wavelengths is calculated and the result is expressed as per cent of the control cell (untreated) absorbance.

CELL CYCLE ANALYSIS (III)

The effect of HdCDT intoxication on cell cycle progression in the cell lines; 3DO54.8, X16, MLE12 and HeLa was monitored using flow cytometry analysis of the DNA content [140]. Cells were seeded in 24-well plates at a density of $1x10^5$ cells /ml and intoxicated with different concentrations of HdCDT (50, 100 and 500 ng) for 24 h, followed by centrifugation, one wash

with PBS and fixation in 1 ml ice cold 70% ethanol on ice for 15 min. The cells were then re-suspended in 300µl PI solution (0.05 mg/ml PI; 0.02 mg/ml RNase; 0.3% Igepal; 1 mg/ml sodium citrate) and re-incubated for 1 h on ice. Flow cytometry analysis was performed with FACSCalibur (Becton Dickinson, San Jose, CA, USA). Data was collected from 10^4 cells and analyzed using FlowJo (Becton Dickinson).

HdCDT internalization by confocal microscopy (III)

The internalization of Alexa Fluor 488 labeled HdCDT in mouse and human epithelial cell lines was followed by Confocal Laser Scanning Microscopy. MLE12, m-IC_{c12} and HeLa cells at a density of $1x10^5$ were seeded in glass bottom microwell dishes (MatTek Corporation. Ashland, USA). After overnight incubation the cells were washed with PBS and incubated for 5 or 10 min at 37°C with Alexa Fluor 488 labeled HdCDT, (HeLa 10 µg HdCDT for 5 min, MLE12 and m-IC_{c12} 30 µg HdCDT for 10 min), thereafter unbound toxin was removed by washing twice with medium. The internalization of the toxin in live cells was followed at 37° for 48 h using Confocal Laser Scanning Microscopy (Swegene Center for Cellular Imaging, Gothenburg University, Sweden).

Immunization of mice (II and III)

Paper II

Six to eight week old NMRI (wild-type), BALB/c, C57BL/6, athymic nu/nu mice obtained from B&K Universal AB, Sollentuna, Sweden. Toll-like receptor 4 knockout (TLR4 KO) and myeloid differentiation factor 88-knockout (MyD88 KO) mice on a C57BL/6 background, 6-8 weeks of age, were derived by Shizuo Akira (Department of Host Defense, Research Institute for Microbiological Diseases, Osaka University, Japan) and provided by Dr. Mary-Jo Wick (Department of Microbiology and Immunology, Gothenburg University, Sweden). Mice in groups of 5-12 were immunized subcutaneously (sc) on three occasions at 2-week intervals with purified HdLOS7470, OS, LOS-H (5 µg/dose); lacto-N-neotetraose-APD-HSA and monosialyl-lacto-N-neotetraose-APD-HSA conjugates (2.5 µg sugar/dose); bovine serum albumin (BSA) (5 µg/dose) alone or together with HdLOS7470 (1 µg or 5 µg/dose); or were hyper-immunized with *H. ducreyi* 7470 bacteria that were boiled for 1 h. The hyper-immunization was performed with increasing amount of bacteria, injected 9 times, two times sc and 7 times intraperitoneally.

Paper III

Balb/c mice (female) at 6-8 weeks of age obtained Taconic (Denmark). Mice in groups of 5-12 were immunized subcutaneously (sc) 2 - 4 times, 2 weeks apart with; BSA (5 µg/dose) alone or together with HdCDT (5 µg/dose); *H. ducreyi* 4438 (10^6 CFU/dose) alone or together with

HdCDT (10µg/dose); HdCDT (5µg/dose) alone or together with HdLOS7470 (5µg/dose); HdLOS7470 (5µg/dose), HdCDT toxoid (10µg/dose); HdCDT toxoid (10µg/dose) + RIBI adjuvant system (Monophosphoryl Lipid A and synthetic trehalose dicornomycolate, Corixa, USA) prepared and mixed according to manufactures instructions or HdCDT toxoid (10µg/dose) + Imject® Alum (aluminum hydroxide and magnesium hydroxide, Pierce, USA), diluted 1:5 and mixed 1:2 with toxoid preparation according to manufactures instructions.

Mice were sacrificed 10 days after last immunization, sera and genital tissue were collected. Sera were stored at-20°until used. Genital tissue was stored at -20° in 270 µl PBS solution containing 2mM phenylmethylsulfonylflouride (Sigma-Aldrich Inc, St Louis, USA), 0.1 mg/ml soybean trypsin inhibitor (Sigma-Aldrich Inc, St Louis, USA) and 0.05 mM EDTA. Antibodies were extracted from the tissue through permeabilization by addition of saponin (Sigma), to a final concentration of 10% and incubation at 4°over night followed by centrifugation (15.5 k x g, 10 min) [161]. Pre-immune sera were obtained from naive mice.

During experiments animals were maintained at the Department of Experimental Biomedicine, at University of Gothenburg under pathogen free conditions. Experiments were carried out with the approval of the Ethical Committee for Laboratory Animals in Gothenburg.

ANTIBODY ANALYSIS

ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA) (II AND III)

Antibodies to BSA, HSP, HdLOS, HdCDT and whole bacteria were assayed by ELISA, as described [153, 162]. Briefly, 96-well Maxisorp plates were coated with 10 µg/ml of BSA, 1.5 µg/ml HSP, 10 µg/ml HdLOS, 1.5µg/ml of HdCDT or 5 µg/ml sialyl/neolactotetra conjugated to HSA, in phosphate-buffered saline (PBS). For the estimation of antibodies to bacteria, plates were pre-coated with poly-lysine (10ug/ml) for 30 min, then 10^{10} *H. ducreyi* bacteria/ml were added and fixed with 0.5% glutaraldehyde solution, as described [162]. Sera were added at a1:10, 1:20 or 1:100 dilution in the first well and then diluted three-fold in 0.1 % BSA–PBS or 0.05% Tween-PBS (for BSA and HdLOS). In Paper I was a pool of three sera induced by HdLOS (diluted 1:100) selected as reference serum for estimation of anti-LOS antibodies, and assigned a value of 100 units/ml (corresponding to an end point titer of about 1:700). Similarly, for estimation of anti-BSA antibodies were a pool of three sera against BSA (diluted 1:500) used as reference (100 units/ml corresponding to an end point titer of 1:50 000). The antibody concentrations were evaluated using reference line analysis according to the reference sera, and 10 units/ml was the lowest value giving reproducible results. The levels of

IgG subclasses and of the antibodies to sialyl/Neo-lactotetra are expressed as end-point titer, a reciprocal serum dilution that gives an absorbance of 0.3 at 405 nm. A titer of $\leq$10 was considered to be a negative value.

In Paper II was a pool of mouse immune serum containing antibodies against BSA, HSP, HdLOS7470 or HdCDT selected as a reference and included on all the plates coated with BSA, HSP, HdLOS7470 or HdCDT, respectively. For detection of IgG antibodies alkaline phosphatase conjugated AffiniPure Goat–anti–mouse IgG (high+low) (Jackson ImmunoResearch Laboratories Inc. USA) was used at a dilution of 1:2000 and for the detection of IgG subclasses alkaline phosphatase conjugated Goat Anti–Mouse IgG1, IgG2a, IgG2b and IgG3 (SouthernBiotech, Birmingham, Alabama, USA) was used at a dilution of 1:1000. The plates were developed by the addition of *p*-nitrophenyl phosphatase (NPP) substrate (Sigma-Aldrich Inc, St Louis, USA). The absorbance was measured at 405 nm. Results are expressed as end point titer which is the value that corresponds to the serum dilution giving an absorbance value of 0.2 above background.

LOS ANTIBODY INHIBITION STUDY (II)

Inhibition was performed by mixing of diluted anti-HdLOS 7470 serum with an equivalent volume containing 5, 25 or 100 μg of HdLOS7470; HdLOS4438; Lacto-N-neotetraose; 3'sialyllactose as inhibitor or PBS as control. The antibodies in the mixtures were first incubated for 2 h at 37°C and then for 24 h at +4°C in order to allow the adsorption of the antibodies to the antigen, followed by centrifugation and antibody estimation of the supernatant by ELISA. The percentage of inhibition was defined as % of OD in diluted and absorbed serum compared to the same serum diluted and mixed with PBS (1-A_{405} of adsorbed serum/A_{405} of serum with PBS) × 100%.

THIN-LAYER CHROMATOGRAM (TLC) BINDING ASSAYS (II)

The binding of the anti-LOS antiserum to purified glycosphingolipids with structures related to the terminal carbohydrate sequences found in HdLOS was tested with;

GM3 ganglioside (NeuAcα3Galβ4Glcβ1Cer; terminal sialyl-lactose sequence);

lactosylceramide (Galβ4Glcβ1Cer; terminal lactose sequence); sialyl-neolactotetraosylceramide (NeuAcα3Galβ4GlcNAcβ3Galβ4Glcβ1Cer; terminal sialyl-neolactotetra sequence);

neolactotetraosylceramide (Galβ4GlcNAcβ3Galβ4Glcβ1Cer; terminal neolactotetra sequence); and the negative reference globotetraosylceramide (GalNAcβ3Galα4Galβ4Glcβ1Cer) [163].

HdLOS7470 and HdLOS4438, as well as lipid A-Kdo-Kdo were used as positive references in the binding assays.

Polyclonal rabbit anti-mouse antibodies (DAKO, Glostrup, Denmark) were labelled with ^{125}I using $Na^{125}I$ (100 µCi/ml; Amersham Pharmacia Biotech, Little Chalfont, UK), according to the protocol (Pierce, Rockford, IL), giving approximately 5×10^3 cpm/µg protein. Binding of anti-LOS serum to glycoshingolipids separated on TLC was performed as described [164].

Inhibition of TNF-α production by LOS-stimulated human PBMCs using a specific HdLOS-antiserum (II)

PBMCs from four donors were treated separately with purified *E. coli* Ra strain LOS (Sigma), HdLOS4438 or HdLOS7470 at concentrations that ranged from 0.01 - 10 ng/ml. The LOS samples were pre-mixed with an equal volume of anti-LOS serum diluted 1:10, 1:50 or 1:100 before they were added to the PBMCs. After 18 h, cells were centrifuged and supernatants were collected. The concentration of TNF-α in supernatant was measured with ELISA according to manufacturer (DuoSet ELISA Development System; R&D Systems, Abingdon, UK).

Toxin neutralization assay (III)

The level of HdCDT neutralizing antibodies was assayed as described before [69, 115]. Briefly, eight two-fold dilutions of sera in 50 µl of culture medium containing 2% FCS was incubated, in 96-well tissue culture plates with the addition of 50 µl of HdCDT at a concentration of 100µg/ml, for 2h and 15min. 100 µl of the mixture was then added to 24 h semi-confluent culture of HeLa cells (approximately 5×10^4 cells/ml) in culture medium containing 8% FCS, after 2h of incubation at 37° C, medium was discarded and fresh medium was added. Toxin titrations, as well as control cells, were included in each test. The cells were stained with Giemsa after 72 h of incubation and examined under the microscope. The neutralization titer was defined as the dilution of serum that gave 50% cell growth as compared to the control.

Statistical analysis

Statistical analyses were done using Prism Software System (GraphPad Software Inc., San Diego, CA). The relationship between ELISA antibody titers and neutralizing titers was evaluated using Pearson correlation test. Comparisons of different mice groups were performed using an unpaired *t*-test and values of $P<0.05$ were considered statistically significant.

RESULTS AND COMMENTS

1. THE FUNCTION AND VIABILITY OF HUMAN MONOCYTE-DERIVED-DENDRITIC CELLS, MACROPHAGES AND $CD4^+$ T-CELLS AFTER INTERACTIONS WITH *H. DUCREYI* BACTERIA, HDLOS AND HDCDT, *IN VITRO* (PAPER I)

The specific antibody response is dependent on the presentation of antigens by antigen presenting cells (APCs), followed by the activation of a T-cell response. These are the initiating steps in the development of immune responses. In order to investigate the function and viability of APCs after interactions with *H. ducreyi* bacteria, HdLOS and HdCDT human derived cells were used, *in vitro.*

1.1. PHAGOCYTOSIS OF *H. DUCREYI* BY DCS AND MQS

The antigen presentation and the ability to activate a T-cell response are dependent on the capacity of APCs to phagocytose and present pathogen components. In order to evaluate the phagocytic ability of human monocyte derived DCs and MQs, three non-opsonized FITC-labeled *H. ducreyi* strains, *H. influenzae* and *E. coli* were used. The DCs were found to ingest all three *H. ducreyi* strains and no significant differences were found in the percentages of phagocytosis between the different *H. ducreyi* strains, but there were significant differences in this respect between *H. ducreyi* 4438 and *H. influenzae* and *E. coli* (P = 0.03) (Tabel 2.). The correlation between phagocytosis by DCs and MQs was high (r = 0.9). The level of phagocytosis by DCs and MQs of the three *H. ducreyi* strains and the non-capsulated *H. influenzae* strain were similar, although the MQs had slightly better rates of bacterial uptake (Tabel 2.).

Table 2. The percentages of DCs and MQs that phagocytosed FITC-labeled bacteria at 37° C for 60 min as determined by FACS analysis

Bacteria	Mean* % of DCs with bacteria ± S.D.	Mean* % of MQs with bacteria ± S.D.
H. ducreyi 7470	15 ± 2.1	22 ± 11.1
H. ducreyi 4438	27 ± 10.0 [a]	26 ± 15.5
H. ducreyi 35000	13 ± 5.0	22.5 ± 6.3
H. influenzae	8 ± 0.7 [b]	14.5 ± 7.7
E. coli	6 ± 2.8 [c]	17 ± 12.7

* Mean of three independent experiments; a versus b and c, $P < 0.05$.

1.2. PRO-INFLAMMATORY CYTOKINE PRODUCTION BY DC AND MQS AFTER INDUCTION BY *H. DUCREYI* BACTERIA, HDLOS AND HDCDT

The activation of DC and MQs caused by *H. ducreyi*, HdLOS and HdCDT was studied by analyzing cell supernatant for the secretion of IL-1β, IL-6, IL-8, TNF-α, and IL-12 after 24 h of incubation, a non capsulated strain of *H. influenza* and *V. cholerae* LPS were used for comparison.

All of the *H. ducreyi* strains induced strong secretion by DCs of IL-1β, IL-6, IL-8, TNF-α, and IL-12 as compared to unstimulated DCs ($P \leq 0.05$) (Fig 5A-E). In general, the *H. ducreyi* strains and *H. influenzae* induced similar secretion of cytokines. Although the induced cytokine level varied slightly among DCs from different donors the patterns of the cytokine responses to different antigens were similar. The level of IL-12 production by DCs stimulated for 24 h with either *H. ducreyi* or *H. influenzae* were high, ranging from 32 000 to 55 000 pg/ml; the corresponding level in HdLOS-stimulated DCs was about 8000 pg/ml (Fig 5E). The secreted level of IL-8 and TNF-α by DCs pulsed with bacteria were significantly higher than obtained after contact with LOS ($P \leq 0.05$), indicating that components other than HdLOS are involved in determining the level of DC activation and production of cytokines. In addition, the induction of cytokine production by HdLOS and *V. cholerae* LPS were comparable (data not shown).

Stimulated MQs secreted high levels of cytokines e.g. a threefold increase in IL-6 and a fivefold increase in IL-8 were noted after 24 h of incubation with *H. ducreyi*, the level of IL-1β were overall low (data not shown). Moreover, MQs secreted a lower level of all the cytokines as compared with DCs.

The *H. ducreyi* strains used in this study were gentamycin-killed and consequently no HdCDT were secreted, therefore DCs and MQs were incubated with addition of purified HdCDT preparation. The only cytokine production induced by HdCDT was IL-8 and the secreted level was low (Fig 5C).

Our results indicated that HdCDT inhibited the secretion of IL-12 by DCs. IL-12 is an important cytokine since it activates NK cells and induces the differentiation of $CD4^+$ T-cells into Th1. Therefore, HdCDT treatment of DCs prior to bacterial stimulation was evaluated for a possible effect on the induction of IL-12 production by bacteria stimulated DCs. The highest dose of HdCDT used (0.5 µg) caused about 52% inhibition of IL-12 secretion as compared with bacteria-stimulated DCs (paper II). If the DCs instead were stimulated with bacteria first and then treated with 0.5 µg of HdCDT only 13% of the total IL-12 production was inhibited (data not shown), suggesting that complete inhibition of cytokine production in stimulated cells requires a high concentration of HdCDT.

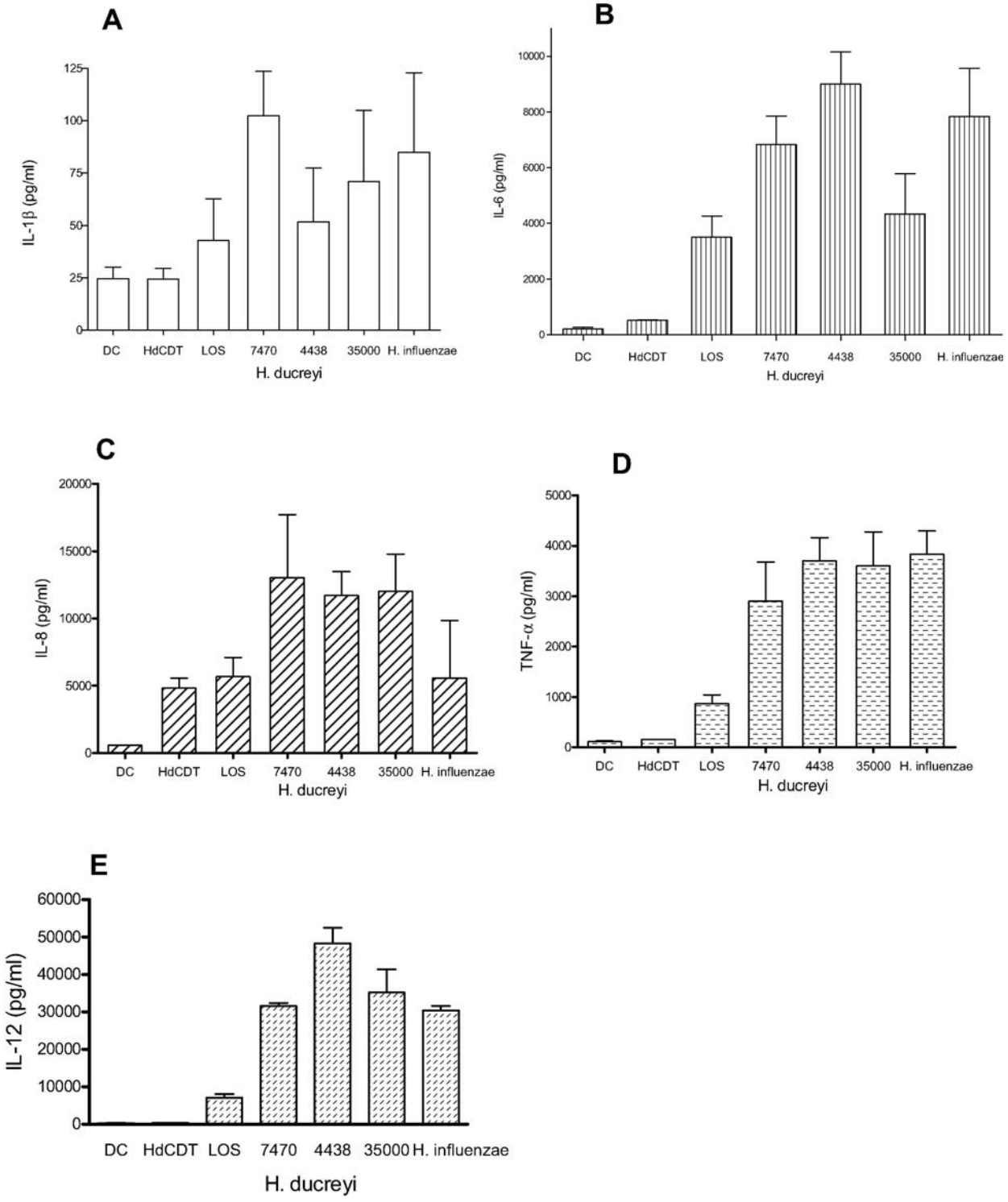

Figure 5. IL-1β (A), IL-6 (B), IL-8 (C), TNF-α (D), and IL-12 (E) production by monocyte-derived DCs that were co-cultured with different antigens. Cell viability, determined at the start of each experiment by the trypan blue exclusion test was greater than 90%. The following antigens were used: *H. ducreyi* CCUG 7470, CCUG 4438, ATCC 35000, *H. influenzae*, *H. ducreyi* LOS (1 μg/ml) or HdCDT (0.5 μg/ml). The culture supernatants were tested after 24 h for IL-1β, IL-6, IL-8, TNF-α, and IL-12. The data represent the mean ± S.D. from three independent experiments. For the supernatants of bacteria-pulsed DCs versus non-pulsed DCs, $P \leq 0.05$; for non-pulsed DCs versus LOS-pulsed DCs, $P \leq 0.05$.

1.3. HdCDT action on DC viability and apoptosis

In order to investigate the effect of HdCDT treatment on APCs, the viability of treated cells was investigated by trypan blue exclusion test and early and late apoptosis was detected by a combination of annexin V-FITC and PI staining. HdCDT treatment resulted in a slow successive decrease in DC viability over time. However, 50% of cells were still not stained with trypan blue after 3 days of incubation with 0.5 µg/ml of HdCDT. When apoptosis was evaluated, a significant and successive increase in the number of cells stained with annexin V-FITC was found, resulting in about 60% of the cells being stained with annexin V-FITC after 72h of HdCDT treatment (Fig 6). During the experimental period, only a low percentage of cells were stained exclusively with PI (necrotic cells).

These results indicate that HdCDT treatment induces early and late apoptosis in DCs, albeit slowly. It must though be mentioned that a high concentration of toxin was used in the experiment.

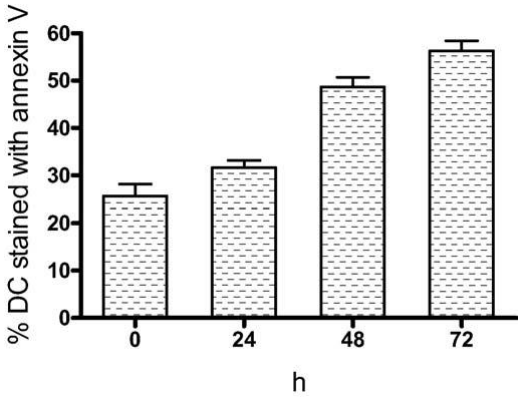

Figure 6. Induction of early and late apoptosis in monocyte-derived DCs treated with 0.5 µg/ml HdCDT over 3 days. The data are expressed as the percentages of annexin V-stained cells in the total cell population, and represent the mean ± S.D. of triplicates. For the percentages of annexin V-stained cells at time 0 versus after 24 and 48 h, respectively, $P < 0.05$ and $P < 0.001$.

1.4. Activation of T-cells by DCs and MQs pulsed with *H. ducreyi*, HdLOS and HdCDT

H. ducreyi and HdLOS induced secretion of regulatory and inflammatory cytokines by DCs and MQs. In order to evaluate the capability of the stimulated DCs and MQs to activate $CD4^+$ T-cells, proliferation and cytokine secretion by the $CD4^+$ T-cells were measured.

1.4.1. INDUCTION OF CD4+ T-CELL PROLIFERATION

The viability of the DCs and MQs incubated with bacteria (*H. ducreyi or H. influenzae*) or HdLOS were not affected. DCs stimulated with *H. ducreyi* or *H. influenzae* significantly increased the $CD4^+$ T-cell proliferation as compared with unstimulated or HdLOS stimulated DCs (Fig 7A). In addition, MQs stimulated with HdLOS induced higher level of T-cell proliferation than MQs stimulated with *H. ducreyi*. Proliferation was not improved in $CD4^+$ T-cells incubated with the antigens alone (data not shown). In general proliferation of $CD4^+$ T-cells were significantly induced by stimulated DCs, as compared with stimulated MQs. DCs and MQs stimulated with HdCDT were unable to induce T-cell proliferation (Fig 7).

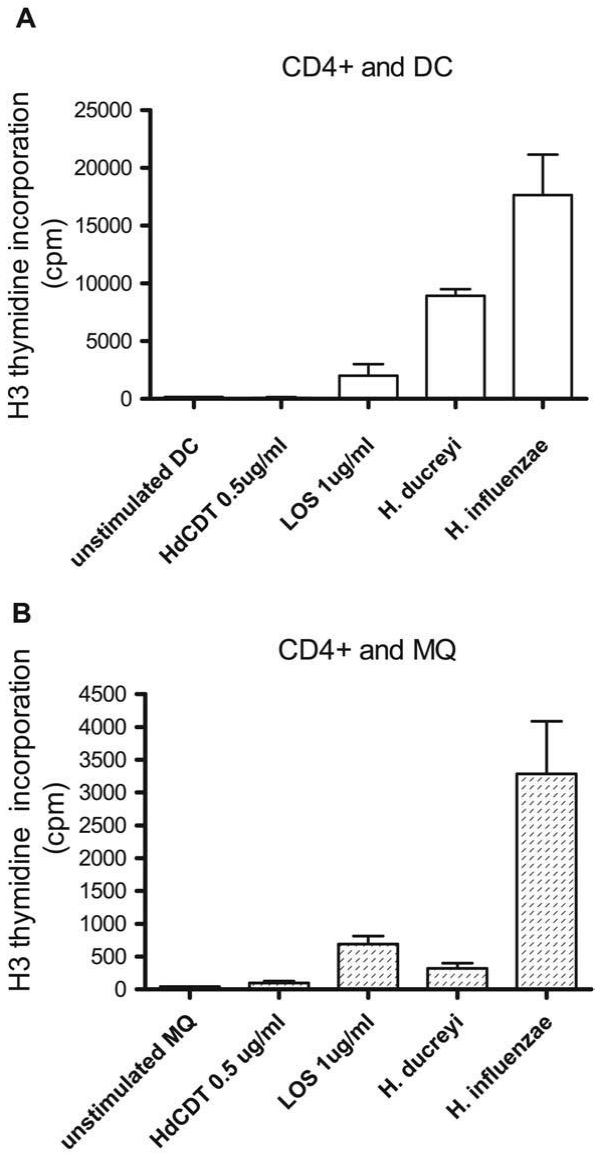

Figure 7. T-cell proliferation in response to DCs (A) and MQs (B) that were stimulated with bacteria or bacterial antigens. APCs were stimulated with gentamycin-killed *H. ducreyi* CCUG 7470, non-capsulated *H. influenzae*, *H. ducreyi* LOS (1 μg/ml), and HdCDT (0.5 μg/ml) for 2 days. Antigenpulsed DCs or MQs were washed, and cell viability was determined before co-culturing with T-cells as being 85% after pipetting and harvesting of the cells. The $CD4^+$ T-cells (1×10^5 per well) were co-cultured with APCs. After 6 days. T-cell proliferation was measured by the incorporation of [^{3}H]-thymidine. The data represent the mean ± S.D. from three independent experiments. For T-cell proliferation induced by bacteria-pulsed DCs or MQs versus non-pulsed DCs or MQs, $P \leq 0.03$; for T-cell proliferation induced by *H. influenzae*-pulsed DCs versus *H. ducreyi*, $P \leq 0.05$; for T-cell proliferation induced by bacteria-pulsed DCs versus LOS-pulsed DCs, $P \leq 0.05$.

In order to investigate whether HdCDT activity on antigen-stimulated APCs caused down regulation of the T-cell proliferation, DCs and MQs were first stimulated with HdLOS for 24h and then two different concentrations of HdCDT were added. The result indicated that the APC induced T-cell proliferation was partially blocked or abolished by the highest HdCDT dose (0.5 μg/ml) (Fig 8).

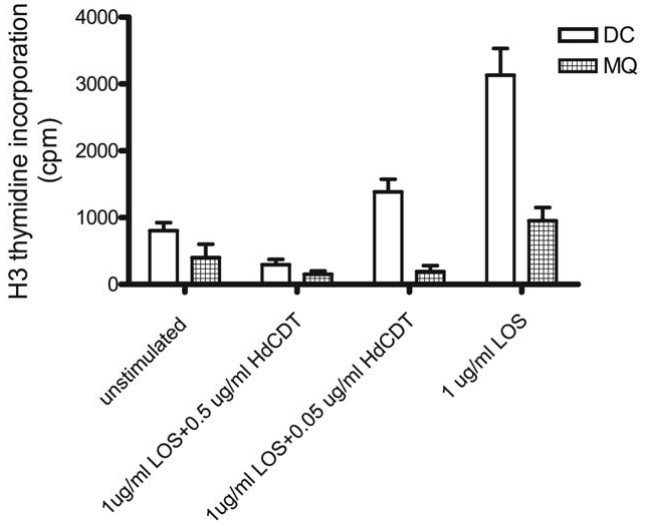

Figure 8. Abolition of T-cell proliferation after contact with APCs that were stimulated with LOS and then treated with HdCDT. $CD4^+$ T-cells (1×10^5 per well) were co-cultured with DCs or MQs (1×10^4 per well) that were treated with purified LOS (1 μg/ml for 24 h) and then treated with two concentrations (0.5 or 0.05 μg/ml) of HdCDT. The pulsed DCs were washed and transferred to a new tissue culture plate before co-culturing with the T-cells. After 6 days of co-cultivation, T-cell proliferation was assessed by measuring the incorporation of [^{3}H]-thymidine. The data represent the mean ± S.D. from three independent experiments. For T-cell proliferation induced by DCs that were treated with LOS alone versus DC that were treated with LOS and different concentrations of HdCDT, $P \leq 0.01$.

The specificity of the effect caused by HdCDT on the DCs capacity to activate T-cell proliferation was investigated by neutralizing of active HdCDT with toxin specific hyper-immune rabbit serum. DCs were pulsed with the neutralized HdCDT before co-cultivation with the $CD4^+$ T-cells. The T-cell proliferation was increased 1.6–2.3-fold when active HdCDT was neutralized, indicating that the effect was HdCDT specific (data not shown).

In summary, these results indicate that HdCDT might influence the function of the APCs by causing apoptosis and decreases in cytokine production especially IL-12, provided a sufficient amount of toxin is available.

1.4.2. CYTOKINE SECRETION BY $CD4^+$ T-CELLS

To determine if the T-cell response generated by *H. ducreyi* and its antigens was of a Th1-type or a Th2-type the secretion of IL-4, IL-13 and IFN-γ was measured in the culture supernatants of $CD4^+$ T-cells co-cultured with DCs or MQs previously stimulated with bacteria, HdLOS, or HdCDT (Fig 9).

High levels of IFN-γ (up to 3300 pg/ml), but not of IL-4 or IL-13 were secreted by $CD4^+$ T-cells after contact with DCs stimulated with *H. ducreyi* bacteria. DCs alone produced low levels of IFN-γ, ranging from 400 to 600pg/ml, after stimulation with different *H. ducreyi* strains (data not shown), which indicates that this cytokine was secreted mainly by $CD4^+$ T-cells. Low levels of cytokines were detected in the culture supernatants of $CD4^+$ T-cells that were co-cultured with DCs stimulated with HdLOS or HdCDT (Fig 9A).

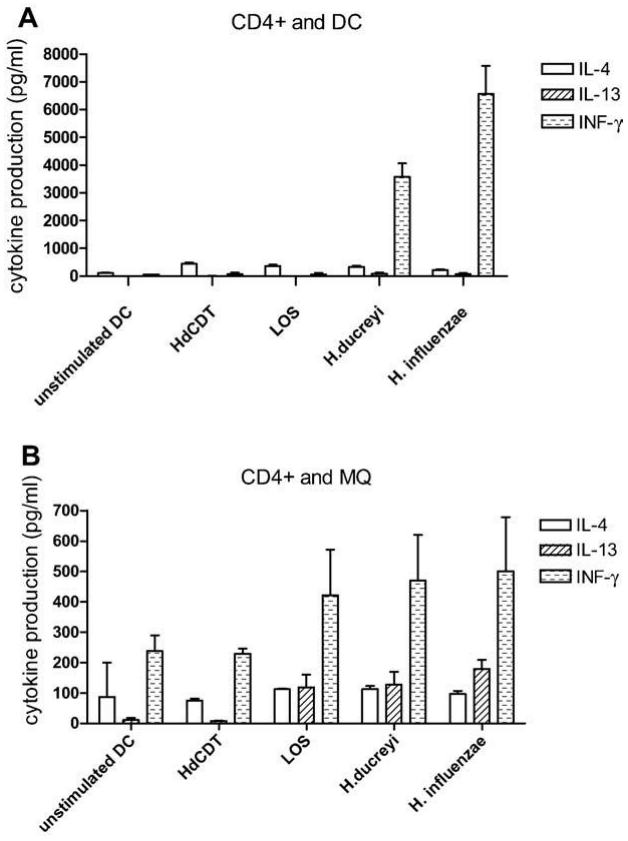

Figure 9. IL-4, IL-13 and IFN-γ production by $CD4^+$ T-cells that were co-cultured with DCs (A) or MQs (B) that had been pulsed with the following antigens: gentamycin-killed *H. ducreyi* CCUG 7470, non-capsulated *H. influenzae*, *H. ducreyi* LOS (1 μg/ml) or HdCDT (0.5 μg/ml) for 2 days. Antigen-pulsed DCs or MQs were washed; cell viability was determined by trypan blue exclusion test, being 85% after pipetting and harvesting of cells. The $CD4^+$ T-cells (1×10^5 per well) were then co-cultured with antigen pulsed DCs or MQs (1×10^4 per well). The culture supernatants were tested after 48 h of co-culturing for the presence of IL-4, IL-13, and IFN-γ. The supernatants from unstimulated DCs or MQs that were co-cultured with T-cells were used as the negative controls. The data represent the mean ± S.D. of three independent experiments. For IFN-γ production induced by bacteria-pulsed DCs or MQs that were co-cultured with T-cells versus non-pulsed DCs or MQs, $P \leq 0.05$.

Similarly, $CD4^+$ T-cells that were co-cultured with MQs stimulated with HdLOS, *H. ducreyi* or *H. influenzae* bacteria, secreted significantly higher concentrations of IFN-γ than of IL-4 and IL-13 (Fig 9B). However, $CD4^+$ T-cells that were co-cultured with stimulated MQs secreted about 10-fold lower levels of all the cytokines tested, as compared with co-culture with stimulated DCs. Furthermore, no significant increase in the levels of IL-4, IL-13, and IFN-γ were detected in the culture supernatants of $CD4^+$ T-cells that were co-cultured with HdCDT treated DCs or MQs.

IFN-γ was found to be the predominantly secreted cytokine by $CD4^+$ T-cells after co-culture with APCs stimulated with *H. ducreyi* bacteria and this result strengthens the hypothesis of a Th1-type immune response at least in the early stages of *H. ducreyi* infection.

2. Immunogenic and adjuvant properties of HdLOS (paper II and III)

LPS is a T-cell independent antigen that is able to directly activate B-cells in the absence of T-cells. Antibodies directed against the O-side part of the LPS are bactericidal for many Gram-negative bacteria. HdLOS induces inflammatory responses, as described above. In addition specific antibodies may play a role in protection against bacteria, therefore the immunogenic and adjuvant properties of HdLOS were evaluated.

2.1. Immunogenicity of purified HdLOS

To investigate the immunogenicity of HdLOS and host parameters involved in the antibody response, mice of different strains were used; the outbreed strain NMRI and the inbreed strains BALB/c (good antibody producer and Th2 responder) and C57BL/6 (good cell mediated immunity and Th1 responder), and the genetically modified strains nu/nu (athymic mice), TLR4-KO (Toll-like receptor 4 receptor deficient) and MyD88-KO (TLR adaptor deficient).

Immunization with purified HdLOS7470 or HdLOS4438 induced strong serum IgG antibody responses of 10 000-12 000 units/ml in NMRI and BALB/c mice while a significantly lower responses (P=0.002) was detected in C57BL/6 mice (Fig 10A). As expected, the athymic nu/nu mice respond with IgM antibodies only (Fig 10B).

Furthermore, the IgG subclass distribution in sera was investigated for NMRI and BALB/c mice and the highest levels were noted for IgG1/IgG2b and IgG2b, respectively. Fig 10C shows the IgG subclass distribution in BALB/c mice. In addition, no antibody response to HdLOS was detected in mice that lacked either functional TLR4 or MyD88 (Fig. 10A and B).

In summary, HdLOS was found to be an immunogenic T-cell independent antigen, which was dependent on the TLR4 and MyD88 signaling pathway.

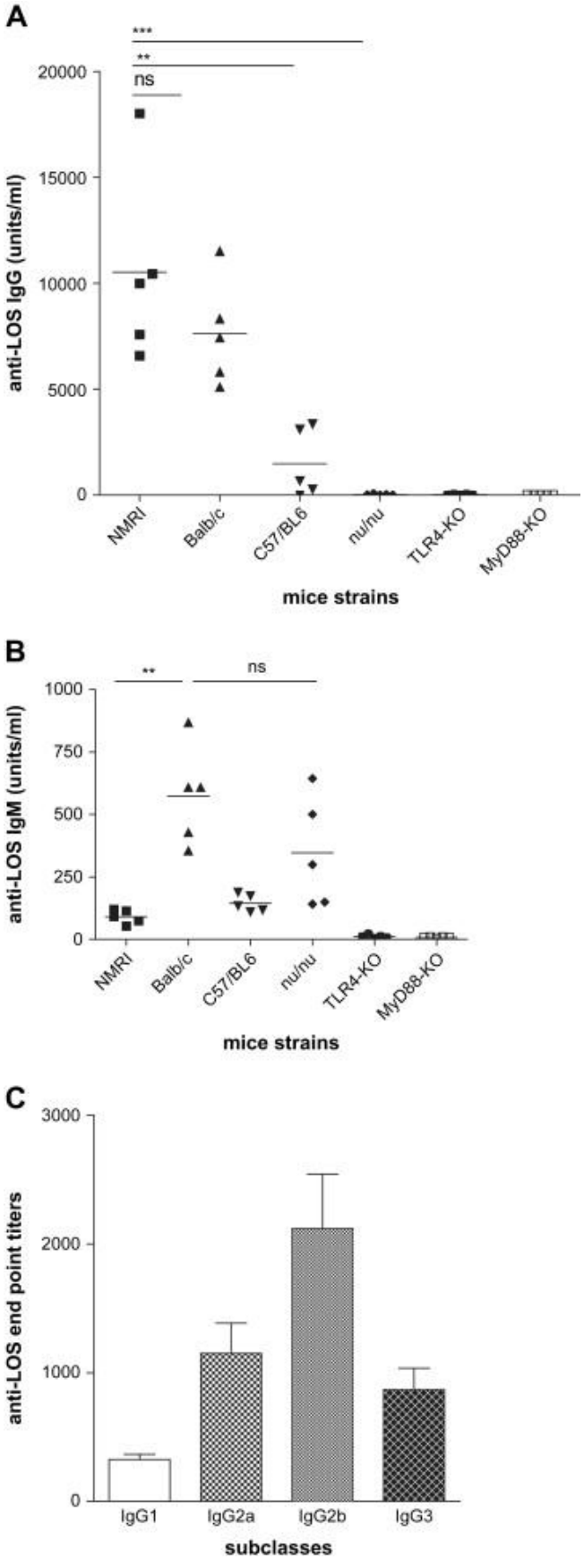

Figure 10. Antibody response in mice immunized with purified LOS 7470. (A) IgG and (B) IgM antibody response in different mice strains after immunization with purified 7470LOS and (C) IgG subclass response in BALB/c mice immunized with LOS 7470. Outbreed, NMRI; inbred, BALB/c (good antibody producers and Th2 responders), C57BL/6 (good cell mediated immunity and Th1 responders), nu/nu (athymic mice), TLR4-KO (Toll-like receptor 4 receptor deficient) and MyD88-KO (TLR adaptor deficient) were immunized sc three times with 5 μg of purified LOS. The antibodies to LOS were estimated by ELISA and expressed in units per milliliter (A and B) or end point titers (C) according to the reference serum. In (A) and (B) the data are plotted as values for individual mice (n = 5) after subtraction of the respective mean values for the pre-immune animals. A horizontal bar represents the mean value. In (C) the IgG subclass antibodies to 7470LOS are expressed as end point titer and represent the mean ± SEM (n = 10) after subtraction of a pre-immune mean value. NMRI vs. BALB/c, ns; NMRI vs. C57/BL6 $P < 0.001$ (**); NMRI vs. nu/nu, $P < 0.0001$ (***) (unpaired t-test); BALB/c vs. NMRI, $P < 0.0001$ (***); BALB/c vs. nu/nu, ns (not significant) (unpaired t-test).

In order to characterize the LOS structure responsible for the induction of the anti-LOS antibody response, BALB/c mice were immunized with the oligosaccharide fraction of HdLOS7470 (OS) or with HdLOS7470 that had been hydrolyzed with anhydrous hydrazine in order to remove the *O*-linked fatty acids from lipid A (LOS-H) (Fig 11). Neither the oligosaccharide part nor the hydrazine treated HdLOS were able to induce a significant antibody response, as compared with pre-immune mice.

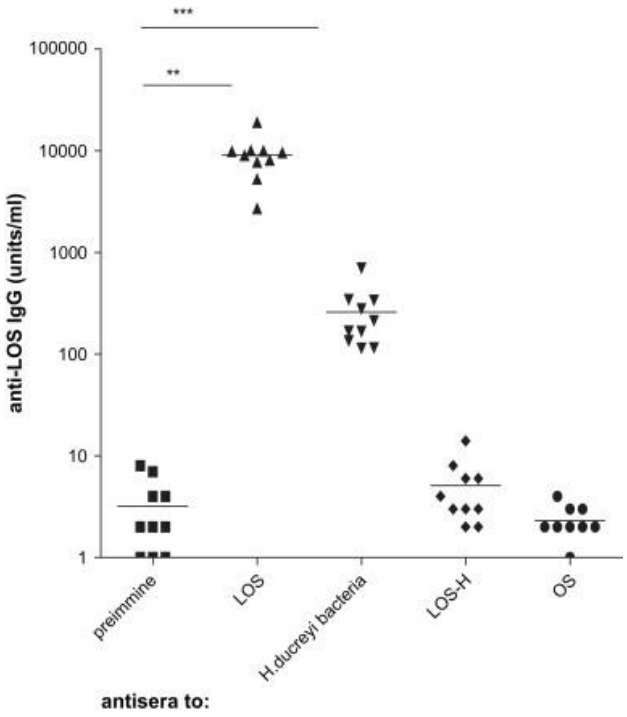

Figure 11. The IgG antibody response to LOS7470 in BALB/c mice immunized with LOS7470, OS, LOS-H, or boiled *H. ducreyi* bacteria. Mice (n = 10) were immunized sc three times with 5 μg of different preparations of LOS and nine times with boiled *H. ducreyi* 7470 bacteria (in total 2×10^{12} bacteria during 4 weeks of immunizations). The levels of antibodies to LOS7470 were estimated by ELISA and are expressed in units per milliliter according to the reference serum. The data are plotted as values for individual mice (n = 10) after subtraction of the respective mean values for the pre-immune animals. A horizontal bar represents the mean value. Pre-immune vs. anti-LOS serum P = 0.0001 (***); pre-immune vs. antiserum to bacteria serum P = 0.001 (**) (unpaired t-test).

These results indicated that the immunogenicity of HdLOS was dependent on both the oligosaccharide, which probably requires a correct presentation by lipid A, and an intact lipid A. Interestingly, hyper-immunization of mice with boiled *H. ducreyi* bacteria resulted in an anti-HdLOS response that was weaker than that induced by purified HdLOS (Fig 11). However, this might depend on many factors e.g. the total amount of bacterial LOS injected, the presentation of LOS on the bacterial surface, the release of LOS to the environment and an eventual conformational exposure in LOS structure etc.

2.2. SPECIFICITY OF ANTIBODIES INDUCED BY HDLOS

To investigate the specificity of the generated HdLOS antibodies an inhibition study was performed. Anti-HdLOS sera was adsorb with HdLOS4438, HdLOS7470, lacto-*N*-neotetraose (LNnT), or a 3'sialyllactose and the majority (97-98%) of antibodies induced by HdLOS7470 were absorbed by homologous or heterologous HdLOS and only about 15%± 5% (mean ± SD) of antibodies were absorbed by LNnT, or the 3'sialyllactose moiety (unpublished data). In order to detect and quantify antibodies with specificity for the non-reducing sialylated end of the HdLOS, ELISA plates coated with either sialyl-LNnT or LNnT conjugated to human serum albumin (HAS) as antigen was used. Low but significant levels of antibodies specific for lacto-*N*-neotetraose moiety in the anti-LOS sera were detected (paper II). For the detection of antibodies to the distal epitopes of HdLOS exposed on the bacterial surface, the antibody levels in anti-HdLOS7470 sera were estimated using whole cell ELISA. We found that the mean antibody titer (n=10) was about 150 times lower than when the sera were tested against purified HdLOS (data not shown). When the binding capacity of anti-HdLOS7470 antibodies to selected human glycosphingolipids was tested on thin-layer chromatograms, the antibodies bound to HdLOS7470, HdLOS4338 and strongly to LipidA-Kdo-Kdo, but no binding of the antibodies to any of the glycosphingolipids were detected (paper II). It should though be noted that the solvent system used was optimized for the separation of glycosphingolipids, while the resolution of the LOS compounds was relatively poor.

These results indicated that majority of antibodies generated against HdLOS were specific for a fragment common to the LOS of both strains, i.e., the inner core of the LOS and that antibodies to the distal part of the HdLOS are not induced in sufficient amounts to bind to the related glycosphingolipid tissue structures. In addition, anti-HdLOS antibodies strongly bound to the Lipid A-Kdo-Kdo structure.

2.3. ANTI-INFLAMMATORY ACTIVITY OF ANTI-HDLOS ANTIBODIES

In the first paper HdLOS was shown to induce production of TNF-α by monocytes derived DCs and MQs. With the purpose to exemplify the inflammatory effects caused by HdLOS7470 or HdLOS4438 and evaluate anti-inflammatory activities of antibodies specific to HdLOS, the TNF-α release from PBMCs was chosen as a parameter to be measured. For comparison was the LOS of an *E. coli* Ra strain (with similar MW) used. Both of the HdLOS preparations, as well as the *E. coli* LOS were found to be potent inducers of TNF-α secretion (Fig 12). The concentration of TNF-α secreted by PBMCs was 8 times higher than detected for DCs in paper I (Fig 5). This difference in cytokine production can probably be explained by the mixed

composition of cells in the PBMC culture. The anti-inflammatory activity of the HdLOS specific antibodies were assessed as the inhibition of TNF-α secretion from HdLOS stimulated PBMC, but no significantly inhibition of the TNF-α secretion was detected in three out of four PBMC preparations, even at the highest concentration of antiserum (Fig 12).

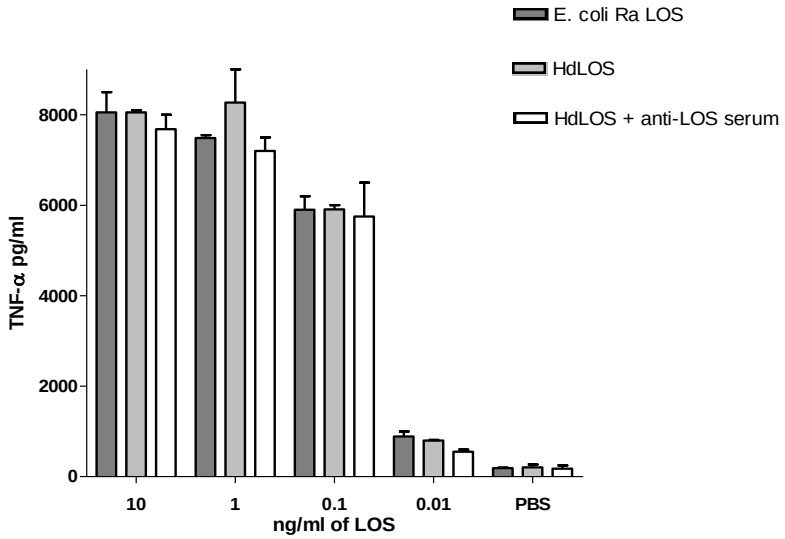

Fig 12. Antiserum to HdLOS fails to inhibit TNF-α release from stimulated PBMC. The PBMC from one donor were stimulated for 18 h with different amounts of *E. coli* Ra LOS. HdLOS-stimulated PMBC were treated with LOS in addition to homologous anti-LOS antiserum (final dilution 1:10) or pre-immune serum (control). After 18 h, the supernatants were collected and TNF-α was measured by ELISA. The TNF-α levels were calculated relative to the reference values, and are expressed in pg/ml (mean ± SEM of 3 estimations) with the control values being extracted.

2.4. ADJUVANT ACTIVITY OF HDLOS

The generation of antibodies to HdLOS was found to be dependent on an intact lipid A moiety, therefore the possibility of an adjuvant activity of HdLOS was investigated, based on the fact that Monophosphoryl Lipid A is a regularly used as adjuvant e.g. in the production of monoclonal antibodies in animals. We used BSA as a parameter of the antibody production and immunized BALB/c mice with BSA alone or with the addition of 1 μg or 5 μg of HdLOS7470 (Fig 13A). After three sc immunizations with BSA and HdLOS together were the total IgG antibody levels 9-10-fold higher than those observed after immunization with BSA alone. Both doses of HdLOS showed adjuvant capacities, with 5 μg of LOS having the most potent activity. The predominant IgG subclass raised against BSA was IgG1, followed by IgG2a and an approximately 30-fold increase in IgG2b and a 5-fold increase in IgG2a were noted when HdLOS was added.

These results indicate that the addition of HdLOS not only increased the antibody response to BSA, but also changed the IgG distribution pattern to resemble that obtained by immunization of mice with purified HdLOS.

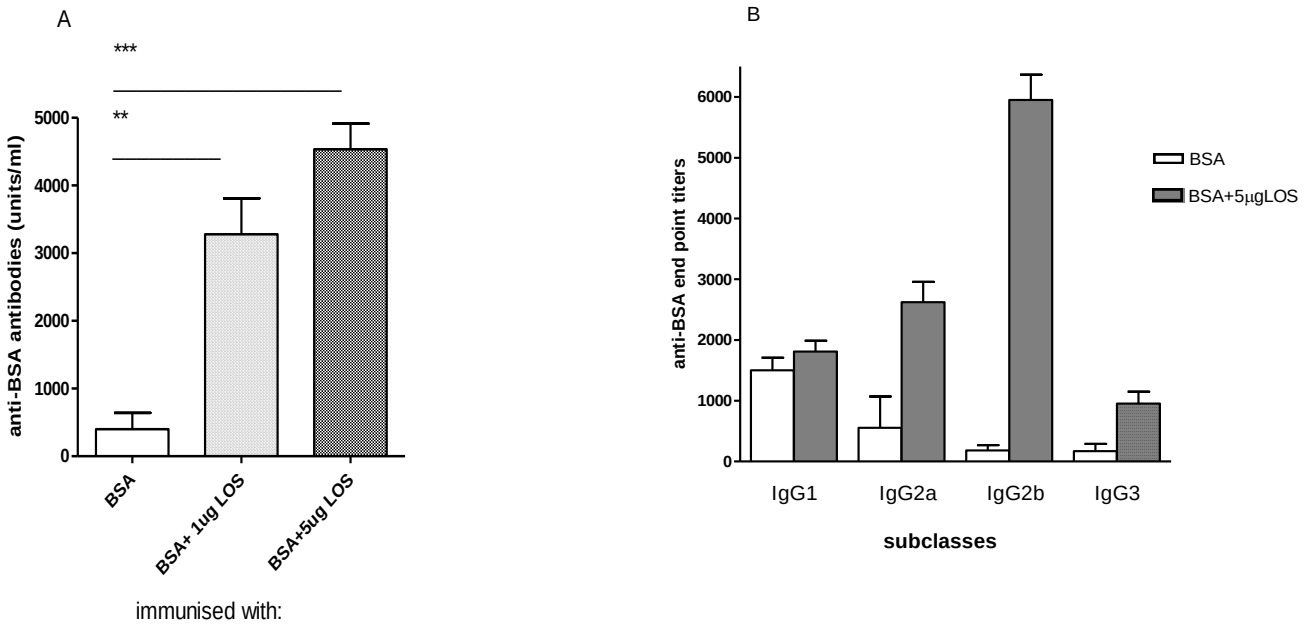

Figure 13. Adjuvant activity of HdLOS7470 on antibody responses to BSA in BALB/c mice. (A) Total IgG response to BSA. (B) IgG1, IgG2a, IgG2b, and IgG3 subclasses to BSA. BALB/c mice (n = 10) were immunized sc three times with 5 µg of BSA with or without the addition of 1 µg or 5 µg of HdLOS7470 as adjuvant. Ten days after the last injection the induced antibody levels were measured by ELISA the levels of total IgG anti-BSA are expressed as units/ml (1 units correspond with an end point titer of 1:500). The levels of IgG1, 2a, 2b, and 3 subclasses were measured by ELISA and the results are expressed as antibody end point titer, mean $\pm$ SEM. BSA vs BSA + 1 µg HdLOS, P = 0.001 (**); for BSA vs.. BSA + 5 µg HdLOS, P = 0.0001 (***).

Question arises, if HdLOS could act as an adjuvant for the stimulation of HdCDT antibody production in the same manner as for BSA. The sera were analyzed for HdCDT specific antibodies by ELISA and three sc immunizations with HdCDT and HdLOS together resulted in a significantly increase in the anti-HdCDT IgG antibody level (P=0.007), as compared with the levels observed after immunization with HdCDT alone (paper III). In contrast to the subclass switch reported for the anti-BSA antibodies, the dominating IgG subclass in the anti-HdCDT sera were still IgG1 and the addition of LOS only slightly elevated the IgG2b response to HdCDT (paper III).

In summary purified HdLOS significantly increased the antibody responses to BSA and HdCDT, thus working as an adjuvant.

3. Evaluation of HdCDT impact on the serum antibody responses using a mouse model (paper III)

3.1. Mouse model

As a first step the effect of HdCDT intoxication in different mouse cell lines was investigated in order to justify for the use of mouse as a model for our studies of the HdCDT effect on serum antibody response. We found that the mouse cell lines tested were sensitive to HdCDT intoxication and that the G2 cell cycle arrest was similar as previously reported for both human and rabbit cells, though the concentration of toxin needed for intoxication of mouse cells was higher. Furthermore the internalization of Alexa Fluor 488 labeled HdCDT in the two different epithelial mouse cell lines MLE and m-IC_{c12} was compared with the epithelial human cell line HeLa (paper III). By the use of confocal microscopy the HdCDT internalization pattern was visualized and found to be the same in the mouse cell lines and HeLa, provided a higher dose of HdCDT in combination with a longer period of incubation with the toxin was used.

Taken together, these results indicated that mouse cells involved in immune response are sensitive to HdCDT and are intoxicated in a similar manner as human cells, provided a higher amount of HdCDT. Therefore we concluded that different impacts of HdCDT on antibody responses could be investigated in mice.

3.2. HdCDT influence on antibody response to HSP, HdLOS and BSA

In order to investigate the effect caused by HdCDT on the antibody response, mice were immunized 2-4 times with viable non-toxin producing strain of *H. ducreyi* 4438 alone or together with 10 µg active HdCDT. The serum antibodies to HSP were analyzed by ELISA and no significant differences in the antibody response were detected (Fig 14A).

The effect caused by HdCDT on the antibody response to purified HdLOS and BSA was evaluated as well. Subsequently mice were immunized with purified HdLOS or BSA (5 µg/dose) alone or together with active HdCDT (5 µg/dose). The addition of toxin did not affect the antibody response to HdLOS (data not shown), but resulted in an enhanced antibody response to BSA, with a significant increase in antibody titer (ELISA) after the second immunization (P= 0.007) (Fig 14B).

3.3. ANTIBODY RESPONSE TO HDCDT

When antibody response against HdCDT in sera from mice immunized with *H. ducreyi* 4438 together with 10 µg active HdCDT was analyzed, by ELISA. A significant increase of anti-HdCDT antibodies was detected in sera after 3 immunizations with bacteria and toxin together, giving the end point titer of about 1:2500 (Fig 14C). In the same serum antibodies induced to HSP, which is exposed on bacteria the HSP specific antibody titers were 12 times higher. However, immunization with 5 µg HdCDT or BSA, a protein with similar molecular weight as HdCDT holotoxin, resulted in antibody endpoint titer to HdCDT of 1:800 (paper III, Fig 3 A) and BSA of 1:50 000 (fig 14), the last about 60 times higher. This result indicates that HdCDT is not as immunogenic as the other proteins.

HdCDT neutralizing antibodies were detected after three immunizations in some of the sera and after 4 sc. immunizations with bacteria and 10 µg HdCDT neutralizing antibodies were detected in all sera, however the levels were low (Fig 14D). In addition no anti-HdCDT antibodies were detected in the saponin extract from the genital tissue (not shown)

Taken together the active HdCDT was proven to be poorly immunogenic and did not significantly impair the serum antibody response to HdLOS or protein. Furthermore at least three injections with high 10µg of active HdCDT were needed to generate an anti-HdCDT antibody response in serum, though with infrequent naturalizing capacity.

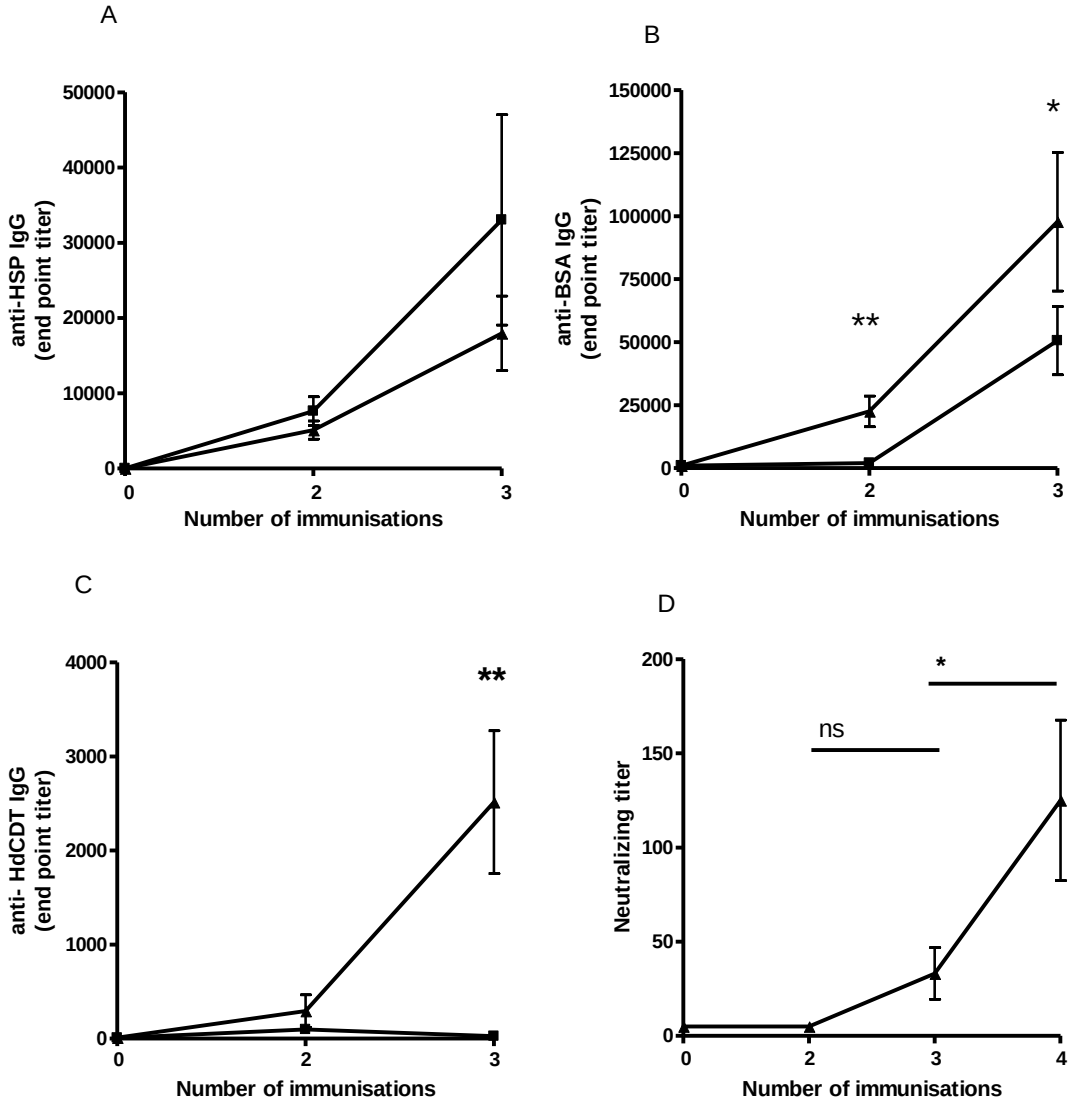

Fig 14. Impact of HdCDT on serum antibody response. Balb/c mice immunized sc 2 or 3 times with; A) and C), 10^6 CFU *H. ducreyi* 4438 (■) or 10^6 CFU *H. ducreyi* 4438 + 10µg HdCDT (▲), B) 5 µg BSA (■) or 5µg BSA+ 5µg HdCDT (▲). Serum IgG antibodies to A) purified *H. ducreyi* HSP, B) BSA and C) HdCDT. Antibody levels were estimated using ELISA and expressed as end point titer, the values represent the mean ± SEM (n=5-6). D) HdCDT neutralizing antibodies in detected in sera from Balb/c mice immunized sc 2- 4 times with 10^6 CFU *H. ducreyi* 4438 + 10µg HdCDT. A) ns., B) BSA vs. BSA + HdCDT after 2 immunizations, P=0.007 (**), after 3 immunizations P=0.03 (*) and C) *H. ducreyi* 4438 vs. *H. ducreyi* 4438 + HdCDT after 3 immunizations, P=0.008 (**). D) *H. ducreyi* 4438 + HdCDT 3 vs. 4 immunizations $P= 0.04$(*), (unpaired *t*- test).

4. GENERATION OF ANTIBODIES TO HDCDT IN THE GENITAL TISSUE USING A MOUSE MODEL (PAPER III)

Even though mice were immunized with 10 µg of active HdCDT in combination with bacteria, no HdCDT specific antibodies were detected in the genital tract. In another study we showed that formaldehyde treatment of HdCDT increased the immunogenicity and decreased the toxicity of the toxin [153]. Therefore formaldehyde detoxified HdCDT was used in an attempt to generate toxin antibodies with neutralizing capacity. HdCDT specific antibodies were detected in sera and genital tissue after three immunizations, as estimated ELISA. The level of HdCDT specific antibodies generated in response to the HdCDT toxoid in sera were about 30 times higher than the level of antibodies generated to active HdCDT. The addition of adjuvants increased in the generation of anti-HdCDT IgG antibodies in both sera and genital tissue, as compared with HdCDT toxoid alone (Fig 15A). In addition, only very low levels of anti-HdCDT IgA were detected in sera and genital tissue (data not shown).

The IgG antibody levels in sera and genital tissue, showed high correlation (r=0.8) (Fig 15B).

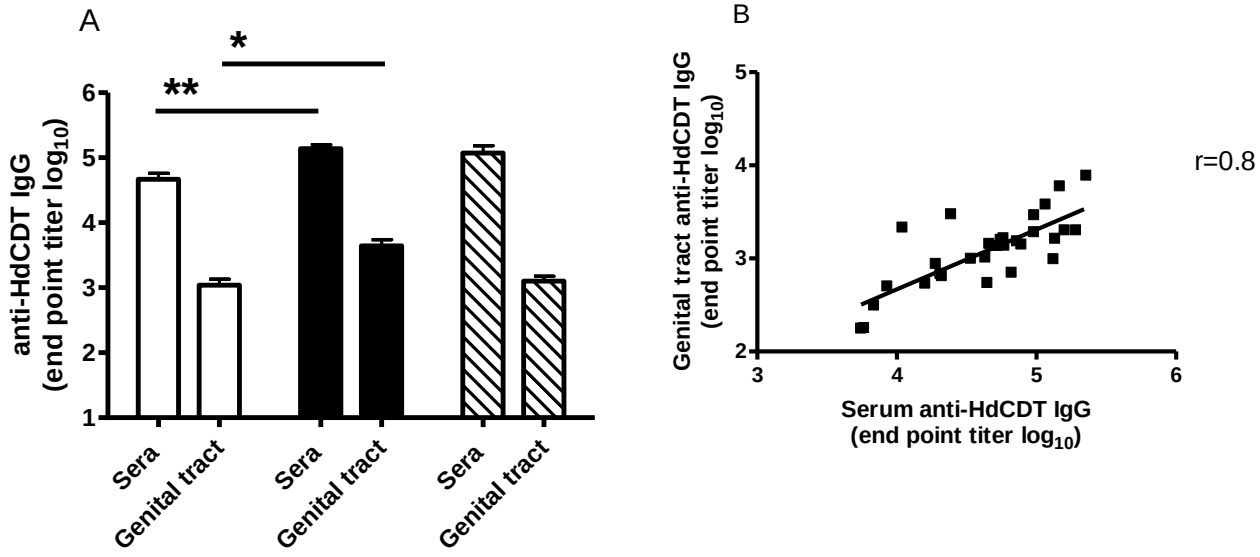

Fig 15. A) IgG antibodies to HdCDT in sera and genital tract in Balb/c mice immunized 3 times sc with HdCDT toxoid (white bar), HdCDT toxoid + RIBI (black bar) or HdCDT toxoid + Alum (hatched bar) estimated by ELISA and expressed as end point titer, bars represent the mean ± SEM (n=5). B) Correlation between serum anti-HdCDT IgG and vaginal tract anti-HdCDT IgG. A) Toxoid vs. toxoid + RIBI, sera P= 0.002(**), genital tract P= 0.02 (*) (unpaired t- test). B) Pearson correlation r = 0.8.

When the neutralizing capacity of the anti-HdCDT sera was tested, only sera with high ELISA titer, about 1:20 000, manifested neutralizing capacity and the correlation between the neutralizing capacity of the antibodies and ELISA anti-HdCDT IgG level in the sera was high (r=0.7) (Fig 16). This correlation was increased in the sera with the highest antibody levels (r= 0.8).

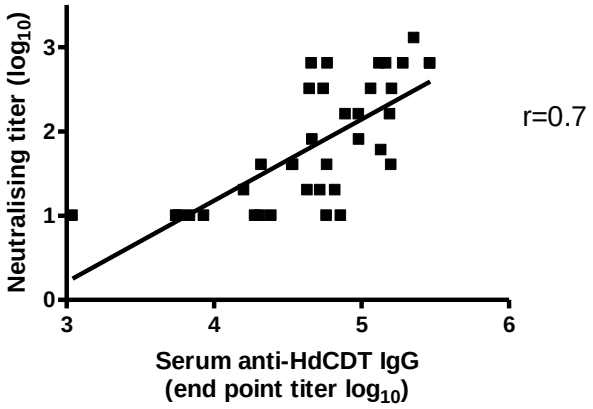

Fig 16. Correlation between serum anti-HdCDT IgG and HdCDT neutralizing titers in sera from Balb/c mice immunized 3 times sc with HdCDT toxoid, HdCDT toxoid + RIBI or HdCDT toxoid + Alum. Serum anti-HdCDT IgG were estimated by ELISA and expressed as end point titer.
Neutralizing titer was estimated using toxin neutralization assay.
Pearson correlation r = 0.7.

These results indicate that in order to generate antibodies in the genital tissue, parental immunization is sufficient, provided the induced antibody levels are high. Furthermore, the neutralizing capacity of the antibodies was found to correlate with high levels of anti-HdCDT antibodies in sera.

GENERAL DISCUSSION

This thesis evaluates the role of lipooligosaccharide and the cytolethal distending toxin produced by *Haemophilus ducreyi* (HdCDT and HdLOS) in antibody responses. The decision to focus on the two toxins, LOS (endotoxin) and CDT was based on the potential importance of them in the immunopathogenesis of chancroid. HdLOS is reported to be involved in ulcer production in animal models [34-35, 40]. Endotoxin produced by Gram–negative bacteria is an important inducer of the inflammatory responses in the early stages of infection and antibodies directed against the O-side chain of the polysaccharide may efficiently eliminate bacteria through antibody dependent complement-mediated bactericidal activity [76-78, 80]. HdCDT is most likely an important factor in the pathogenesis of *H. ducreyi*. The toxin efficiently kills cells involved in the immune response and is reported to contribute to the retardation of healing and thereby to the persistence of infection, *in vivo* [38-39, 124, 126, 136, 138]. To investigate the induction of antibody responses against *H. ducreyi* antigens and to evaluate if a possible protective role of antibodies is important for the future development of a vaccine against this extracellular bacterium.

As a first step, the early stage in the immune response to *H. ducreyi* was evaluated using human APCs, *in vitro*. *H. ducreyi* and HdLOS induced human monocyte-derived DCs and MQs to produce pro-inflammatory cytokines in a similar way as other Gram-negative bacteria [157, 165-167]. The production of cytokines by antigen presenting cells is an important factor in the differentiation of naïve and uncommitted T-cells into effectors of a Th1- or Th2-type [63]. In our study DCs and MQs pulsed with *H. ducreyi* bacteria and MQs pulsed with HdLOS were found to produce e.g. IL-12 and induced $CD4^+$ T-cells to produce IFN-γ which is a typical marker of a Th1-type immune response. It should be pointed out that in our study only APCs derived from monocytes purified form human peripheral blood were employed, while in skin or genital mucosa other residential antigen presenting cells may be involved in early responses. However, our *in vitro* results supports the previous finding that the early response to *H. ducreyi* in human experimental infection was of the Th1-type [168]. The Th1-type immune responses in infection are dominated by intense phagocytic activity and low antibody production, later it switches to a Th2-type immune response which is characterized by high antibody titers and re-establishment of homeostasis to protect the host from tissue destruction (reviewed in [63]). Antibodies are generally considered to be of great importance in the defence and protection against extracellular pathogens and their toxins. *H. ducreyi* infection is persisting without antibiotic treatment, suggesting that bacteria are not easily eliminated by the host defences [7].

It was postulated that the Th1-type response might dominate throughout the *H. ducreyi* infection and as mentioned previously humans mount what appears to be a delayed type hypersensitivity (DTH) reaction in response to chancroid and experimental *H. ducreyi* infection [27, 29, 64, 168]. Anyhow, antibodies against bacterial antigens are produced during infection with *H. ducreyi* and it is of great interest to evaluate the importance of antibodies in the defense against *H. ducreyi* [67-69, 94-95].

HdCDT is reported to cause DNA damage and death of DCs and our results confirmed this report since HdCDT decreased the viability and slowly induced progressive apoptosis of human monocyte-derived DC [125]. In addition, HdCDT is reported to efficiently induce cell cycle arrest and apoptosis/necrosis of other cells involved in the immune response [138, 140]. In our *in vitro* studies with human cells high doses of HdCDT resulted in inhibited cytokine secretion and inability of APCs to induce T-cell activation. The lack of T-cell proliferation in response to HdCDT pre-treated APCs is probably due to the fact that intoxicated DCs undergo cell death and only live DCs are able induce T-cell proliferation [169]. However, it should be pointed out that the inhibited cytokine secretion and the inability of human APCs to induce T-cell activation *in vitro* was dependent on high doses of HdCDT and the biological relevance of these findings needs further evaluation. Thus, HdCDT might influence the antigen-presenting capacity of APCs and thereby inhibit the *in vivo* immune responses to *H. ducreyi*, provided a sufficient amount of toxin is produced during infection, with consequences as prolonged ulcer healing and decreased production of antibodies that can protect the host against subsequent infections. The toxin effects observed *in vitro* raise the question of an eventual impact of HdCDT on the antibody responses during *H. ducreyi* infection, studied here in mice [27, 29, 64].

Specific antibodies to *H. ducreyi* heat shock protein (HSP) and HdLOS were generated in mice after subcutaneous immunization with live *H. ducreyi* bacteria or purified HdLOS, respectively. HSP is an dominating protein with extra-cellular localization and are expressed in high amounts when *H. ducreyi* bacteria are grown, *in vitro* [159]. HdLOS is another surface localized antigen and HdLOS antibodies were detected in sera from chancroid patients [94-95].

In our experimental model as much as 10 µg of active HdCDT protein per dose was used to induce an antibody response and still low titers were noted. Immunization of mice with BSA, a protein with similar molecular weight as the HdCDT holotoxin induced a 60 fold higher antibody level. The different subunits of HdCDT are of low MW, in our toxin preparation they are produced separately in different recombinants and reconstituted using GST immobilized

CdtB with the addition of CdtA and CdtC [38, 120]. The purified toxin preparation showed two low molecular peaks, indicating that the holotoxin with 70 kDa MW is not assembled in solution [153]. In previous studies low levels of toxin specific antibodies were detected in sera from patients with proven chancroid or experimental *H. ducreyi* infection in humans [69-70, 168, 170-171]. The duration of bacterial persistence in lesions it not known nor is the amount of toxin produced during infection known. These factors may influence the antibody response to HdCDT.

In order to evaluate if HdCDT could influence the generation of antibodies, we first proved that mouse cells were sensitive to HdCDT intoxication in a similar way as previously reported for human and rabbit cells [120, 124, 130, 132, 140, 172]. We considered a mouse model as relevant for our HdCDT studies based on the cell results and studies concerning other CDT producing bacteria e.g. *H. hepaticus* and *C. jejuni* [173-174].

We used active HdCDT in a dose of 10μg protein, which was about 100 times the amount of toxin causing a 50% decrease in viability of mouse immune cells, *in vitro*, in order to assure that local and distal immune cells could be affected. The decision to use this amount of toxin was also based on the report that 10μg was the lowest dose of HdCDT that induce inflammation in rabbit skin [38].

CDT produced by *H. ducrey*i and other bacteria is suggested to act as an immunotoxin (reviewed in [112]). However, our results indicated that the serum antibody responses to unrelated protein antigen (BSA) and bacterial proteins were not significantly down-regulated in mice by simultaneous injection of HdCDT, despite the fact that a very high concentration of toxin was used and T-cells, B-cells and antigen presenting cells are proven to be sensitive to active HdCDT *in vitro* [136, 138]. It is possible that HdCDT is transported to lymph nodes already bound to cells from the site of infection and thus unable to influence T-cells and B-cells in the lymph nodes and if so the toxin probably only has a local cellular effect in lesions.

Purified HdLOS possessed adjuvant activity which resulted in a significantly increased antibody response to BSA and HdCDT, including a shift of the IgG subclass composition from IgG1 towards IgG2 for BSA. The immune response to BSA was modulated in a similar way that different LOS structures from *Neisseria meningitidis* serogroup B modulated the response to outer membrane proteins [79]. However, in infectious situation this activity may not significantly influence the antibody response to other bacterial antigens, including CDT.

When the biological activity of the antibodies generated against HdLOS was evaluated, they were found to be incapable of neutralizing the endotoxic activity generated by HdLOS, as measured by TNF-α released by PBMCs. In addition, the complement mediated serum

bactericidal activity, which is an important primary host defense against Gram-negative bacteria, was not enhanced by the high levels of anti-HdLOS antibodies in mouse sera, in agreement with a previous report regarding human and rabbit anti-HdLOS antibodies. In addition, this report indicated that the HdLOS antibodies were somehow blocked from LOS accessibility [97]. Our studies indicated that LOS, being a T-cell independent antigen was very immunogenic in contrast to e.g. *Campylobacter jejuni* LPS which was of low immunogenicity [175]. The antibodies generated were found to be directed against the core of the oligosaccharide and not to the distal part. The specificity partly explains the low biological activity of the antibodies, since the core region of the LOS is not exposed on the surface of bacteria and thus not accessible for antibody binding. The distal part of LOS is reported to be immunochemically identical with paragloboside, a glycoshingolipid precursor of human blood antigens and this molecular mimicry of host structure by HdLOS is another suggested mechanism for *H. ducreyi* to evade the specific antibody response [86]. In addition, *H. ducreyi* produces the following virulence factors; ducreyi serum resistance A (DsrA), ducreyi lectin A (DltA), and major outer membrane proteins (MOMP), which are reported to contribute to serum resistance probably by hiding of epitopes [45, 47, 56]. As evidence from above, the biological activity of the HdLOS specific antibodies in disease most certainly needs further evaluation.

An important aim of this thesis was to generate toxin neutralizing antibodies in the genital tract, since those antibodies are of great importance in the defense against sexually transmitted pathogens. The presence of IgG antibodies in female genital secretions reflect that of serum, suggesting that IgG originates from the circulation. However, reports that genital IgG antibody does not always correlate with that of serum imply that at least some IgG originates from local sources. Regardless of the source, however, the route of passage into the lumen, whether by passive (paracellular) diffusion or through an active transport mechanism is not known [176].

Different routes of mucosal antigen delivery and adjuvants for the generation of antibodies in the genital tract of mice have been extensively studied [177-180]. The demonstrated induction of protective antibodies against human papillomavirus (HPV) was of special interest for us, since HPV, similarly to *H. ducreyi*, acts on the genital skin and mucosal epithelium, and causes persistent infection with lesion development as consequence. HPV vaccines based on L1 virus-like particle (VLP) are reported to prevent genital HPV infection after intramuscular injections with aluminum salt. The combination of the adjuvants MLP and aluminum salt was reported to be more efficient in the induction of a persistent immune response against HPV16/18 in human as compared to aluminum salt alone [181]. Local administration of HPV16 L1 VLPs was

dependent on the use of potent adjuvant in order to generate neutralizing antibodies in serum and in genital, rectal and oral secretions in mice [180].

In an attempt to induce HdCDT antibodies in the genital tract a toxoid based on formaldehyde detoxified HdCDT developed in our laboratory was used in the present investigation. The detoxification procedure cross-linked the three protein subunits, resulting in a protein complex of 70 kDa produced a very immunogenic toxoid [153]. In this study parenteral immunization of mice with HdCDT toxoid alone or in combination with RIBI or aluminum salt resulted in the generation of high anti-HdCDT serum antibody levels. The high levels of HdCDT specific antibodies in sera correlated with the level of antibodies extracted from genital tissue. In addition, only serum with high anti-HdCDT antibody titers possessed toxin neutralizing capacity. The antibodies extracted from genital tissue were not tested by neutralizing assay, since saponin used in the extraction protocol is toxic to cell cultures [182]. But, it is most likely that antibodies with toxin neutralizing capacity in sera can be transferred to the genital tract and give raise to neutralizing activity there.

Antibodies with toxin neutralizing capacity are of great importance in the host defense against bacterial toxins and are protective in several toxin mediated diseases e.g. tetanus, diphtheria and whooping cough [183-184]. However, in chancroid patients only low levels of toxin neutralizing antibodies are detected in sera and their protective capacity in chancroid is not clear [69-70].

Furthermore, in the case of localized aggressive periodontitis, caused by another CDT producing bacterium, *A. actinomycetemcomitans*, the majority of patients were unable to mount a significant anti-AaCDT antibody response, which provided a partial explanation for their susceptibility to the disease [152]. The neutralizing antibodies are often specific to epitopes situated on the receptor binding subunits of AB toxins [185]. In the case of HdCDT, a monoclonal antibody directed against CdtC was reported to possess toxin neutralizing activity [69]. In addition, Wising *et al*. reported that immunization of rabbits with the HdCDT holotoxin or the individual protein subunits CdtA, CdtB or CdtC resulted in generation of antibodies with neutralizing capacity and the antibodies to the individual subunits exerted lower neutralizing capacity than the antibodies generated to the holotoxin [38]. In order to shorten the infection and allow epithelialisation, HdCDT neutralizing antibodies are most likely needed at the site of bacterial infection. In the case of chancroid HdCDT neutralizing antibodies in the genital region should for that reason be of great value. However, the importance of HdCDT neutralizing antibodies in a *H. ducreyi* infection is difficult to evaluate since no model is available for the study of persistent infection.

Based on these results high level of toxin neutralizing antibodies achieved by parental immunization with HdCDT toxoid might be sufficient to exert toxin neutralizing activity in the genital tissue and therefore the toxoid is recommended to be tested as a component of a future vaccine candidate against *H. ducrey*i and other CDT producing bacteria.

CONCLUSIONS

1. Interactions of *H. ducreyi* bacteria and HdLOS with human monocyte-derived DCs and MQs stimulated a pro-inflammatory cytokine response. $CD4^+$ T-cells responded to bacterial or HdLOS activated DCs and MQs with increased proliferation and secretion of INF-γ, but not of IL-4 and IL-13, indicating that a Th1-type immune response occurs in the early stages of *H. ducreyi* infection.

HdCDT decreased the viability and caused progressive apoptosis of the DCs, provided a high toxin concentration was used. In addition, HdCDT directly or indirectly inhibited the secretion of inflammatory cytokines by DCs and MQs and intoxication subsequently resulted in failure of the APCs to activate $CD4^+$ T-cells.

2. Purified HdLOS is an immunogenic T-cell independent antigen, dependent on the TLR4 signaling pathway for induction of specific HdLOS antibodies in mice. The majority of the anti-HdLOS antibodies are specific for the inner core of LOS and those antibodies are not significantly involved in the neutralization of HdLOS endotoxic activity. Purified HdLOS exerted adjuvant properties and significantly increased the antibody responses to proteins tested (BSA and HdCDT), and stimulated an increase in antibodies of the IgG2b subclass.

3. Active HdCDT does not down-regulate the humoral antibody response to *H. ducreyi* antigens, as tested for different proteins and purified lipooligosaccharide.

4. The active HdCDT is weakly immunogenic and as many as four immunizations with 10 µg of protein is needed in mice to induce toxin neutralizing antibodies. Parenteral immunizations with HdCDT toxoid alone and in combination with aluminum salts or lipid A based adjuvants induce high levels of serum and genital HdCDT specific IgG antibodies, including neutralizing antibodies.

SWEDISH SUMMARY - POPULÄRVETENSKAPLIG SAMMANFATTNING PÅ SVENSKA

Den här avhandlingen är fokuserad på två toxiner (gift) som bakterien *Haemophilus ducreyi* producerar. *H. ducreyi* är en gramnegativ bakterie som orsakar den sexuellt överförbara könssjukdomen chancroid, vilken kännetecknas av svårläkta sår på de yttre könsorganen. De två toxinerna, lipooligosackarid (LOS) och cytoletalt distanserande toxin (CDT) bidrar till bildandet av de chancroida såren. Liksom andra könssjukdomar har chancroid visat sig öka smittspridningen av HIV och i dagsläget finns det inget vaccin mot sjukdomen utan den botas med antibiotika.

Lipopolysackarid (LPS) är en huvudsaklig komponent i gramnegativa bakteriers cellvägg och består av en inre fettsyra och en yttre kolhydratkedja. Det LPS som *H. ducreyi* producerar har till skillnad från många andra bakteriers LPS en mycket kort kolhydratkedja och benämns därför LOS. CDT är ett protein som består av tre subenheter. Det är sedan tidigare känt att CDT skadar cellers DNA, vilket leder till celldöd. Vid en bakteriell infektion kan antikroppar riktade mot en särskild komponent, som exempelvis ett toxin, på ett effektivt sätt neutralisera detta. Det är ännu inte klarlagt vilken betydelse antikroppar har i försvaret mot chancroid och om dessa två toxiner påverkar bildandet av antikroppar under en *H. ducreyi* infektion. Det huvudsakliga målet med arbetet var därför att undersöka hur de båda toxinerna påverkar bildandet av *H. ducreyi*–specifika antikroppar.

Vi fann att levande *H. ducreyi* och framrenat LOS inducerade mänskliga celler involverade i vårt immunförsvar att producera inflammatoriska cytokiner, dvs. substanser som signalerar till kroppen att den är infekterad. Vi undersökte om det bildades antikroppar mot LOS och antikropparnas specificitet, dvs. vilken del av LOS som de kände igen, om de hade någon funktion samt om LOS kunde påverkade bildandet av andra antikroppar. Det visade sig att mycket antikroppar bildades mot LOS och att de kände igen den inre strukturen på LOS. Tyvärr var dessa inte kapabla att inaktivera den skadliga effekten av LOS. Det visade sig att LOS fungerade som ett immunostimulerande ämne (adjuvant) som hade förmågan att öka antikroppsproduktionen mot andra bakteriella komponenter.

När CDTs förmåga att påverka immunförsvarets antikroppsproduktion undersöktes, upptäckte vi att endast mycket lite antikroppar var riktade mot aktivt CDT och att de antikroppar som bildades inte var särskilt bra på att neutralisera toxinet. Toxinet orsakade inte en minskning av antikroppsproduktionen mot andra komponenter, trots att det kan döda celler inblandade i immunförsvaret. För att undersöka om antikroppar mot CDT är av betydelse för skyddet mot *H. ducreyi* definierade vi ett sätt att inducera produktionen av antikroppar mot CDT med neutraliserande kapacitet. Genom att behandla aktivt CDT med formaldehyd kopplades de tre subenheterna i toxinet samman och toxinet förlorade sin förmåga att döda celler. Möss som immuniserades med det förändrade CDT i kombination med ett adjuvant, bildade höga nivåer av antikroppar mot CDT med förmågan att neutralisera toxinet.

Vi föreslår att man utvärderar om det går att använda inaktiverat CDT som en komponent i ett vaccin mot *H. ducreyi* och andra CDT producerande bakterier.

ACKNOWLEDGMENTS - TACKORD

Ett stort tack till alla er som på något sätt bidragit till denna avhandling.
Ett alldeles särskilt tack vill jag framföra till:

Teresa, för att jag har fått vara doktorand hos dig. En spännande resa som ibland varit krokig, men framför allt mycket givande och utvecklande. Din sprudlande entusiasm för forskningen är beundrans värd och du är verkligen en outtömlig källa till kunskap och inspiration. Jag vill synnerligen tacka för ditt fantastiska stöd inför min halvtids kontroll och med denna avhandling.

Karin, det finns bara en som DU, Kul ibland Arg mycket Rolig och Intelligent och Noggrann. Tack för all ovärderlig hjälp och vänskap under mina år som doktorand.

Vivi, för all hjälp med toxinet, det har varit mycket trivsamt att arbeta med dig.

Catharina, för all din hjälp och dina uppmuntrande ord under min doktorand tid. Du har fått mig att tro på att det faktiskt är genomförbart.

Hinda, **Tingting and Judica**, previous co-workers in "the Ducreyi group", it has been a great pleasure working with you and I wish you all the best in the future.

Leif, jag är verkligen tacksam för all hjälp med Paper III och avhandlingen.

Gudrun, **Karin** & **Erik** för att ni förstår vikten av bra kaffe.

Petra, **Maria**, **Susanna, Anna** och **Carl-Fredrik**, att arbeta på samma lab som er har varit toppen.

Anna-Karin, **Helena** & **Erik**, att dela doktorand rum med er har varit underbart, jag hoppas verkligen att traditionen med "kaka, på en torsdag" kan leva vidare.

Sara, vännen, alltid lika entusiastisk och engagerad vad det än gäller, hoppas att vi får arbeta ihop igen någon gång i framtiden.

Ali, for introducing me to research, long time ago….

Susanne och **Harald**, för att ni på bästa sätt fixar så att allting bara fungerat.

Gudrun, **Gun**, **Malin R**, **Margareta**, **Marianne L**, **Lasse** och **Karin S**, för att ni är så otroligt hjälpsamma.

Samuel, för hjälp med FACS inställningar och FACS figurer.

Julia, at Swegene Center for Cellular Imaging, for help with imaging equipment.

Vincent, for revising the English text of Paper II.

Mike, for revising the English text of Paper III.

And to all of **You** previous, present and future co-workers at the department of Medical Microbiology and Immunology, never forget that you are the ones creating the wonderful atmosphere at this department.

Nina & Kerstin, ni är verkligen de mest fantastiska vännerna man kan tänka sig att hitta på en biologutbildning, jag kan inte föreställa mig hur doktorandtiden hade varit utan er.

Pappa, för att du är du, och för att du alltid finns där när det stormar.

Mamma, utan all din uppmuntran och ditt stöd hade det aldrig varit möjligt för mig att slutföra denna avhandling.

Fredrik, **Tove** & **Nora** min underbara familj som gör varje dag i livet speciell, ni är det bästa som finns. Jag älskar er!

The work in this thesis was supported by grants from the Swedish Agency for Research Cooperation with Developing Countries (Sida/SAREC), the Swedish Research Council, the Swedish cancer foundation and ALF/LUA, Göteborg.

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