The influence of gene expression on the motility of dendritic cells infected by *Toxoplasma gondii*

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SUMMARY

Previous studies have shown that several host genes are up-regulated, within 8 hours, in mouse bone-marrow derived dendritic cells (DC) infected by Toxoplasma gondii. In order to determine whether the increase in expression is linked to the hypermotility of the infected DCs, the expression of specific genes was knocked down by utilizing RNA interference. To assess the influence of the specific gene expression, the hypermotility of the infected dendritic cells was studied by using a transmigration assay. The specific genes examined in this study were those encoding, Cathepsin L, Cathepsin B, Cathepsin S, and C-type lectin domain family 4 member E (CLEC4E). The results from this work indicate that a reduction in mRNA expression of Cathepsin L and Cathepsin S resulted in a decrease in the number of transmigrating infected DCs as compared to the negative control. Since the dissemination of the parasite is highly dependent on dendritic cell migration in vivo, greater understanding of the role played by Cathepsin L and S during Toxoplasma infection could contribute to a therapeutic target of clinical interest in the future.
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<td>APC</td>
<td>Antigen presenting cell</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CD4⁺/CD8⁺</td>
<td>Cluster of differentiation 4/8</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>DAPI</td>
<td>4’,6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cell</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified eagle medium</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>CLE4E</td>
<td>C-type lectin domain family 4 member E</td>
</tr>
<tr>
<td>GMCSF</td>
<td>Granulocyte-monocyte colony-stimulating factor</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>HFF</td>
<td>Human foreskin fibroblast</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon gamma</td>
</tr>
<tr>
<td>IL-12</td>
<td>Interleukin 12</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
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<tr>
<td>NK cells</td>
<td>Natural killer cell</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
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<td>PCR</td>
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<td>RNA interference</td>
</tr>
<tr>
<td>TAE</td>
<td>Tris-acetate-EDTA</td>
</tr>
<tr>
<td>T-cells</td>
<td>Thymus derived lymphocyte</td>
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</table>
1 INTRODUCTION

1.1 *Toxoplasma gondii*

*Toxoplasma gondii* is an intracellular parasite and belongs to the Apicomplexa phylum. The definitive host of *T. gondii* is member of the Felidae family (cats); however, all warm blooded vertebrates including humans can be infected as intermediate hosts. *T. gondii* is one of the most widespread human parasites, infecting up to one-third of the global population [8]. The life cycle of *T. gondii* includes two phases. The sexual phase, in which the cat is the host, results in the release of oocysts via faeces. The second phase [15], asexual, take place in the intermediate host (Figure 1.1).

The infection of humans by *T. gondii* can occur following ingestion of tissue cysts in infected meat or contaminated water with oocysts [23], (Figure 1.1). After ingestion of the parasite by the intermediate host, the tachyzoites (a rapidly multiplying stage in the development of the tissue phase) invade a variety of cell types, such as epithelial and blood leukocyte cells including T cells, natural killer cells, and dendritic cells (DC). The parasite initially crosses the intestinal epithelium and rapidly disseminates into the deep tissues by migration to distant sites, such as the central nervous system. The parasite can cross different biological barriers including the blood-brain barrier, blood-retina barrier, and the placenta. The most severe damage takes place at the biological barriers [5]. After rapid dissemination, the parasite’s life cycle changes into a slow growing stage, the bradyzoit (a slowly multiplying encysted form of sporozoan parasite) in the peripheral tissues, where it can remain for the entire lifetime of the host. Reactivation of the infection causes severe neurological complications in immune-compromised patients. Such complications can cause congenital infections in developing fetuses or ocular manifestations in otherwise healthy individuals [8].

There are three different types (I, II, and III) of clonal linages of *T. gondii* spread around the globe, mainly in Europe, North America, and Africa, with type II the being most prevalent in humans [8]. Type I infections are virulent in mice, whilst type II and III strains are not lethal (50% of mouse infected with ~1000 parasites can die), but result in chronic infection. It has been shown that DC hypermotility is especially elevated when the host is infected by *T. gondii* type II [15].
Figure 1.1: The life cycle of *T. gondii*. The cat is the definitive host of *T. gondii* and becomes infected by ingesting sporulated oocysts or infected material. Most mammals (such as sheeps, pigs and humans) and birds are intermediate hosts and can become infected through oocysts. Humans can be infected by ingesting contaminated food or water with oocysts or by ingestion of raw meat. *T. gondii* can cross different barriers as blood brain and blood placenta barriers. [Illustration made by Oranous Fathi Moeen, 2010]

1.2 Apicomplexa phylum

Apicomplexa parasites actively invade and multiply within eukaryotic cells (Figure 1.2a). Members of this group lack motility organelles such as flagella and cilia [5]. Furthermore, they share a conserved group of transmembrane organelles (Figure 1.2b). This complex structure is involved in penetration of the host cells. *Plasmodium* and *T. gondii* are two of the best characterized parasites in this family [6].

Figure 1.2: Apicomplexa structure and function. a) Toxoplasma tachyzoite actively penetrate host cell, human fibroblast, using gliding motility, b) Apicomplexa parasites share a conserved group of organelles; polar ring, conoid (The conoid defines the apical end of the parasite and is associated with the penetration of the host cell.), and micronemes (secretory organelle) [Illustration made by Oranous Fathi Moeen, 2010].
1.3 The host immune response

Ingested parasite cysts invade the intestine and differentiate to tachyzoites followed by rapid replication and dissemination throughout the whole body. During the innate immunity response, neutrophils are the most important immune cells [25]. They are responsible for infection control, before the adaptive immunity is stabilized. Two important immune cells for the infection control of *T. gondii* in the late phase of immunity are the T helper cells (CD4^+^) and cytotoxic T-lymphocytes (CD8^+^) [19]. The most important cytokines leading to control of the proliferating parasites are interferons (IFN) and interleukin-12 (IL-12). The main sources of production of these cytokines are natural killer (NK) and T cells [25].

1.4 Dendritic cells

Dendritic cells are elements of the mammalian immune system and a very specialized type of antigen-presenting cell (APC). They are present in lymphoid organs and in many different parts of the body, such as in the epithelial layer of skin, gastrointestinal, and respiratory tracts. There are several different types of dendritic cells. Langerhans dendritic cells are present in the epidermis, where they trap antigens and migrate to lymph nodes and present them to T cells (Figure 1.3). Follicular dendritic cells are found in lymph follicles, and help in B cell maturation by presenting the intact antigen to the B cells. This occurs in the germinal centers of peripheral lymphoid organs and induces class switching and proliferation. Morphologically, dendritic cells are identified by their membranes, or spine-like projections [1].

![Figure 1.3: Dendritic cells migration. Dendritic cells present in skin (Langerhans-dendritic cells) uptake the antigen and migrate to lymph nodes and present antigen to T cells. [Illustration made by Oranous Fathi Moeen, 2009]](image)
All dendritic cells originate from hemopoietic stem cell precursors of myeloid lineages. The activated DCs migrate to the lymphoid tissues to present the antigen to the T cells for activation of the adaptive immune response [12]. Dendritic cells have an important role during the infection and dissemination of *T. gondii*. Recent studies indicate infected DCs can transport *T. gondii* tachyzoites. This implies that *T. gondii* make use of the DC as a "Trojan horse", for protection against the host immune system and to promote parasite dissemination *in vivo* [15].

### 1.5 Small interfering RNA

Small interfering RNAs (siRNAs) form a class of double-stranded RNA molecules, 20-25 nucleotides in length [2]. SiRNAs are involved in the RNA interference pathway, where they interfere with the expression of a specific gene. SiRNAs were first discovered as part of post-transcriptional gene silencing in plants and shortly thereafter, synthetic siRNAs were shown to be able to induce RNAi in mammalian cells [10]. SiRNAs can be introduced into the cells by different transfection methods to target specific genes and knocking down gene expression (Figure 1.4). Theoretically any gene can be targeted by siRNA if it sequence is known. This has made siRNAs important for gene expression modulation that gives scientists a tool for down regulating targeted genes, allowing study the phenotypic changes in cells.

![Figure 1.4: Mechanism of siRNA silencing. Double-stranded RNA (dsRNA) is spliced by Dicer in an ATP-dependant way. After binding to RISC (RNA-induced silenced complex), siRNA strands are separate. SiRNA/RISC complex cleavage mRNA and silence the target gene. [Illustration made by Oranous Fathi Moeen, 2010]](image-url)
1.6 Cathepsin L, Cathepsin B, Cathepsin S and C-type lectin 4

The Cathepsin proteins belong to the group of cystine protease papain family, which can be found in the endosomes and lysosomes of several different cell types [14]. The Cathepsin proteases play important roles in many physiological and pathological processes [14]. Digestion of endocytosed and endogenous proteins entering lysosomes and assisting in antigen presentation, hormone processing, matrix remodeling and tumor invasion are some of the important functions of Cathepsin proteases inside the cells [7]. It has been shown that Cathepsin L has a significant influence in tumor invasion, metastasis, growth regulation, and antigen presenting processes [24]. Cathepsin L also contributes to murine melanoma cell invasion by affecting cell migration influence. Cathepsin B has also been implicated in tumor progression [24]. Cathepsin S is involved in the process of degradation of antigenic proteins to peptides for presentation on MHC class II in human and mouse [21]. Furthermore, the inhibition of Cathepsin S has important functional consequences in modulating the autoimmune response in murine models [21]. Current studies show that the up-regulation of Cathepsin S expression has a significant role in human astrocytomas metastasis [9].

C-type lectins are a large family of calcium-dependent carbohydrate-binding molecules. They are expressed on the plasma membrane of macrophages, dendritic cells, and other leukocytes. The C-type lectins have an important role in variety of cellular response as inducers or inhibitors of cellular activation [10]. Different C-type lectins have different functions. Cell to cell signaling, cell adhesion and glycoprotein-return are some of the functions of the C-type lectins. C-type lectins are also involved in the immune system response in that some C-type lectins act as antibacterial peptides in the innate immunity [22].

1.7 Aim

Dendritic cells infected with *Toxoplasma gondii* manipulate the expression of host genes, influencing the ways in which the dendritic cell functions. The aim of this work was to study the influence of host gene expression with respect to the hypermotility phenotype demonstrated by dendritic cells following infection with *Toxoplasma* tachyzoites.
2 RESULTS

2.1 Transfection with specific siRNA

The first objective of this project was to determine the time point of maximal knockdown of gene expression. The DCs were transfected with specific siRNA and incubated for 6 and 24 hours. After incubation the RNA was purified and cDNA was synthesized. The cDNA corresponding to the Cathepsin genes, the C-type lectin 4E gene and as a control the housekeeping gene GAPDH were amplified by polymerase chain reaction (PCR) and the PCR products were then processed using electrophoresis. The results shown in figure 2.1 suggest an up and down regulation of gene expression. The transfection of DCs with Cathepsin L and Cathepsin S siRNA resulted in a reduction of gene expression after 6 h. In contrast, attempted knockdown of Cathepsin B siRNA led to an increase in gene expression after 6 h compared to the negative control. No clear difference could be measured in gene expression after 24 h incubation time. The transfection of DCs with C-type lectin 4 siRNA resulted in a reduction in expression after 24 h compared to the negative control. No clear difference could be measured in gene expression after 6 h incubation time. The negative control (Nc) siRNA has no homology to any known murine gene, essentially measuring the effects of the transfection procedure on the expression of each gene target.

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as reference for the experiments. The GAPDH is also known as housekeeping protein as it is well characterized and usually expressed constitutively. No difference could be observed in the expression levels of GAPDH in any of the experiments (Figure 2.2).

Figure 2.1: The effect of siRNA on gene expression in dendritic cells. Dendritic cells were transfected with cognate siRNA. The cDNA was prepared after 6 or 24 h and amplified by PCR. Resulting amplicons were analyzed on 1 % agarose gels, stained with Gel Red. Band intensities were quantified using Image J. a) Cathepsin L (CtL), b) Cathepsin B (CtB), c) Cathepsin S (CtS), and d) C-type lectin 4E (C-4).
Figure 2.2: Gel electrophoresis images from siRNA transfection experiments. Dendritic cells were transfected with cognate siRNA. The cDNA was prepared after 6 or 24 h and amplified by PCR using reference primers GAPDH. Electrophoresis image prepared in 1 % agarose gel and stained with Gel red for a) Cathepsin L (CtL) and b) Cathepsin S (CtS).

2.2 In vitro migration Assay

The next objective was to assess the migratory capacity of the infected dendritic cells after siRNA treatment. The DCs were treated differently and loaded into a transwell membrane to migrate. After an incubation time of 16 h, the DCs remaining on the upper surface of the membrane were removed, and migrated cells were quantified using a counting chamber. The results are shown in figure 2.3-2.6. As a control, migration of non infected DCs was compared to infected DCs. The infected DCs were compared to infected DCs treated with gene specific siRNA and negative control which was a heterogous siRNA. There was a 39% reduction in the number of infected DCs that migrated across the insert following treatment with Cathepsin L siRNA compared to infected DCs treated with negative control siRNA (Figure 2.3). For Cathepsin B there were no differences between infected DC treated with siRNA compared to the negative control siRNA (Figure 2.4). Infected DCs treated with Cathepsin S siRNA showed an 8% reduction of transmigration in comparison to DCs treated with negative control siRNA (Figure 2.5). For C-type lectin 4 there were no differences between the infected DC treated with siRNA compared to negative control siRNA (Figure 2.6).

Figure 2.3: Effect of siRNA antagonizing Cathepsin L on migration by dendritic cells. The green bars illustrate non infected DCs and red bars illustrate the infected DCs. The migrated cells were quantified using a counting chamber. The percentages of number of loaded DC and number of migrated cells after 16h incubation are shown in the graph. The experiment was done in triplicate and repeated three times.
Figure 2.4: Effect of siRNA antagonizing Cathepsin B on migration by dendritic cells. The green bars illustrate non infected DCs and red bars illustrate the infected DCs. The migrated cells were quantified using a counting chamber. The percentages of number of loaded DC and number of migrated cells after 16h incubation are shown in the graph. The experiment with Cathepsin B was done once in triplicate.

Figure 2.5: Effect of siRNA antagonizing Cathepsin S on migration by dendritic cells. The green bars illustrate non infected DCs and red bars illustrate the infected DCs. The migrated cells were quantified using a counting chamber. The percentages of number of loaded DC and number of migrated cells after 16h incubation are shown in the graph. The experiment was done in duplicate, and repeated three times.
Figure 2.6: Effect of siRNA antagonizing C-type Lectin-4 migration by dendritic cells. The green bars illustrate non infected DCs and red bars illustrate the infected DCs. The migrated cells were quantified using a counting chamber. The percentages of number of loaded DC and number of migrated cells after 16h incubation are shown in the graph. The experiment for the C-type lectin 4 was done once in a single well.

To ensure that the siRNA only targeted the Cathepsin L gene in the DCs and not T. gondii, the sequence of the siRNA was blasted against the mouse and T. gondii genomes respectively. The selected siRNA targeted only the mouse derived Cathepsin gene, (Figure 2.7). In contrast, there was no significant match between the siRNA sequence and Cathepsin L of T. gondii.

Figure 2.7: Comparison of the Cathepsin L gene from mouse with the Cathepsin L from Toxoplasma. The siRNA part of Cathepsin L gene from mouse was blasted against the Cathepsin L from T. gondii.
2.3 Immunofluorescence staining

In order to detect any changes in the morphology between dendritic cells treated with Cathepsin L siRNA and DCs treated with negative control siRNA, immunofluorescence staining was performed using Alexa Fluor 594 phalloidin. Alexa Fluor 594 phalloidin is a high-affinity probe for F-actin that is made from a mushroom toxin conjugated with bright and photostable Alexa Fluor 594 dye. This probe is used for labeling, identifying and quantitating F-actin in formaldehyde-fixed and permeabilized tissue sections, cell cultures or cell-free experiments. Using this probe is a powerful tool for investigating the distribution of F-actins in the cells [16]. However, no difference between the DCs with and without treatment of Cathepsin L siRNA was observed (figure 2.8).

![Immunofluorescence staining of dendritic cells. The images were taken by Leica microscope at 100 magnification a) Dendritic cells stained by DAPI (blue) and Phalloidin (Red), b) DCs, treated with Cathepsin L RNA interference and c) DCs treated with negative control siRNA.](image-url)
Previous studies in the Barragan laboratory indicated that there is a correlation between increment in motility of the dendritic cells and the over-expressed genes. Several over-expressed genes were selected for this study such as Cathepsin L, Cathepsin B, and Cathepsin S and C type lectin 4. The hypermotility of DCs were investigated with respect to specific overexpression genes. The hypermotility of the DCs were assayed by knocking down targeted genes with the use of RNA interference.

In the first part of the experiments, optimal incubation times were investigated. This step involved use of siRNA for knocking down targeted genes. The best incubation times were 6 h and 24 h depending on the gene under study. The results I observed suggested a regulation of gene expression by siRNA. In order to get more quantitative data, a quantitative assay was needed. However, the data showed that there was a down regulation of Cathepsin L and S, but up regulation of Cathepsin B, by siRNA after 6 h, but no effect after 24 h. There was a down regulation of C type lectin after 4 h and no effect after 6 h. RNA interferences are usually used for knock down of genes in order to characterize gene function [13]. The overexpression of Cathepsin B could be explained by microRNA activation and the interaction of siRNA and a part of the gene that can act as a promoter resulting in overexpression of that gene [20]. Infected DCs treated with siRNA targeting Cathepsin L and S exhibited a reduction in migration. This phenotype indicates the involvement of T. gondii in targeting Cathepsin L and S genes for increment in motility of the DC, thus dissemination of the parasite in the host. Finding the host genes that play a role in the hypermotility of infected DCs is highly important for understanding the mechanism of the parasite dissemination in the host. T. gondii invades leukocytes and by doing so it evades cellular immunity, allowing the infected cells to act as "Trojan horses" for dissemination in the host [18]. This makes it one of the most widespread human pathogens.

The aim of this study was to characterize what genes are involved in the hypermotility of the DCs infected with T. gondii. From the result obtained in this study I could conclude that targeting Cathepsin L and S can reduce the motility of dendritic cells. Since the dissemination of the parasite is highly dependent on dendritic cell migration in vivo, Cathepsin L and S could contribute to a therapeutic target of clinical interest in the future. A possible future experiment could involve the treatment of infected DCs with combined siRNA targeting, Cathepsin L and S. The combination therapy could be more effective at reducing the motility of the DCs, indicating that the up regulation of several genes is required to initiate and maintain the hyper migratory phenotype. Another promising research can be the study of migration for other antigen presenting cells such as macrophage, which are target for T. gondii as well as DCs, and compare the result together. The results in this work were obtained using an in vitro model. For a complete investigation of the influence of the targeted genes, an in vivo study must also be carried out.
MATERIALS AND METHODS

4.1 Parasites and Animals

Toxoplasma tachzyoites of type II strain ”PTG-GFP luc” [11] with the serial 2-day passage in human foreskin fibroblast monolayer were used in this study. Type II strain infection can be controlled and at the same time increase the migration of DCs than other strain [2]. Female C57/BL6 mice were obtained from the MTC animal facility, Karolinska Institute. The bone marrow extracted from C57/BL6 mice was used for the production of dendritic cells.

4.2 Cell Culture

Human foreskin fibroblasts (HFF) were obtained from Antonio Barragan [4], (All cells types are described in table 4.1), with one week passage growing in D10 medium (Dulbecco’s modified Eagle medium (DMEM) (Invitrogen) with 10 % heat-inactivated phosphate buffered saline (1x PBS, containing 800 ml H2O, 8 g NaCl, 0.2 g KCl, 1.44 g Na2HPO4, 0.24 g KH2PO4, pH ≈ 7.4), 1 % HEPES, 1 % glutamine and 0.2 % gentamicin).

Dendritic cells were produced from the bone marrow of C57/BL6 mice. The legs were removed from the mouse, and washed in 70 % ethanol. After removing the muscle tissue surrounding the femur, the bone marrow was flushed with a long needle (23G) using DMEM medium. Collected bone marrow was filtered with 100 micron strainer and centrifuged at 950 x g for 10 min. The pellet was resuspended in D10 medium (Invitrogen) supplemented with Granulocyte-monocyte colony-stimulating factor (GM-CSF) (important cytokine for differentiation of stem cell to DC and macrophage) secreted from a myeloma cell line (X63) (20 %). The cells were seeded into six-well plates. Fresh medium were added every 2nd day to each well for 6 days (1 mouse provided ≈ 10^7 DCs).

GM-CSF cytokines were prepared as follows: First approximately 3 million myeloma cells transfected to produce the GM-CSF [17] were thawed and put into a small T25 flask and incubated in D10 medium at 37°C for three days. The cells were flushed and transferred into three medium size flasks (T75 cm²) growing in 60 ml X63 medium (D10 medium with 2 % geneticin). After five days incubation at 37°C, they were transferred to tubes and centrifuged at 3800 x g for 10 min. The supernatant was discarded and the pellet containing the cells was washed four times with 10 ml D10 medium. After washing the pellet, it was transferred to a large T175 flask with 200 ml of D10 medium and incubated at 37°C. After 48 h, the cells were collected and centrifuged at 550 x g for 10 min. The supernatant containing GM-CSF was collected and stored at −20°C until the day of use.

| Table 4.1: Cell type and their origin |
| Cell type | Abbreviation | Origin |
| Human foreskin fibroblast | HFF | As described in [4] |
| Dendritic cells | DC | Bone marrow of C57/BL6 mice |
| Myeloma cells | Myeloma cells | As described in [17] |
4.3 Transfection with siRNA

Dendritic cells were plated in 24-well plates at a density of $2 \times 10^5$ cells per well. 6 μl siRNA (100 nM siRNA which was suggested in protocol) (Qiagen) mixed with 6 μl ”HiPerFect transfection Reagent”. The solution was added to the DCs and the mixture was incubated for 6 and 24 h at 37°C. After incubation, the cells were collected, centrifuged at 42000 x g for 2 min and resuspended in 100 μl TRizol reagent (Invitrogen), in preparation for RNA extraction. The siRNA sequences used for different gene are shown in the table 4.2.

Table 4.2: siRNA sequences used in this study

<table>
<thead>
<tr>
<th>Gene</th>
<th>siRNA sequence (5'-3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cathepsin L</td>
<td>AAG CTACCTCAAATTATAAA</td>
</tr>
<tr>
<td>Cathepsin B</td>
<td>TCCAGAAAGGATGAACATTAA</td>
</tr>
<tr>
<td>Cathepsin S</td>
<td>AAGGATAAGAATGAAGAAGAA</td>
</tr>
<tr>
<td>C-type lectin domain family 4, member E</td>
<td>CACCAGATGTGTCGTAACATA</td>
</tr>
</tbody>
</table>

4.4 RNA extraction

The RNA was extracted from DCs using ”TRIzol Plus RNA Purification Kit” (Invitrogen). According to manufacturer’s instruction lysate obtained with the TRIzol Reagent was incubated at room temp for 5 minutes to allow complete dissociation of nucleoprotein complexes. Chloroform (0.2 ml /1 ml) was added to samples, followed by shaking by hand for 15 seconds. After 2-3 min incubation the sample was centrifuged at 95000 x g for 15 min at 4°C. The colorless, upper phase containing the RNA was transferred to a fresh RNase-free tube. An equal volume of 70 % ethanol was added to obtain a final ethanol concentration of 35 %. After vortexing, the sample was transferred to a spin cartridge with a collection tube followed by centrifugation at 95000 x g for 15 seconds at room temp. The flow-through was discarded and the spin cartridge was reinserted into the same collection tube. The sample was washed once with 700 μl Wash Buffer I (Invitrogen) and twice with 500 μl Wash Buffer II (Invitrogen) with ethanol, and centrifuged at 95000 x g for 15 seconds. The spin cartridge was centrifuged at 95000 x g for 1 min at room temp to dry the membrane with attached RNA. The collection tube was discarded and spin cartridge was inserted into a recovery tube. RNase-free water (30 μl) was added to the center of the spin cartridge followed by incubation at room temp for 1 min. The spin cartridge with recovery tube was centrifuged for 2 min at 95000 x g at room temp. The purified RNA was stored at −20°C if it was to be used within a few days, and stored long-term at −70°C.

To remove any remaining DNA, a DNase digest using the ”TURBO DNA-free kit” (Ambion) was performed. First 0.1 volume 10X TURBO DNase Buffer and 1 μl TURBO DNase was added to RNA (30 μl) followed by mixing and incubation at 37°C for 30 min. After incubation, 0.1 volume DNase Inactivation Reagent was added to the solution, which was incubated at room temp for 2 min. The solution was centrifuged at 95000 x g for 2 min and the RNA was transferred into a fresh tube. The RNA concentration was measured 260 nm spectrophotometrically (Bio RAD smart spec Plus spectrophotometer). All extracted RNA was stored at −70°C.
4.5 Synthesis of cDNA

For cDNA synthesis “Ready-TO-GO PCR Beads” (GE Healthcare) was used. Each bead contains all reagents necessary for PCR (dNTP, PCR buffer, and MgCl₂). The purified RNA was mixed with the beads and 1 μl of 0.5 μg/μl “First strand oligo dT” and “RNase free water” to a volume of 50 μl. The mixture was incubated at 42°C for 30 min followed by an incubation time of 5 min at 95°C. The rest of the purified RNAs were stored at −70°C.

4.6 Primers and sequences

Specific primer pairs used for PCR for amplification are shown in Table 4.3.

<table>
<thead>
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<th>Gene</th>
<th>Primer Sequence (5'→3')</th>
<th>PCR product length (bp)</th>
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<tbody>
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<td>Cathepsin L</td>
<td>Forward ATGGCACGAATGAGGAAGAG</td>
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<tr>
<td></td>
<td>Reverse GAAAAAACCTCCCTTTCTTG</td>
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</tr>
<tr>
<td>Cathepsin B</td>
<td>Forward GAAGAAGCTGTGTGGCACTG</td>
<td>197</td>
</tr>
<tr>
<td></td>
<td>Reverse GTTCGGTCAGAAATGGGCTTC</td>
<td></td>
</tr>
<tr>
<td>Cathepsin S</td>
<td>Forward AAGGATAAGAATGAAGAAGAA</td>
<td>80</td>
</tr>
<tr>
<td></td>
<td>Reverse CAAGAACACCATGGTCACATTCATGC</td>
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<td>C-type lectin 4E</td>
<td>Forward AGCCAAATTGACCATCCTCAG</td>
<td>176</td>
</tr>
<tr>
<td></td>
<td>Reverse GCCCTTTGATGGGATTCCAG</td>
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<tr>
<td>GAPDH</td>
<td>Forward ACCCAGAAGACTGTGGATGG</td>
<td>69</td>
</tr>
<tr>
<td></td>
<td>Reverse AACGAGAGATGATGTCTGG</td>
<td></td>
</tr>
</tbody>
</table>

4.7 Polymerase chain reaction

Mixtures contained cDNA (9.5 μl cDNA from 126 ng/μl RNA for Cathepsin L, and Cathepsin B, 6 μl cDNA from 425 ng/μl RNA for Cathepsin S, 9.5 μl cDNA from 156 ng/μl RNA for C type lectin4) were analyzed using PCR. The mixtures included 12.5 μl ”Master mix ”(containing Mg²⁺, KCl, dNTP (Deoxyribonucleotide triphosphate), and PCR buffer) (Invitrogen), 1 μl of each primer (to a final concentration of 1 μM), ”RNase free water” to a total amount of 25 μl. Different PCR programs were used for different genes as described in Table 4.4.
Table 4.4: Polymerase chain reaction program for different genes.

<table>
<thead>
<tr>
<th>Gene</th>
<th>PCR program</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cathepsin L</td>
<td>95°C for 1 min, 35 cycle of (95°C for 30 sec, 68°C for 1 min) and 68°C for 1 min</td>
</tr>
<tr>
<td>Cathepsin B</td>
<td>95°C for 2 min, 35 cycle of (95°C for 30 sec, 65°C for 30 sec, 60°C for 30 sec) and 72°C for 7 min</td>
</tr>
<tr>
<td>Cathepsin S</td>
<td>95°C for 2 min, 35 cycle of (95°C for 30 sec, 60°C for 30 sec, 72°C for 45 sec) and 72°C for 7 min</td>
</tr>
<tr>
<td>C-type lectin 4E</td>
<td>95°C for 2 min, 35 cycle of (95°C for 30 sec, 55°C for 30 sec, 72°C for 45 sec) and 72°C for 7 min</td>
</tr>
</tbody>
</table>

4.8 Agarose electrophoresis

Gel contained 1% agarose in "1xTAE buffer" (containing a mixture of 242 g Tris base, 57.1 ml of glacial acetic acid, 100 ml of 0.5 M EDTA (pH 8.0) in 1000 ml H2O) stained with 1 μl Gel red (10,000X in water) (Biotium) were run for 35 min at 100 V. The results were documented using gel Documentation System (Bio Rad Chemi Doc).

4.9 Immunofluorescence assays

Individual cover-slips were placed into each well in a 24-well plate. 400 μl lysine was added to each well and incubated for 10 min on a shaker at room temperature. After washing with 500 μl sterile water for 10 min, the coverslips were dried at room temp for approximately 30 min. DCs (2 x 10^5 cells per well) were transfected with Cathepsin L siRNA, incubated for 6 h and transferred to wells with coverslips and infected with T. gondii parasite (6 x 10^5 parasites per well) overnight at 37°C. The cells were washed for 10 min in 500 μl warm PBS, then fixed in 400 μl 4% warm paraformaldehyde (PFA) for 20 min followed by two more washing steps with PBS. After fixation the cells were blocked using 300 μl blocking solution (PBS containing 3% bovine serum albumin and 0.25% Triton-X100) and incubated for 30 min at room temperature. After washing three times with 500 μl PBS, the cells were stained with 2 μl Alexa Fluor 594 phalloidin (Invitrogen), followed by an incubation time for 1 h at room temperature in dark. After the last incubation the cells were washed again three times with 500 μl PBS and once with 500 μl sterile water (all steps were done at room temp). The cover slips were mounted on a slide with one drop DAPI (4',6-diamidino-2-phenylindole) and incubated at room temperature for 2 h. The cover slips were stored at 4°C in dark until they were analyzed using a Leica fluorescence microscope.

4.10 In vitro migration assays

The motility of the infected dendritic cells was measured using the transwell quantification method. Dendritic cells were plated at a density of 2 x 10^5 cells per well. The amount of siRNA required to knock down expression varied depending on the specific gene under study (the recommended amount in the protocol was 100 nM siRNA for 2 x 10^5 DCs, 100 nM siRNA showed a knock down of gene expression for Cathepsin L, B and S but it wasn’t enough for CLE4E, after using a double amount of siRNA (200 nM siRNA ) a knockdown of CLE4E expression was detected) and was added to the wells, followed by an incubation time
of 6 h for Cathepsin L, B and S and 24 h for CLE4E at 37°C. After incubation, the cells were infected with *T. gondii*, approximately $6 \times 10^5$ parasites for every $2 \times 10^5$ DCs (3:1 ratio) followed by 2 h incubation at 37°C. The DCs were transferred to transwell filters (Becton Dickinson) with 8 μm pore size and incubated for 16 h at 37°C. After incubation, the DCs remaining on the upper surface of the membrane were removed, and DCs that have migrated through the pores were counted. For quantification of the migrated DCs a counting chamber was used.

![Figure 4.1: Cell migration assay. A cell culture of DCs is placed on a transwell membrane to migrate through an 8 μm pore membrane. After an incubation time of 16 h, the DCs remaining on the upper surface of the membrane are removed, and DCs that have migrated through the pores are counted. [Illustration made by Oranous Fathi Moeen, 2009]]
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References


