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The M1 protein of *Streptococcus pyogenes* triggers an innate uptake mechanism into polarized human endothelial cells

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Running title:

M1 protein-mediated invasion of *S. pyogenes* into human endothelial cells

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Abstract

Serotype M1 *S. pyogenes* is a major human pathogen associated with severe invasive diseases causing high morbidity and mortality. In a substantial number of patients invasive disease develop in previously healthy individuals with no obvious port of entry. This led to the hypothesis that the source of streptococci in these cases is a transient bacteraemia. Therefore, this study focusses on the analysis of interaction of tissue-invasive serotype M1 *S. pyogenes* with human endothelial cells (EC) of the vascular system. We identify the M1 surface protein of *S. pyogenes* as the EC invasin which is recognised by polarized human blood endothelial cells, thereby triggering rapid phagocytosis-like uptake of streptococci into polarized EC layers. Upon internalization M1 *S. pyogenes* are incorporated into phagosomes which traffic via the endosomal/lysosomal pathway. However, a proportion of streptococci successfully evades this innate killing process and mediates its escape into the cytoplasm of the host cell. The results of this study demonstrate that blood endothelial cells possess an efficient uptake mechanism for serotype M1 *S. pyogenes*. Despite efficient phagocytosis, streptococcal survival within EC constitutes one potential mechanism which favours intracellular persistence and, thus, facilitates continuous infection and dissemination from the primary side of infection into deep tissue.

Introduction

Streptococcus pyogenes (group A streptococcus, GAS) is an exclusively human pathogen that causes a wide variety of diseases. In addition to mild and superficial infections of the upper respiratory tract and the skin, *S. pyogenes* also causes severe invasive diseases including necrotizing fasciitis, septicemia and streptococcal toxic shock syndrome [1,2]. Despite antibiotic therapy these invasive conditions are the cause for substantial morbidity and mortality worldwide [2]. Since the late 1980s a re-occurence of severe invasive diseases due to *S. pyogenes* infections has been observed. Among the over 120 serotypes known various M types have been associated with invasive diseases. Serotypes M1 and M3, however, are the most prevalent and together are responsible for up to 50% of the cases of severe invasive diseases [3].

Although *S. pyogenes* has been considered as an extracellular pathogen, various reports have shown that *S. pyogenes* invades into different cell types *in vitro* including epithelial cells [4-6], neutrophils [7,8] and macrophages [9,10]. Internalization of *S. pyogenes* into eukaryotic cells is considered to be one successful mechanism to evade host immune responses as well as penicillin treatment and, thus, might promote carriage and dissemination from the primary side of infection. The *in vivo* relevance of an intracellular habitat in the persistence within the human host has been demonstrated by a study of Osterlund and colleagues. The authors re-isolated viable *S. pyogenes* from biopsies of patients with tonsillitis [11].

Adhesion and invasion into host epithelial cells represent first steps in the pathogenesis of streptococcal infections and these processes are quite well understood [1]. It has been shown that serotype M1 *S. pyogenes* mediates its uptake into epithelial cells by binding of the M1 surface protein to host cell integrins, thereby, triggering a PI3K-dependent signalling cascade, which leads to actin polymerization and zipper-like uptake of bacteria into epithelial cells [12-16]. A pre-requisite for the development of deep tissue infections, however, is the transition from the pharyngeal epithelial surface into deeper, naturally sterile, tissue. Thereby, the

pathogen has to overcome different cellular and tissue barriers. Disruption of the epithelial cell layer, e.g. following physical injury, provides entry ports for pathogenic streptococci [17]. However, in the majority of patients with deep tissue infections no obvious port of entry is detectable. This led to the hypothesis that streptococcal spread is associated with a transient bacteraemia originating from the oropharynx [3,18].

Classical phagocytic cells represent important barriers that cross the way of bacteria into deep tissue. Hertzén *et al*. showed that serotype M1 *S. pyogenes* exploits phagocytic cells as an intracellular reservoir to ensure streptococcal survival. The authors demonstrated that GAS survives and even replicates within macrophages, egresses out of the cells and re-infects new cells [10]. Another potentially important cell type are blood endothelial cells lining the lumen of the blood vessels. We previously identified a new invasion mechanism of serotype M3 *S. pyogenes* into endothelial cells [19], and identified the fibronectin-binding protein FbaB as a major invasin of M3 *S. pyogenes* [20]. However, a direct interaction of the most invasive serotype M1 *S. pyogenes* with endothelial cells has not been shown before. In this study we analyse the molecular interaction of serotype M1 *S. pyogenes* with primary human blood endothelial cells of the umbilical cord (HUVEC) in further detail. Here, we show that M1 GAS is efficiently internalized by EC. We identify the M1 surface protein of M1 GAS as the target protein which triggers a phagocytosis-like uptake process of M1 GAS into EC. Intracellular streptococci traffic via the classical endocytic pathway, but may also escape from the phagocytic vacuole to reside and replicate within the cytoplasm, thereby being protected from innate host immune defences and finding a niche for survival and persistence.

Material and Methods

Bacterial strains and culture conditions

Serotype M1 GAS strains A527 (KTL3), A270 and A271 are clinical isolates from the blood of patients with invasive diseases and were from the culture collection of the Department of Medical Microbiology of the Helmholtz-Centre of Infection Research in Braunschweig, Germany. *S. pyogenes* SF370 (A302) is a genome sequenced serotype M1 GAS which was described elsewhere [21]. *S. pyogenes* serotype M1 strain 90-226 (A766) and its isogenic knock-out mutant lacking the M1 surface protein, strain ΔM1 90-226 (A767), were previously described [22]. The M1 GAS mutant strains KTL3 GRAB-ko, KTL3 prtS::spc, KTL3 slo::spc and KTL3 ΔsagA were from the culture collection of the Department of Medical Microbiology of the Helmholtz-Centre of Infection Research in Braunschweig. Streptococcal strains were cultured in tryptic soy broth (TSB, BD Biosciences) with antibiotics where appropriate (kanamycin 150 µg ml^{-1}, spectinomycin 80 µg ml^{-1}) at 37°C and 5% CO_2 without agitation. To obtain electro-competent GAS cells, strains were grown in tryptic soy broth supplemented with yeast extract (TSBY, BD Biosciences) containing 1% glycine. *E. coli* DH5α was routinely grown in Luria broth (LB) at 37°C with agitation. To obtain competent E. coli cells, bacteria were cultures in SOB medium at 37°C with agitation. *Lactococcus lactis* (*L. lactis*) strains pP59 (-M1) and pLM1 (+M1) were kindly provided by Prof. Patrick Cleary [23] and were cultured in M17 medium (BD Biosciences) supplemented with 0.5% glucose and 5 µg ml^{-1} erythromycin at 30°C without agitation.

Reagents

Phospho lipase C gamma inhibitor edelfosine (ET-18-OCH_3) and the calcium chelator BAPTA-AM were purchased from Calbiochem. Calphostin C, inhibiting protein kinase C, was obtained from Enzo.

Complementation of *S. pyogenes* M1 knock out mutant

The full length *emm1* gene (including 51 nucleotides upstream of the start codon and 84 nucleotides downstream from the stop codon) was amplified from chromosomal DNA of the genome sequenced M1 GAS strain SF370 using the primer pair M1_fwd_*KpnI* **(**5'-GCGGTACCCCTGAAAATGAGGGTTTCTTC-3') and M1_rev_*XbaI* (5'-GCGTCTAGACTCCTTAACCTCATTCTTCTATAC-3'). The amplification product was digested with *KpnI* and *XbaI* and ligated into the shuttle vector pDC_{erm} [24]. Following transformation of the corresponding plasmid into *E. coli* DH5α, transformants were selected on LB agar plates containing 500 µg ml^{-1} of erythromycin. The presence of the *emm1* gene was confirmed by PCR, restriction enzyme analysis and DNA sequencing. Plasmid DNA was isolated and then subcloned into electro-competent *S. pyogenes* ΔM1 90-226 (A767). Positive clones (complemented ΔM1 GAS) were selected on TSBY agar plates in the presence of 2 µg ml^{-1} erythromycin and verified by PCR. In order to assess surface expression of the M1 protein, streptococcal cultures were analysed by immunofluorescence staining using a M1 specific primary antibody and a labelled secondary antibody.

Endothelial cell culture and *in vitro* transwell infection assay

Primary human endothelial cells from the umbilical cord (HUVEC) were obtained from Lonza and cultured in EGM-2 medium (PromoCell) according to the manufacturer's protocol at 37°C and 5% CO_2. For infection assays cell culture inserts with a pore size of 3.0 or 0.4 µm (BD Biosciences) were coated with 1% gelatine and subsequently cross-linked with 0.5% glutaraldehyde. Cross-linked gelatine served as a substrate to generate a semi-artificial basement membrane [25]. $3x10^4$ HUVEC (passage 3-5) were seeded per transwell and grown at 37°C and 5% CO_2 until confluence was reached. The formation of a confluent cell layer was determined by measurement of the transendothelial impedance using the CellZscope® system (NanoAnalytics), electron and fluorescence microscopy. One day prior to infection

HUVEC were washed with EBM-2 (PromoCell) and, until infection, were further incubated in antibiotic free EGM-2 medium (PromoCell). On the day of infection streptococcal overnight cultures were sub-cultured in TSB medium and cultivated until exponential growth phase (OD_{600} 0.4). Prior to infection streptococci were harvested by centrifugation and diluted in EBM-2 medium (PromoCell) containing 2.5% foetal calf serum (FCS, PAA). HUVEC were infected with M1 GAS strains with a MOI of 50 for different time intervals. After washing, cells were fixed with 4% paraformaldehyde (PFA) in PBS. Infections assays using *L. lactis* were performed according to the protocol described above for streptococci with the exception that *L. lactis* strains were cultured in M17 medium supplemented with 5% glucose and 5 µg ml^{-1} erythromycin at 30°C. The infection assays itself were carried out at 37°C and 5% CO_2 for 3 h. Internalization rates were determined by differential staining of intra- and extracellular cocci as described below.

To determine whether host cell signalling molecules of the classical phagocytic uptake machinery were involved in streptococcal uptake, function interfering assays using specific chemicals were conducted. HUVEC were pre-treated with 20 µM edelfosine, 2 µM calphostin C or 25 µM BAPTA-AM for 30 min to inhibit phospholipase C gamma, protein kinase C and intracellular calcium release, respectively, and were then infected with M1 GAS A527 for 3 h according to the standard infection protocol as described above. DMSO treated cells served as a control to exclude any toxic effects on HUVEC or streptococci due to the solvent itself.

To analyse the effect of IgG on streptococcal internalization, HUVEC were infected with M1 GAS strain A527 for 3 h according to the standard infection protocol in the presence of 100 ng ml^{-1} or 200 ng ml^{-1} purified mouse monoclonal control IgG (R&D Systems).

Survival assay

HUVEC were seeded on cell culture transwell inserts and infected with M1 GAS strain A527 as described above. After an initial period of 3 h of infection, infected HUVEC layers were

washed with EBM-2. Afterwards, remaining extracellular streptococci were killed by incubation of samples in EBM-2 medium containing 2.5% FCS, 5 µg ml^{-1} penicillin and 100 µg ml^{-1} gentamicin for defined time periods. After 2 h, 4 h, 6 h, 9 h HUVEC layers were washed at least five times with EBM-2 medium and then incubated with 100 µl 0.025% Trypsin/ 0.01% EDTA (PromoCell) per well for 5 min at 37°C to detach cells from the transwell membrane. Detached cells were permeabilized by incubation with 400 µl 1% saponin in PBS for 20 min at 37°C. Serial dilutions of cell suspensions were prepared in EBM-2 medium. 25 µl of the undiluted cell suspension or the dilutions were spotted on THY (Todd-Hewitt yeast broth, BD Biosciences) plates and incubated at 37°C, 5% CO_2 for up to 48 h. Following incubation, bacterial colonies were counted and the colony forming units (cfu) per ml were calculated. The experiment was performed in triplicates and repeated at least three times on different days.

Immuno fluorescence

Following fixation, samples were washed with PBS, blocked with PBS containing 10% FCS (PAA) for 30 min and subsequently stained for intra- and extracellular streptococci as described previously [6]. Briefly, extracellular streptococci were stained with a rabbit polyclonal antibody recognizing *S. pyogenes* (anti-GAS, 1:100 of the stock solution) [26] followed by an anti-rabbit ALEXA Fluor® A488 coupled secondary antibody (Invitrogen, 1:200 dilution). After permeabilization of cells with 0.1% Triton-X-100, streptococci were labelled with the anti-GAS primary antibody and a secondary anti-rabbit ALEXA Fluor® 568 labelled secondary antibody (Invitrogen, 1:300 dilution) to stain intracellular streptococci. According to their respective label intracellular bacteria appeared red and extracellular streptococci green to yellow. Staining of intra- and extracellular lactococci was performed according to the protocol described for streptococci except that a rabbit anti-*L. lactis* antibody (1:100 dilution) was used as primary antibody. The anti-*L. lactis* antibody was obtained from

Pineda by immunising a rabbit with heat killed *L. lactis* cells according to the standard immunization procedure of the manufacturer. To visualise the actin cytoskeleton, cells were permeabilized with 0.1% Triton-X-100, streptococci were stained with a species specific anti-GAS antibody and an ALEXA Fluor® coupled secondary antibody (Invitrogen) as described above. Subsequently the actin cytoskeleton was visualised with ALEXA Fluor® 488 or 568 coupled phalloidin (Invitrogen, diluted 1:100). To analyse co-localization of streptococci with Lamp-1, cells were directly permeabilized with 0.1% Triton-X-100. Following permeabilization streptococci were stained with a rabbit anti-GAS antibody and a species specific ALEXA Fluor® 568 coupled antibody (Invitrogen). The Lamp-1 positive compartment was labelled with a 1:50 dilution of a mouse anti-human Lamp-1 (CD107a) primary antibody (BD Pharmingen) and subsequently stained with a goat-anti-mouse ALEXA Fluor® A488 secondary antibody (Invitrogen, 1:200 dilution). In order to visualize the β_1-integrin subunit on HUVEC, unpermeabilized cells were incubated with 5 µg per well of an anti-β_1-integrin antibody (Chemicon International) for 1h at room temperature followed by a goat-anti-mouse ALEXA Fluor® 488 coupled secondary antibody. For visualization of the Arp2/3 complex, cells were permeabilized with 0.1% Triton-X-100 and the Arp2/3 complex was stained with a mouse-anti-Arp2/3 (p16 #323H3) antibody (kindly provided by Dr. C. Erck, HZI, Braunschweig, 1:100 dilution) and a goat-anti-mouse ALEXA Fluor® 488 coupled secondary antibody (1:200 dilution). Polarization and confluency of HUVEC layers were tested by visualization of the zona occludens protein (ZoI). Infected cell layers were fixed with -20°C 100% methanol for 10 min at 4°C. Following permeabilization with 0.1% Triton-X-100, the zona occludens protein was stained using an anti-ZoI rabbit antibody (Santa Cruz) and a goat-anti-rabbit ALEXA Fluor® 488 coupled secondary antibody (Invitrogen). Viability staining of intracellular streptococci was performed using the LIVE/ DEAD® Baclight™ Bacterial Viability Kit (Invitrogen) according to the manufacturer's protocol. Following a final wash step, stained transwell membranes were cut out of the transwell insert and mounted

on glass slides using ProLong® Gold anti-fade reagent containing DAPI (Molecular probes). Stained samples were examined using a Zeiss Axiophot with an attached Zeiss AxioCam HRc digital camera and Zeiss Axiovision software 4.8 or a Zeiss Axio Imager A2 with a Zeiss Axio Cam MRm camera and ZEN 2011 software. Contrast and brightness of images were adjusted using Adobe Photoshop CS3 Extended. Microscopic analyses have been performed of three independent experiments and representative images are shown.

Determination of internalization rates of M1 GAS into EC

Following immunofluorescence staining of infected EC layers, internalization rates of bacterial strains were evaluated by enumerating intracellular bacteria. A minimum of 100 cells were analysed per assay and internalization rates were expressed as intracellular bacteria per 10 cells or % intracellular bacteria. Each assay was repeated at least three times on different days. Results represent mean values (± SD) of one representative experiment.

Field emission scanning electron microscopy (FESEM)

For FESEM analysis HUVEC were infected with streptococci for 3 h as described above, washed with EBM-2 medium or TE-buffer and subsequently fixed with 2% glutaraldehyde and 5% formaldehyde in 0.1 M cacodylate buffer (0.1 M cacodylate, 0.01 M $CaCl_2$, 0.01 M $MgCl_2$, 0.09 M sucrose, pH 6.9). Samples were prepared as described previously [27]. Briefly, samples were washed with TE-buffer and were then dehydrated in a grade series of ethanol (10%, 30%, 50%, 70%, 90%) with 10 min incubation on ice for each step. Samples were incubated twice in 100% ethanol for 15 min at room temperature. Following critical-point drying with liquid CO_2 (CPD30, BalTec; CPD300, Leica), samples were coated with a thin gold-palladium film (SCD 500, BalTec) and examined in a field emission scanning electron microscope Zeiss DSM 982 Gemini (Zeiss, Germany) with an acceleration voltage of 5 kV or

a field emission scanning electron microscope Merlin (Zeiss). Brightness and contrast of images were adjusted with Adobe Photoshop CS3 Extended.

BSA-gold loading of HUVEC lysosomes and transmission electron microscopy (TEM)

In order to prepare 15 nm BSA-gold particles, 10 ml colloidal gold solution (pH 6.0) was incubated with 100 µg BSA for 30 min at room temperature. Following incubation, the BSA-gold solution was centrifuged for 15 min at 20.000 rpm (TLD100, Beckman), followed by washing with PBS containing 0.5 mg/ml PEG 20000. To pre-load HUVEC lysosomes, HUVEC were fed with BSA-gold 18 hours prior to infection as described [6]. On the following day, cells were infected with streptococci (for 4 h, 5 h, 16 h or 18 h) as described above, washed and fixed with 2% glutaraldehyde and 5% formaldehyde in 0.1 M cacodylate buffer for 1 h on ice. For TEM analysis samples were processed as previously described [27]. Briefly, samples were washed with 0.1 M cacodylate buffer, contrasted with 1% aqueous osmium tetroxide, dehydrated in a grade series of ethanol and embedded in Spurr resin. Subsequently, ultra-thin sections of infected cell layers were prepared and counterstained with uranyl acetate and lead citrate. Processed samples were analysed with a TEM EM 910 (Zeiss). Images were recorded digitally with a Slow-Scan CCD-Camera (ProScan, 1024 x 1024) with ITEM-Software (Olympus Soft Imaging Solutions). Contrast and brightness of images were adjusted with Adobe Photoshop CS3 Extended.

In order to visualize cellular membranes, a combined ferrocyanide and osmium tetroxide staining technique was applied. Fixed HUVEC layers were washed with 0.1 M cacodylate buffer, incubated with 1.5% potassiumferrocyanide in 1% aqueous osmium tetroxide and then treated with 1% aqueous thiocarbocyanide. After a second incubation with 1.5% potassiumferrocyanide in 1% aqueous osmium tetroxide, samples were washed with 0.1 M cacodylate buffer, dehydrated and processed as described above.

Statistical analysis

Data was analysed using Microsoft Office Excel 2010 software. Statistical comparison was performed using the Student's paired t-test. Results were expressed as mean values ± SD of one representative experiment p values of 0.05 or less were considered significant.

Results

Serotype M1 *S. pyogenes* isolates trigger uptake into polarized human endothelial cells (EC)

Since a direct interaction of the most invasive serotype M1 *S. pyogenes* with endothelial cells (EC) of the vascular system has not been studied before, we aimed to assess the invasion potential of different M1 GAS strains on primary human endothelial cells of the umbilical cord (HUVEC). In order to assess the invasiveness of M1 GAS on EC, we established a complex *in vitro* transwell infection model. In this infection model blood endothelial cells of the umbilical cord were cultivated on an artificial semi-permeable membrane of a transwell insert, thereby generating an artificial barrier model in which the apical side represents the blood stream and the basolateral side represents the tissue side. Confluence and polarization of the cell layer was determined by electron microscopic analysis (Suppl. Fig. 1a), staining of the zona occludens protein ZoI (Suppl. Fig. 1b) and measurement of the transendothelial impedance (data not shown). All techniques confirmed that HUVEC layers are indeed polarized. Confluent polarized HUVEC layers were then infected with four different clinical strains of serotype M1 GAS (A527, A302, A270, A271) isolated from patients with invasive diseases. Immuno fluorescence staining of infected HUVEC layers show that all four strains tested are successfully internalized into EC. Approximately 80% of bacteria are found intracellular and localized within the perinuclear region of the cell. Extracellular bacteria were found to be localized in the inter-cellular junction region, along or at the end of actin stress fibers (Fig. 1a-d). Three of the M1 GAS isolates show similar internalization rates into HUVEC (Fig. 1e), with more than 100 intracellular bacteria per 10 HUVEC cells 4 h post infection. Strain A271 exhibits lower infection rates than the other strains (Fig. 1e). Since all M1 GAS strains trigger uptake into EC, the following experiments were performed with strain A527 (KTL3) only. In summary, these results demonstrate an active uptake of M1 GAS by EC. Furthermore, the confluence status as well as polarization of the EC layer proofed to be

essential for efficient internalization of M1 GAS into HUVEC since HUVEC grown on coverslips showed no internalization of M1 GAS strains (data not shown). Thus, we have established a suitable infection system which was subsequently applied to study the interaction of M1 GAS on HUVEC in further detail.

Internalization of M1 *S. pyogenes* is characterized by actin rearrangements and trafficking of internalized streptococci along the classical endocytic pathway with lysosomal destination

To further characterize the uptake mechanism of M1 GAS by HUVEC we next analysed the mode of internalization and intracellular fate of M1 GAS within HUVEC. Internalization of M1 GAS into HUVEC starts within the first hour of infection and increasing numbers of intracellular bacteria are detected 3 h, 4 h and 5 h post infection (Suppl. Fig. 2). Following attachment of streptococci to the host cell, M1 GAS triggers the formation of membrane protrusions that tightly engulf the bacterial chain (Fig. 2a). Finally, streptococci are completely surrounded by the host cell membrane and incorporated into endothelial cells (data not shown). On the cellular level the formation of membrane protrusions is based on a re-organization of the actin cytoskeleton. These processes are initiated by engagement of a cellular receptor and subsequent activation of specific intracellular signalling cascades which lead to actin remodelling processes. Internalization of M1 GAS into HUVEC is accompanied by clustering of the β_1-integrin subunit on the host cell surface in the near vicinity of invading bacteria (Fig. 2b). In contrast, β_1-integrins are statistically distributed in areas where no streptococcal invasion occurs (Fig. 2b). Receptor engagement and subsequent activation of downstream signalling events trigger F-actin accumulation around internalized streptococci (Fig. 2c). Moreover, during internalization of M1 GAS into HUVEC an aggregation of the actin related protein complex 2/3 (Arp2/3) is detectable around invading streptococci which coincides with actin recruitment (Fig. 2d). By function-interfering experiments we identify the

host cell signalling molecules phospholipase C gamma (PLCɣ), protein kinase C (PKC) and intracellular calcium as key molecules involved in the internalization process. Blocking either of those significantly reduced or completely abolished streptococcal internalization (Fig. 2e). To assess the subsequent trafficking route of M1 GAS within the cell we next investigated the acquisition of the late endosomal-lysosomal marker protein Lamp-1. Immunofluorescence analysis of infected HUVEC layers show that M1 GAS co-localizes with Lamp-1 positive compartments 3 h post infection (Fig. 2f). To investigate if phagosomes containing streptococci eventually fuse with terminal lysosomes, HUVEC were fed with BSA-gold particles 18 h prior to infection with M1 GAS. BSA-gold particles are internalized into the host cell, traffic along the endocytic pathway and are subsequently delivered into terminal lysosomes. Following pre-incubation, HUVEC were infected with M1 GAS and samples were processed for transmission electron microscopy (TEM). TEM analysis of ultra-thin sections of infected HUVEC layers reveal co-localization of M1 GAS with gold particles (visible as black electron dense material) within the same intracellular compartment (Fig. 2g), indicating fusion of the M1 GAS containing phagosome with terminal lysosomes.

Taken together our results clearly demonstrate efficient phagocytosis-like uptake of M1 GAS into HUVEC. Internalized streptococci are incorporated into phagosomes which travel along the classical endocytic route and, in the end, fuse with terminal lysosomes.

The M1 surface protein of M1 GAS is the invasin which mediates uptake into EC

Next we identified the bacterial factor that mediates M1 GAS entry into EC. Unlike serotype M3 GAS, which expresses the EC invasin FbaB [20], M1 GAS lacks this specific protein on its cell surface. Therefore, we tested a series of pre-existing knock-out mutants in the M1 background for their ability to be internalized into EC (Fig. 3a) and identified the M1 surface protein as one promising candidate. We decided to analyse its role during internalization into EC in further detail. The M1 knock-out mutant, which lacks the M1 surface protein (ΔM1

GAS), is not internalized by EC (Fig. 3b, c), whereas the M1 wt GAS strain (A766) is successfully phagocytosed (Fig. 3b). By complementing the M1 knock-out mutant with M1 protein, uptake into EC is restored, yielding internalization rates comparable to that of the wt M1 GAS strain (Fig. 3b, d). To finally prove that the M1 protein is sufficient to trigger uptake into HUVEC, its properties as a key factor for phagocytosis were tested by heterologous expression of M1 in *L. lactis*. *L. lactis* is a non-pathogenic bacterium and is naturally non-invasive in eukaryotic cells. Infection assays of HUVEC with the parental *L. lactis* strain, which lacks the M1 protein on its surface, demonstrate that *L. lactis* is not internalized by endothelial cells (Fig. 3e, right bar labelled -M1). In contrast, heterologous expression of M1 converts *L. lactis* into an efficiently internalized strain (Fig. 3e, left bar labelled +M1). In summary, our data clearly identify the M1 surface protein of GAS as a key factor which is sufficient to mediate uptake of bacteria into EC.

Viable M1 GAS escape from the phagolysosomal compartments

Incorporation of foreign material e.g. pathogens into phagosomes and subsequent phagolysosomal fusion leads to degradation of foreign material by hydrolytic enzymes and their elimination [28]. However, here we show that serotype M1 GAS has developed strategies to counteract the innate killing mechanisms of the host cell and to ensure streptococcal survival. Transmission electron microscopic analysis of ultra-thin sections of infected HUVEC layers reveal streptococcal escape from the membrane bound compartment of the phagolysosomes into the cytoplasm of the host cell (Fig. 4a). Ferrocyanide and osmium tetroxide staining confirms the absence of any cellular membranes around intracellular streptococci (Fig. 4b, white arrows) which are, for example, localized in close proximity to the endoplasmic reticulum (Fig. 4b, black arrow). This gives further evidence of the cytoplasmic localization of M1 GAS and, thus, streptococcal escape from the phagolysosome. Viability staining of M1 GAS demonstrates live intracellular bacteria after 5 h of infection

(Fig. 4c) and also 10 h post infection the majority of intracellular streptococci is viable (Fig. 4d). Quantification of survival rates by plating of HUVEC lysates following infection with M1 GAS also confirms streptococcal survival, yielding $2x10^4$ cfu/ml 2 h post infection, $1,5x10^4$ cfu/ml 4 h post infection, $1x10^4$ cfu/ml 6 h post infection and $5x10^3$ cfu/ml after 9 h of infection (Fig. 4e). Furthermore, transmission electron microscopic analysis of infected EC layers reveal morphologically intact streptococcal cells as well as ongoing streptococcal replication within the cytoplasm (Fig. 4a), which supports viability of intracellular streptococci. Taken together these data demonstrate intracellular streptococcal survival within endothelial cells. This process is mediated by escape from the acidic environment of the phagolysosome into the cytoplasm of the host cell which ensures protection from innate host defences.

Discussion

Internalization into host cells represents one successful mechanism of *S. pyogenes* to evade innate host immune defences. Several reports have shown that serotype M1 *S. pyogenes* efficiently invades and survives within various cell types including non-phagocytic cells as well as phagocytic cells [4-6,8,10,12,19,26]. In this study we provide evidence that serotype M1 streptococci efficiently invade human blood endothelial cells and that the M1 protein is a key molecule in this process. For this, we established a complex *in vitro* infection assay mimicking the endothelial cell barrier which allowed us to study the internalization mechanisms close to *in vivo* conditions: EC were cultivated on a semi-artificial basement membrane on transwell inserts to form a confluent and polarized endothelial cell barrier (Suppl. Fig. 1) which proofed to be crucial for efficient uptake of M1 streptococci into EC. M1 expressing *S. pyogenes* trigger an innate phagocytosis-like uptake into EC (Fig. 1), accompanied by the formation of membrane protrusions that tightly engulf the streptococcal chain resulting in zipper-like uptake of streptococci into EC (Fig. 2a). Typical features for phagocytosis in professional phagocytic cells [28-31] and also key features of the M1 invasion process are i) the accumulation of F-actin in the near vicinity of internalized bacteria, ii) coincident aggregation of the Arp2/3 complex around internalized streptococci, and iii) requirement of phospholipase C, intracellular calcium and protein kinase C (Fig. 2c-e). In contrast to this, invasion of M1 *S. pyogenes* into epithelial cells does not require protein kinase C [14]. This demonstrates that distinct signaling cascades are initiated during the internalization processes of M1 GAS into epithelial and endothelial cells, although both are mediated by M1 protein via engagement of subsets of β_1- integrins (Fig. 2b) [12].

Hijacking host cell receptor molecules for efficient invasion is a common strategy of bacterial pathogens to gain access to host cells [32], featuring the bacterial factors as invasins or internalization factors. M1 Protein is such an invasin, but also possesses many other important

functional properties (reviewed in [33]). Gain-of-function experiments in *Lactococcus lactis* as well as loss-of-function experiments in M1 GAS herein identify the M1 protein as a key factor which mediates uptake of GAS into EC (Fig. 3), yet demonstrating another role of this major virulence factor of GAS. Other studies identified the M1 protein as a crucial factor that mediates survival within neutrophils and macrophages [8,10], and this opens a perspective to speculate whether it may also mediate survival within endothelial cells.

Another important GAS invasin for endothelial cells is the fibronectin-binding protein FbaB of M3 *S. pyogenes*, an invasin with unique EC tropism [20]. Although their invasion factors are distinct, M3 and M1 *S. pyogenes* trigger similar uptake processes with phago-lysosomal destination (Fig. 2) [19,20]. However, there are also other important factors that may have a strong impact on the EC invasion of GAS. A very recent study of Ermert *et al*. identified protein H as enhancing factor for M1 *S. pyogenes* endothelial cell adherence and invasion: specific binding of the complement inhibitor protein C4b to protein H resulted in a more than 10 fold increase of invasion/survival of the M1 GAS in HUVEC [34]. This highlights the potential effect of plasma proteins on endothelial cell interaction. Plasma proteins that have receptors on the luminal side of endothelial cell are in general likely enhancers of EC adherence and invasion. For the sake of *in vitro* monolayer integrity our assays were conducted in 2.5% FCS, a condition in which a number of plasma proteins naturally present in human blood were missing. As in case of C4BP, these factors may enhance invasion into EC. For example, non-immune binding of human IgG enhanced EC invasion 1.7 fold (data not shown). On the other hand, it may be assumed that other factors such as fibrinogen that bind to M1 protein lead to large aggregate formation of streptococci that may inhibit uptake by phagocytic cells but also by EC due to the size of the target. Furthermore, secreted M1 protein forms also complexes with fibrinogen leading to platelet aggregation and thrombus formation [35] as well as vascular leakage [36].

In general one has to discriminate between true opsonization of streptococci by host components that lead to phagocytosis and terminate in killing of the pathogen, and the receptor-mediated uptake governed by invasins and host factors. Host cells have developed strategies to ensure killing of invading bacteria, one of which is delivery into terminal lysosomes for subsequent degradation and killing [28,31]. In response to that, mechanisms have evolved in bacterial pathogens that avoid killing and facilitate intracellular survival within host cells [37-40]. Staali *et al*. showed that M1 expressing *S. pyogenes* inhibit fusion of phagosomes with azurophilic granules within human neutrophils [41]. In macrophages, M1 GAS prevents fusion of the phagosome with late endosomal compartments or lysosomes [10]. Here we provide evidence that, in endothelial cells, M1 GAS-containing phagosomes acquire late endosomal lysosomal markers and fuse with terminal lysosomes (Fig. 2f, g). However, our data also revealed that a proportion of M1 GAS escapes from the phagolysosome into the cytoplasm (Fig. 4a, b). Electron microscopic analysis of ultra-thin sections of infected EC layers and viability staining of bacteria demonstrates that intracellular M1 GAS residing within the cytoplasm of the host cell appear morphologically intact and show features of replication (Fig. 4a-d) implicating that M1 GAS is able to escape from a membrane-surrounded compartment like *Listeria* or *Shigella*.

In summary, our results show the potential of EC to internalize M1 GAS and, thus, demonstrate that blood endothelial cells represent a potential reservoir for viable serotype M1 GAS. One future aim will be to determine the role of streptolysin S and O that appear to have an impact on *S. pyogenes* endothelial cell invasion.

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Figure legends

Fig. 1. Serotype M1 *S. pyogenes* clinical isolates trigger uptake into polarized EC

Immuno fluorescence analysis of the invasion potential of four different M1 GAS strains on polarized confluent EC. HUVEC were infected with the M1 GAS strains A527 (a), A302 SF370 (b), A270 (c) and A271 (d) for 5 h. After washing and fixation, cells were stained differentially for extra (green-yellow) and intracellular (red) streptococci. The actin cytoskeleton stained with phalloidin is depicted in green. Representative images of three independent experiments are shown. Bars represent 5µm (a-d).

e. Quantification of internalization rates of different M1 GAS strains on HUVEC 4 h post infection. The graph represents mean values ± SD from one out of three independent experiments.

Fig. 2. M1 *S. pyogenes* induces actin cytoskeleton rearrangements and traffics along the endocytic pathway

a. FESEM image showing uptake of streptococci (GAS A527) into EC 3 h post infection. Upon infection EC form membrane protrusions that tightly engulf the streptococcal chain, representing a zipper-like uptake process. Bar represents 600 nm. Representative images of three independent experiments are shown.

b. Immunofluorescence staining of β_1-integrins on the surface of EC. β_1-integrin clustering (green) is detectable (3 h post infection) in the near vicinity of internalized bacteria (GAS A527) (red). The inserts show split channels for GAS and β_1-integrin of the indicated area of the merged image. EC nuclei are stained in blue. Representative images of three independent experiments are shown. Bar represents 5 µm.

c. Involvement of F-actin in the internalization process of M1 GAS A527. Internalized streptococci (red) co-localize with F-actin (green) 1 h post infection. Inserts show enlarged

sections of split channels of the indicated area from the merged image. Representative images of three independent experiments are shown. Bar represents 5 µm.

d. Aggregation of the Arp2/3 complex (green) is detectable in the near vicinity of internalized streptococci (blue) 1 h post infection. Inserts show enlarged sections of split channels of the indicated area from the merged image. Representative images of three independent experiments are shown. Bar represents 5 µm.

e. Use of specific inhibitors, which interfere with host cell signalling molecules involved in classical phagocytic uptake processes, blocks M1 GAS mediated uptake into EC (3 h post infection). Prior to infection with M1 GAS A527, HUVEC were pre-treated with edelfosine [20 µM], Bapta-AM [25 µM] and calphostin C [2 µM] to inhibit phospholipase C, intracellular calcium release and protein kinase C respectively. Internalization rates are expressed as % intracellular bacteria compared to a DMSO treated control with 100% internalization. The graph shows the mean values ± SD of one representative out of three independent experiments.

f. M1 GAS A527 (red) co-localizes with the Lamp-1 positive (green) late endosomal/lysosomal compartments 3 h post infection. The inserts show split channels for Lamp-1 and GAS of the indicated area in the merged image. Representative images of three independent experiments are shown. Bar represents 5 µm.

g. TEM analysis of ultra-thin sections of infected EC layers. A close association of streptococci with gold particles is detectable (white arrows), indicating fusion of the M1 GAS containing phagosome with BSA-gold loaded terminal lysosomes. Representative images of three independent experiments are shown. Bar represents 500 nm.

Fig. 3. The M1 knock out mutant is deficient for internalization into EC

a. Analysis of the ability of M1 wildtype (wt) and mutant strains to promote internalization into HUVEC. - no internalization, +/+++/++++++ rate of internalization.

b. Quantification of internalization rates of the M1 wt GAS strain 60-226 (A766), the M1 knock out mutant ΔM1 60-226 (A767) and the ΔM1 mutant complemented with *emm1* (compl. ΔM1 GAS) into HUVEC (4 h post infection). In contrast to the M1 wt strain, the ΔM1 knock out mutant is deficient for internalization into HUVEC. Complementation of ΔM1 GAS strain (compl. ΔM1 GAS) restores the invasive phenotype yielding uptake rates comparable to those of the wild type M1 GAS strain. The graph represents mean values ± SD of one representative out of three independent experiments.

c.-d. Fluorescence images of HUVEC infected with the ΔM1 knock out mutant (c) and the complemented ΔM1 knock out mutant (d) 4 h post infection. Intracellular bacteria are stained in red, extracellular bacteria are depicted in green-yellow. EC nuclei are shown in blue. Representative images of three independent experiments are shown. Bars indicate 20 µm.

e. Analysis of the invasion potential of *L. lactis* strains on HUVEC 3 h post infection. The parental *L. lactis* strain (-M1), lacking the M1 protein on its surface, is not internalized. In contrast, heterologous expression of M1 on the lactococcal surface converts *L. lactis* into an efficiently internalized strain (+M1).

Fig. 4. Viable M1 *S. pyogenes* escape from the lysosomal compartment

a. TEM analysis of ultra-thin sections of infected HUVEC layers shows streptococcal escape (arrow) from the phagolysosomal compartment into the cytoplasm of the host cell (4 h post infection). Representative images of two independent experiments are shown. Bar represents 1 µm.

b. Free M1 GAS A527 (white arrows) reside within the cytoplasm of the host cell (5 h post infection) and are localized in close proximity to the endoplasmic reticulum (black arrow). Representative images of two independent experiments are shown. Bar indicates 500 nm.

c.-d. Immunofluorescence analysis of the viability of M1 GAS. Viable intracellular M1 GAS (green) are detectable 5 h (c) as well as 10 h (d) post infection. EC nuclei also absorb the

green dye and, therefore, also appear green. Representative images of three independent experiments are shown. Bars represent 5µm.

e. Quantification of intracellular survival of M1 GAS A527 in HUVEC 2 h, 4 h, 6 h and 9 h post infection. The graph shows mean values ± SD of triplicates from one representative experiment.

Suppl. Fig. 1.

a. FESEM analysis of HUVEC cultivated on a transwell insert shows the formation of a tight confluent polarized EC layer after 5 days of cultivation. Bar represents 20 µm.

b. Immunofluorescence staining of the tight junction protein zona occludens (ZoI, green) of a polarized confluent HUVEC layer cultured on a cell culture transwell insert. EC nuclei are depicted in blue. Bar indicates 10 µm.

Suppl. Fig. 2

Quantification of uptake of M1 GAS strain A527 into HUVEC at different time points. Internalization rates were determined by enumerating intracellular (red) bacteria.

References

1 Cunningham MW: Pathogenesis of group a streptococcal infections. Clinical microbiology reviews 2000;13:470-511.

2 Carapetis JR, Steer AC, Mulholland EK, Weber M: The global burden of group a streptococcal diseases. The Lancet infectious diseases 2005;5:685-694.

3 Stevens DL: Invasive streptococcal infections. Journal of infection and chemotherapy : official journal of the Japan Society of Chemotherapy 2001;7:69-80.

4 LaPenta D, Rubens C, Chi E, Cleary PP: Group a streptococci efficiently invade human respiratory epithelial cells. Proceedings of the National Academy of Sciences of the United States of America 1994;91:12115-12119.

5 Jadoun J, Ozeri V, Burstein E, Skutelsky E, Hanski E, Sela S: Protein f1 is required for efficient entry of streptococcus pyogenes into epithelial cells. The Journal of infectious diseases 1998;178:147-158.

6 Rohde M, Muller E, Chhatwal GS, Talay SR: Host cell caveolae act as an entry-port for group a streptococci. Cellular microbiology 2003;5:323-342.

7 Medina E, Rohde M, Chhatwal GS: Intracellular survival of streptococcus pyogenes in polymorphonuclear cells results in increased bacterial virulence. Infection and immunity 2003;71:5376-5380.

8 Staali L, Morgelin M, Bjorck L, Tapper H: Streptococcus pyogenes expressing m and m-like surface proteins are phagocytosed but survive inside human neutrophils. Cellular microbiology 2003;5:253-265.

9 Thulin P, Johansson L, Low DE, Gan BS, Kotb M, McGeer A, Norrby-Teglund A: Viable group a streptococci in macrophages during acute soft tissue infection. PLoS medicine 2006;3:e53.

10 Hertzen E, Johansson L, Wallin R, Schmidt H, Kroll M, Rehn AP, Kotb M, Morgelin M, Norrby-Teglund A: M1 protein-dependent intracellular trafficking promotes persistence and replication of streptococcus pyogenes in macrophages. Journal of innate immunity 2010;2:534-545.

11 Osterlund A, Popa R, Nikkila T, Scheynius A, Engstrand L: Intracellular reservoir of streptococcus pyogenes in vivo: A possible explanation for recurrent pharyngotonsillitis. The Laryngoscope 1997;107:640-647.

12 Cue D, Dombek PE, Lam H, Cleary PP: Streptococcus pyogenes serotype m1 encodes multiple pathways for entry into human epithelial cells. Infection and immunity 1998;66:4593-4601.

13 Dombek PE, Cue D, Sedgewick J, Lam H, Ruschkowski S, Finlay BB, Cleary PP: High-frequency intracellular invasion of epithelial cells by serotype m1 group a streptococci: M1 protein-mediated invasion and cytoskeletal rearrangements. Molecular microbiology 1999;31:859-870.

14 Purushothaman SS, Wang B, Cleary PP: M1 protein triggers a phosphoinositide cascade for group a streptococcus invasion of epithelial cells. Infection and immunity 2003;71:5823-5830.

15 Wang B, Yurecko RS, Dedhar S, Cleary PP: Integrin-linked kinase is an essential link between integrins and uptake of bacterial pathogens by epithelial cells. Cellular microbiology 2006;8:257-266.

16 Wang B, Li S, Dedhar S, Cleary PP: Paxillin phosphorylation: Bifurcation point downstream of integrin-linked kinase (ilk) in streptococcal invasion. Cellular microbiology 2007;9:1519-1528.

17 Olsen RJ, Shelburne SA, Musser JM: Molecular mechanisms underlying group a streptococcal pathogenesis. Cellular microbiology 2009;11:1-12.

18 Johansson L, Thulin P, Low DE, Norrby-Teglund A: Getting under the skin: The immunopathogenesis of streptococcus pyogenes deep tissue infections. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America 2010;51:58-65.

19 Nerlich A, Rohde M, Talay SR, Genth H, Just I, Chhatwal GS: Invasion of endothelial cells by tissue-invasive m3 type group a streptococci requires src kinase and activation of rac1 by a phosphatidylinositol 3-kinase-independent mechanism. The Journal of biological chemistry 2009;284:20319-20328.

20 Amelung S, Nerlich A, Rohde M, Spellerberg B, Cole JN, Nizet V, Chhatwal GS, Talay SR: The fbab-type fibronectin-binding protein of streptococcus pyogenes promotes specific invasion into endothelial cells. Cellular microbiology 2011;13:1200-1211.

21 Ferretti JJ, McShan WM, Ajdic D, Savic DJ, Savic G, Lyon K, Primeaux C, Sezate S, Suvorov AN, Kenton S, Lai HS, Lin SP, Qian Y, Jia HG, Najar FZ, Ren Q, Zhu H, Song L, White J, Yuan X, Clifton SW, Roe BA, McLaughlin R: Complete genome sequence of an m1 strain of streptococcus pyogenes. Proceedings of the National Academy of Sciences of the United States of America 2001;98:4658-4663.
22 Zimmerlein B, Park HS, Li S, Podbielski A, Cleary PP: The m protein is dispensable for maturation of streptococcal cysteine protease speb. Infection and immunity 2005;73:859-864.
23 Cue D, Lam H, Cleary PP: Genetic dissection of the streptococcus pyogenes m1 protein: Regions involved in fibronectin binding and intracellular invasion. Microbial pathogenesis 2001;31:231-242.
24 Jeng A, Sakota V, Li Z, Datta V, Beall B, Nizet V: Molecular genetic analysis of a group a streptococcus operon encoding serum opacity factor and a novel fibronectin-binding protein, sfbx. Journal of bacteriology 2003;185:1208-1217.
25 Smeets EF, von Asmuth EJ, van der Linden CJ, Leeuwenberg JF, Buurman WA: A comparison of substrates for human umbilical vein endothelial cell culture. Biotechnic & histochemistry : official publication of the Biological Stain Commission 1992;67:241-250.
26 Molinari G, Talay SR, Valentin-Weigand P, Rohde M, Chhatwal GS: The fibronectin-binding protein of streptococcus pyogenes, sfbi, is involved in the internalization of group a streptococci by epithelial cells. Infection and immunity 1997;65:1357-1363.
27 von Kockritz-Blickwede M, Goldmann O, Thulin P, Heinemann K, Norrby-Teglund A, Rohde M, Medina E: Phagocytosis-independent antimicrobial activity of mast cells by means of extracellular trap formation. Blood 2008;111:3070-3080.
28 Flannagan RS, Jaumouille V, Grinstein S: The cell biology of phagocytosis. Annual review of pathology 2012;7:61-98.
29 Steinberg BE, Grinstein S: Pathogen destruction versus intracellular survival: The role of lipids as phagosomal fate determinants. The Journal of clinical investigation 2008;118:2002-2011.
30 Nunes P, Demaurex N: The role of calcium signaling in phagocytosis. Journal of leukocyte biology 2010;88:57-68.
31 Sarantis H, Grinstein S: Subversion of phagocytosis for pathogen survival. Cell host & microbe 2012;12:419-431.
32 Alonso A, Garcia-del Portillo F: Hijacking of eukaryotic functions by intracellular bacterial pathogens. International microbiology : the official journal of the Spanish Society for Microbiology 2004;7:181-191.
33 Oehmcke S, Shannon O, Morgelin M, Herwald H: Streptococcal m proteins and their role as virulence determinants. Clinica chimica acta; international journal of clinical chemistry 2010;411:1172-1180.
34 Ermert D, Weckel A, Agarwal V, Frick IM, Bjorck L, Blom AM: Binding of complement inhibitor c4b-binding protein to a highly virulent streptococcus pyogenes m1 strain is mediated by protein h and enhances adhesion to and invasion of endothelial cells. The Journal of biological chemistry 2013;288:32172-32183.
35 Shannon O, Hertzen E, Norrby-Teglund A, Morgelin M, Sjobring U, Bjorck L: Severe streptococcal infection is associated with m protein-induced platelet activation and thrombus formation. Molecular microbiology 2007;65:1147-1157.
36 Herwald H, Cramer H, Morgelin M, Russell W, Sollenberg U, Norrby-Teglund A, Flodgaard H, Lindbom L, Bjorck L: M protein, a classical bacterial virulence determinant, forms complexes with fibrinogen that induce vascular leakage. Cell 2004;116:367-379.
37 Meresse S, Steele-Mortimer O, Moreno E, Desjardins M, Finlay B, Gorvel JP: Controlling the maturation of pathogen-containing vacuoles: A matter of life and death. Nature cell biology 1999;1:E183-188.
38 Goldberg MB: Actin-based motility of intracellular microbial pathogens. Microbiology and molecular biology reviews : MMBR 2001;65:595-626, table of contents.
39 Roy CR, Tilney LG: The road less traveled: Transport of legionella to the endoplasmic reticulum. The Journal of cell biology 2002;158:415-419.

40 Portnoy DA, Auerbuch V, Glomski IJ: The cell biology of listeria monocytogenes infection: The intersection of bacterial pathogenesis and cell-mediated immunity. The Journal of cell biology 2002;158:409-414.
41 Staali L, Bauer S, Morgelin M, Bjorck L, Tapper H: Streptococcus pyogenes bacteria modulate membrane traffic in human neutrophils and selectively inhibit azurophilic granule fusion with phagosomes. Cellular microbiology 2006;8:690-703.