The M1 Protein of *Streptococcus pyogenes* Triggers an Innate Uptake Mechanism into Polarized Human Endothelial Cells

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Key Words

Streptococcus pyogenes · M1 protein · Endothelial cells · Phagocytosis

Abstract

Serotype M1 *Streptococcus pyogenes* is a major human pathogen associated with severe invasive diseases causing high morbidity and mortality. In a substantial number of cases, invasive disease develops in previously healthy individuals with no obvious port of entry. This has led to the hypothesis that the source of streptococci in these cases is a transient bacteraemia. This study focuses 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 EC, thereby triggering rapid, phagocytosis-like uptake of streptococci into polarized EC layers. Upon internalization, the M1 *S. pyogenes* serotype is incorporated into phagosomes which traffic via the endosomal/lysosomal pathway. However, some of the streptococci successfully evade this innate killing process and hereby mediate their escape into the cytoplasm of the host cell. The results of this study demonstrate that blood EC 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.

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Introduction

Streptococcus pyogenes [a 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-occurrence of severe invasive diseases due to *S. pyogenes* infections has been observed. Among the over 120 serotypes, various known 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 it in-

vades 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 for evading host immune responses and penicillin treatment, and it may thus promote transmission and dissemination from the primary site of infection. The in vivo relevance of an intracellular habitat in the persistence of *S. pyogenes* within the human host was demonstrated by Osterlund et al. [11]. The authors re-isolated viable *S. pyogenes* from biopsies of patients with tonsillitis.

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 prerequisite for the development of deep-tissue infections, however, is the transition from the pharyngeal epithelial surface into deeper, naturally sterile tissue. The pathogen has then 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 has led to the hypothesis that streptococcal spread is associated with a transient bacteraemia originating from the oropharynx [3, 18].

Classic phagocytic cells represent important barriers that cross the way of bacteria into deep tissue. Hertzén et al. [10] showed that serotype M1 *S. pyogenes* exploits phagocytic cells as an intracellular reservoir to ensure streptococcal survival. They demonstrated that the GAS survives and even replicates within macrophages, egresses out of the cells and re-infects new cells. Another potentially important cell type is the blood endothelial cells (EC) lining the lumen of the blood vessels. We previously identified a new invasion mechanism of serotype M3 *S. pyogenes* into EC [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, i.e. M1 *S. pyogenes*, with EC has not been shown before. In this study, we analyse the molecular interaction of serotype M1 *S. pyogenes* with primary human blood EC of the umbilical cord (HUVEC) in further detail. Here, we show that the M1 GAS is efficiently internalized by EC. We identify the M1 surface protein of the M1 GAS as the

target protein which triggers its phagocytosis-like uptake into EC. Intracellular streptococci traffic via the classic 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.

Materials 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 samples were obtained from the culture collection of the Department of Medical Microbiology of the Helmholtz Centre for Infection Research in Braunschweig, Germany. *S. pyogenes* SF370 (A302) is a genome-sequenced serotype M1 GAS which has been described elsewhere [21]. *S. pyogenes* serotype M1 strain 90–226 (A766) and its isogenic knock-out mutant lacking the M1 surface protein, i.e. strain Δ M1 90–226 (A767), were also described previously [22]. The M1 GAS mutant strains KTL3 GRAB-ko, KTL3 *prtS::spc*, KTL3 *slo::spc* and KTL3 *pJRS233- Δ sagA* were from the culture collection of the Department of Medical Microbiology of the Helmholtz Centre. Streptococcal strains were cultured in tryptic soy broth (TSB, BD Biosciences) with antibiotics where appropriate (kanamycin 150 μ g/ml, spectinomycin 80 μ g/ml) at 37°C and 5% CO₂ 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. *Escherichia coli* DH5 α was routinely grown in Luria Broth at 37°C with agitation. To obtain competent *E. coli* cells, bacteria were cultured in SOB medium at 37°C with agitation. *Lactococcus 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 μ g/ml erythromycin at 30°C without agitation.

Reagents

Phospholipase C gamma inhibitor edelfosine (ET-18-OCH₃) and the calcium chelator BAPTA-AM were purchased from Calbiochem. Calphostin C, inhibiting protein kinase C (PKC), 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* DH5 α , transformants were selected on Luria Broth agar plates containing 500 μ g/ml 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* Δ M1 90–226 (A767). Positive clones (complemented

Δ M1 GAS) were selected on TSBY agar plates in the presence of 2 μ g/ml erythromycin and verified by PCR. In order to assess surface expression of the M1 protein, streptococcal cultures were analysed by immunofluorescence staining using an M1-specific primary antibody and a labelled secondary antibody.

EC Culture and in vitro Transwell Infection Assay

Primary HUVEC were obtained from Lonza and were cultured in EGM-2 medium (PromoCell) according to the manufacturer's protocol at 37°C and 5% CO₂. For infection assays, cell culture inserts with a pore size of 3.0 or 0.4 μ m (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]. 3×10^4 HUVEC (passage 3–5) were seeded per transwell and grown at 37°C and 5% CO₂ 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 up to the exponential growth phase (OD₆₀₀ 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 Laboratories). HUVEC were infected with M1 GAS strains with MOI of 50 for different time intervals. After washing, cells were fixed with 4% paraformaldehyde in PBS. Infection assays using *L. lactis* were performed according to the protocol described above for streptococci, with the exception that the *L. lactis* strains were cultured in M17 medium supplemented with 5% glucose and 5 μ g/ml erythromycin at 30°C. The infection assays were carried out at 37°C and 5% CO₂ for 3 h. Internalization rates were determined by differential staining of intracellular and extracellular cocci as described below.

To determine whether host-cell signalling molecules of the classic phagocytic-uptake machinery were involved in streptococcal uptake, function interfering assays using specific chemicals were conducted. HUVEC were pre-treated with 20 μ M edelfosine, 2 μ M calphostin C or 25 μ M BAPTA-AM for 30 min to inhibit phospholipase C gamma, PKC 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 the M1 GAS strain A527 for 3 h according to the standard infection protocol in the presence of 100 or 200 ng/ml purified mouse monoclonal control IgG (R&D Systems).

Survival Assay

HUVEC were seeded on cell-culture transwell inserts and infected with the 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 μ g/ml penicillin and 100 μ g/ml gentamicin for defined time periods. After 2, 4, 6 and 9 h, HUVEC layers were

washed at least 5 times with EBM-2 medium and then incubated with 100 μ l 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 μ l 1% saponin in PBS for 20 min at 37°C. Serial dilutions of cell suspensions were prepared in EBM-2 medium. The undiluted cell suspension or the dilutions (25 μ l) were spotted on Todd-Hewitt Broth (BD Biosciences) plates and incubated at 37°C and 5% CO₂ for up to 48 h. Following incubation, bacterial colonies were counted and the colony-forming units (cfu) per millilitre were calculated. The experiment was performed in triplicate and was repeated at least 3 times on different days.

Immunofluorescence

Following fixation, samples were washed with PBS, blocked with PBS containing 10% FCS (PAA Laboratories) for 30 min and subsequently stained for intracellular 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 labels, intracellular bacteria appeared red and extracellular streptococci appeared green-to-yellow. Staining of intracellular 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 the 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 was subsequently stained with a goat anti-mouse ALEXA Fluor A488 secondary antibody (Invitrogen, 1:200 dilution). In order to visualise the β_1 -integrin subunit on HUVEC, unpermeabilized cells were incubated with 5 μ g per well of an anti- β_1 -integrin antibody (Chemicon International) for 1 h at room temperature followed by a goat anti-mouse ALEXA Fluor 488-coupled secondary antibody. For visualisation 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). The polarization and confluency of HUVEC layers were tested by visualisation of the zona occludens-1 protein (ZO-1). 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 ZO-1 was stained using an anti-ZO-1 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 Pro-Long® 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 of 3 independent experiments were performed and the 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 3 times on different days. Results represent mean values (±SD) of one representative experiment.

Field Emission Scanning Electron Microscopy

For analysis by field-emission scanning electron microscopy, 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 CaCl₂, 0.01 M MgCl₂, 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 and 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 CO₂ (CPD30, BalTec; CPD300, Leica), samples were coated with a thin gold-palladium film (SCD 500, BalTec) and examined under 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

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 h prior to infection as described [6]. On the following day, cells were infected with streptococci (for 4, 5, 16 or 18 h) as described above, and then washed and fixed with 2% glutaraldehyde and 5% formaldehyde in 0.1 M cacodylate buffer for 1 h on ice. For analysis by transmission electron microscopy (TEM), 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, 1,024 × 1,024) with ITEM-Software (Olympus Soft Imaging Solutions). Contrast and brightness of images were adjusted with Adobe Photoshop CS3 Extended.

In order to visualise 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 were analysed using Microsoft Office Excel 2010 software. Statistical comparison was performed using the Student paired t test. Results were expressed as mean values ± SD of one representative experiment, with p values ≤0.05 considered significant.

Results

Serotype M1 S. pyogenes Isolates Trigger Uptake into Polarized Human EC

Since a direct interaction of the most invasive serotype M1 *S. pyogenes* with EC of the vascular system has not been studied before, we aimed to assess the invasion potential of different M1 GAS strains on primary HUVEC. In order to assess the invasiveness of M1 GAS on EC, we established a complex in vitro transwell infection model. In this model, blood EC 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. Confluence and polarization of the cell layer were determined by electron microscopic analysis (online suppl. fig. 1; for all online suppl. material, see www.karger.com/doi/10.1159/000358085), staining of the ZO-1 (online suppl. fig. 1) 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 4 different clinical strains of the serotype M1 GAS (A527, A302, A270, A271) isolated from patients with invasive diseases. Immunofluorescence staining of infected HUVEC layers show that all 4 strains tested are successfully internalized into EC. Approximately 80% of bacteria were found to be intracellular and localized within the perinuclear region of the cell. Extracellular bac-

Fig. 1. Serotype M1 *S. pyogenes* clinical isolates trigger uptake into polarized EC. The figure shows immunofluorescence analysis of the invasion potential of 4 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 extracellular (green-yellow) and intracellular (red) streptococci. The actin cytoskeleton stained with phalloidin is depicted in green. Representative images of 3 independent experiments are shown. a–d Bars: 5 μ m. e Quantification of internalization rates of different M1 GAS strains on HUVEC 4 h after infection. The graph represents mean values $\pm$ SD from 1 of 3 independent experiments.

terea were found to be localized in the intercellular 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 after infection. Strain A271 exhibited lower infection rates than the other strains (fig. 1e). Since all the 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 the M1 GAS by EC. Furthermore, the confluence status as well as polarization of the EC layer proved to be essential for the efficient internalization of the M1 GAS into HUVEC since HUVEC grown on coverslips showed no internalization of M1 GAS strains (data not shown). Thus, we established a suitable infection system which was subsequently applied to study the interaction of the M1 GAS on HUVEC in further detail.

lular (green-yellow) and intracellular (red) streptococci. The actin cytoskeleton stained with phalloidin is depicted in green. Representative images of 3 independent experiments are shown. a–d Bars: 5 μ m. e Quantification of internalization rates of different M1 GAS strains on HUVEC 4 h after infection. The graph represents mean values $\pm$ SD from 1 of 3 independent experiments.

Internalization of M1 S. pyogenes is Characterized by Actin Rearrangements and Trafficking of Internalized Streptococci along the Classic Endocytic Pathway with Lysosomal Destination

To further characterize the uptake mechanism of the M1 GAS by HUVEC, we analysed the mode of internalization and intracellular fate of the M1 GAS within these cells. Internalization of the M1 GAS into HUVEC started within the first hour of infection and increasing numbers of intracellular bacteria were detected 3, 4 and 5 h after infection (online suppl. fig. 2). Following attachment of streptococci to the host cell, the M1 GAS triggered the formation of membrane protrusions that tightly engulfed the bacterial chain (fig. 2a). Finally, streptococci were completely surrounded by the host cell membrane and incorporated into EC (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 engage-

Fig. 2. M1 *S. pyogenes* induces actin cytoskeleton rearrangements and traffics along the endocytic pathway. **a** Field-emission scanning electron microscopy image showing uptake of streptococci (GAS A527) into EC 3 h after infection. Upon infection, EC form membrane protrusions that tightly engulf the streptococcal chain, representing a zipper-like uptake process. Bar: 600 nm. Representative images of 3 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 after infection) in the 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. Representa-

tive images of 3 independent experiments are shown. Bar: 5 μ m. **c** Involvement of F-actin in the internalization process of M1 GAS A527. Internalized streptococci (red) co-localize with F-actin (green) 1 h after infection. Inserts show enlarged sections of split channels of the indicated area from the merged image. Representative images of 3 independent experiments are shown. Bar: 5 μ m. **d** Aggregation of the Arp2/3 complex (green) is detectable in the near vicinity of internalized streptococci (blue) 1 h after infection. Inserts show enlarged sections of split channels of the indicated area from the merged image. Representative images of 3 independent experiments are shown. Bar: 5 μ m.

ment of a cellular receptor and subsequent activation of specific intracellular signalling cascades which lead to actin remodelling processes. Internalization of the M1 GAS into HUVEC was accompanied by clustering of the β_1 -integrin subunit on the host cell surface in the vicinity of invading bacteria (fig. 2b). In contrast, β_1 -integrins were statistically distributed in areas where no streptococcal invasion occurred (fig. 2b). Receptor engagement and subsequent activation of downstream signalling events triggered F-actin accumulation around internalized streptococci (fig. 2c). Moreover, during the internalization of the M1 GAS into HUVEC, an aggregation of the actin-related protein complex 2/3 (Arp2/3) was detectable around invading streptococci which coincided with actin recruitment (fig. 2d). By function-interfering experiments, we identified the host-cell signalling molecules phospholipase C gamma, PKC and intracellular calcium as key molecules involved in the internalization process. Blocking either of these significantly reduced or completely abolished streptococcal inter-

nalization (fig. 2e). To assess the subsequent trafficking route of the 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 showed that the M1 GAS co-localized with Lamp-1-positive compartments 3 h after 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 the 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 the M1 GAS and the samples were processed for TEM. TEM analysis of ultra-thin sections of infected HUVEC layers revealed co-localization of the 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.

Fig. 2. M1 *S. pyogenes* induces actin cytoskeleton rearrangements and traffics along the endocytic pathway. **e** Use of specific inhibitors, which interfere with host-cell signalling molecules involved in classic phagocytic uptake processes, blocks M1 GAS-mediated uptake into EC (3 h after infection). Prior to infection with M1 GAS A527, HUVEC were pre-treated with edelfosine (20 μ M), BAPTA-AM (25 μ M) and calphostin C (2 μ M) to inhibit phospholipase C, intracellular calcium release and PKC, respectively. Internalization rates are expressed as % intracellular bacteria compared to a DMSO-treated control with 100% internalization. The graph shows the mean values $\pm$ SD of 1 representative of 3 independent experiments. **f** M1 GAS A527 (red) co-localizes with the Lamp-1-positive (green) late endosomal/lysosomal compartments 3 h after infection. The inserts show split channels for Lamp-1 and GAS of the indicated area in the merged image. Representative images of 3 independent experiments are shown. Bar: 5 μ m. **g** TEM analysis of ultra-thin sections of infected EC layers. A close association of streptococci with gold particles is detectable (arrows), indicating fusion of the M1 GAS-containing phagosome with BSA-gold-loaded terminal lysosomes. Representative images of 3 independent experiments are shown. Bar: 500 nm.

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

The M1 Surface Protein of the M1 GAS is the Invasin which Mediates Uptake into EC

Next, we identified the bacterial factor that mediates entry of the M1 GAS into EC. Unlike the M3 GAS serotype, which expresses the EC invasins FbaB [20], the 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 (Δ M1 GAS), is not internalized by EC (fig. 3b, c) whereas the M1 wild-type (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 M1 wt 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, demonstrated that *L. lactis* is not internalized by EC (fig. 3e, right bar, labelled -M1). In contrast, hetero-

Fig. 3. The M1 knock-out mutant is deficient for internalization into EC. **a** Analysis of the ability of the M1 wt and mutant strains to promote internalization into HUVEC. The rate of internalization is indicated by + or +++ or ++++++ and no internalization is indicated by -. **b** Quantification of internalization rates of the M1 wt GAS strain 90-226 (A766), the M1 knock-out mutant Δ M1 90-226 (A767) and the Δ M1 mutant complemented with *emm1* (Compl. Δ M1 GAS) into HUVEC (4 h after infection). In contrast to the M1 wt strain, the Δ M1 knock-out mutant is deficient for internalization into HUVEC. Complementation of Δ M1 GAS strain (Compl. Δ M1 GAS) restores the invasive phenotype, yielding uptake rates comparable to those of the M1 wt GAS strain. The

graph represents mean values $\pm$ SD of 1 representative of 3 independent experiments. **c, d** Fluorescence images of HUVEC infected with the Δ M1 knock-out mutant (**c**) and the complemented Δ M1 knock-out mutant (**d**) 4 h after infection. Intracellular bacteria are stained in red, extracellular bacteria are depicted in green-yellow and EC nuclei are shown in blue. Representative images of 3 independent experiments are shown. Bars: 20 μ m. **e** Analysis of the invasion potential of *L. lactis* strains on HUVEC 3 h after 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).

ologous 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 the uptake of bacteria into EC.

The Viable M1 GAS Escapes from the Phagolysosomal Compartments

Incorporation of foreign material, e.g. pathogens, into phagosomes and subsequent phagolysosomal fusion lead to the degradation of foreign material by hydrolytic enzymes and to their elimination [28]. However, here we show that the M1 GAS serotype has developed strategies to counteract the innate killing mechanisms of the host cell and to ensure streptococcal survival. TEM analysis of ultra-thin sections of infected HUVEC layers revealed streptococcal escape from the membrane-bound compartment of the phagolysosomes into the cytoplasm of the host cell (fig. 4a). Ferrocyanide and osmium tetroxide staining confirmed the absence of any cellular membranes around intracellular streptococci (fig. 4b, white arrows) which were localized in close proximity to the endoplasmic reticulum (fig. 4b, black arrow). This gives further evidence of the cytoplasmic localization of the M1 GAS and, thus, streptococcal escape from the phagolysosome. Viability staining of the M1 GAS demonstrated live intracellular bacteria after 5 h of infection (fig. 4c). Ten hours after infection, the majority of intracellular streptococci were still viable (fig. 4d). Quantification of survival rates by plating of HUVEC lysates following infection with the M1 GAS also confirmed streptococcal survival, yielding 2×10^4 cfu/ml 2 h after infection, 1.5×10^4 cfu/ml 4 h after infection, 1×10^4 cfu/ml 6 h after infection and 5×10^3 cfu/ml after 9 h of infection (fig. 4e). Furthermore, TEM analysis of infected EC layers revealed morphologically intact streptococcal cells as well as ongoing streptococcal replication within the cytoplasm (fig. 4a); this supports the viability of intracellular streptococci. Taken together, these data demonstrate intracellular streptococcal survival within EC. 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 phagocytic and non-phagocytic cells [4–6, 8, 10, 12, 19, 26]. In this study, we provide evidence that serotype M1 streptococci efficiently invade human blood EC and that the M1 protein is a key molecule in this process. For this, we established a complex in vitro infection assay mimicking the EC 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 EC barrier (online suppl. fig. 1) which proved to be crucial for the efficient uptake of M1 streptococci into EC. M1-expressing *S. pyogenes* triggers an innate phagocytosis-like uptake into EC (fig. 1), accompanied by the formation of membrane protrusions that tightly engulf the streptococcal chain resulting in the 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 (1) the accumulation of F-actin in the vicinity of internalized bacteria, (2) coincident aggregation of the Arp2/3 complex around internalized streptococci and (3) requirement of phospholipase C, intracellular calcium and PKC (fig. 2c–e). In contrast to this, invasion of M1 *S. pyogenes* into epithelial cells does not require PKC [14]. This demonstrates that distinct signalling cascades are initiated during the internalization processes of the M1 GAS into epithelial cells and EC, although both are mediated by the M1 protein via the 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]). Our gain-of-function experiments in *L. lactis* as well as our loss-of-function experiments in the M1 GAS identify the M1 protein as a key factor which mediates the uptake of the GAS into EC (fig. 3), yet demonstrate another role of this major virulence factor of the GAS. Other studies have identified the M1 protein as a crucial factor that mediates survival within neutrophils and macrophages [8, 10], and this opens up a perspective to speculate on whether it may also mediate survival within EC.

Another important GAS invasin for EC is the fibronectin-binding protein FbaB of M3 *S. pyogenes*, an invasin with unique EC tropism [20]. Although the invasion factors of M3 and M1 *S. pyogenes* are distinct, both trigger similar uptake processes with phago-lysosomal destina-

Fig. 4. Viable M1 *S. pyogenes* escapes 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 after infection). Representative images of 2 independent experiments are shown. Bar: 1 μ m. **b** Free M1 GAS A527 (white arrows) reside within the cytoplasm of the host cell (5 h after infection) and are localized in close proximity to the endoplasmic reticulum (black arrow). Representative images of 2 independent experiments are shown. Bar: 500 nm. **c, d** Immunofluorescence analysis of the viability of M1 GAS. Viable intracellular M1 GAS (green) is detectable 5 h (**c**) and 10 h (**d**) after infection. EC nuclei also absorb the green dye and, therefore, also appear green. Representative images of 3 independent experiments are shown. Bars: 5 μ m. **e** Quantification of intracellular survival of M1 GAS A527 in HUVEC 2, 4, 6 and 9 h after infection. The graph shows mean values $\pm$ SD of triplicates from 1 representative experiment.

tion (fig. 2) [19, 20]. However, there are also other important factors that may have a strong impact on the EC invasion by GAS. The very recent study by Ermer et al. [34] identified protein H as an enhancing factor for M1 *S. pyogenes* EC 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. This highlights the potential effect

of plasma proteins on EC interaction. Plasma proteins that have receptors on the luminal side of EC 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 the 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 the 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 also forms 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 leads to phagocytosis and terminate in the killing of the pathogen and the receptor-mediated uptake governed by invasins and host factors. Host cells have developed strategies to ensure the elimination of invading bacteria, one of which is the delivery into terminal lysosomes for subsequent degradation and killing [28, 31]. In response to this, mechanisms have evolved in bacterial pathogens that avoid killing and facilitate intracellular survival within host cells [37–40]. Staali et al. [41] showed that M1-expressing *S. pyogenes* inhibits fusion of phagosomes with azurophilic granules within human neutrophils. In macrophages, the M1 GAS prevents fusion of the phagosome with late endosomal compartments or lysosomes [10]. Here, we provide evidence that M1 GAS-containing phagosomes in EC acquire late endosomal lysosomal markers and fuse with terminal lysosomes

(fig. 2f, g). However, our data also reveal that a proportion of the 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 demonstrate that the intracellular M1 GAS residing within the cytoplasm of the host cell appears morphologically intact and shows features of replication (fig. 4a–d), implying that it is able to escape from a membrane-surrounded compartment like *Listeria* or *Shigella*.

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

Acknowledgements

The authors thank I. Schleicher for excellent technical assistance with the electron microscopic studies, Prof. Dr. P.P. Cleary for providing the M1 wt GAS strain 90–226 (A766), the M1 knock-out mutant strain 60–226 ΔM1 (A767) and the *L. lactis* strains pP59 and pLM1 and Dr. C. Erck for providing the anti-Arp2/3 antibody. Dr. Ochel greatly acknowledges the President's Initiative and Networking Fund of the Helmholtz Association of German Research Centers (No. VH-GS-202) for supporting this project.

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