M1 Protein-Dependent Intracellular Trafficking Promotes Persistence and Replication of *Streptococcus pyogenes* in Macrophages

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Key Words

Streptococcus pyogenes · Intracellular persistence · Macrophage · M protein · Host-pathogen interaction

Abstract

Streptococcus pyogenes is an important human pathogen that causes a variety of diseases including life-threatening invasive diseases, such as toxic shock and deep tissue infections. Although *S. pyogenes* are classically considered extracellular pathogens, a clinical significance of an intracellular source has been emphasized. In patients with deep tissue infections, an intracellular reservoir of *S. pyogenes* within macrophages was shown to contribute to prolonged bacterial persistence. Here we demonstrate that intracellular survival of *S. pyogenes* in macrophages is associated with an M1 protein-dependent intracellular trafficking in the phagosomal-lysosomal pathway, which results in impaired fusion with lysosomes. The phagocytic vacuoles harbouring M1 protein-expressing bacteria not only served as a safe haven for the bacteria, but also as a replicating niche. An M1 protein-dependent modulation of macrophages was further supported by differences in NF- κ B signalling between cells infected with either the wild-type or M1 protein-deficient strains, thereby indicating a suppressed inflammatory re-

sponse when M1 protein was involved. Evidence of egress of bacteria out of their host cell and subsequent re-infection of new cells emphasize the importance of intracellular bacteria as a reservoir for dissemination of infection and continued tissue injury.

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Introduction

Streptococcus pyogenes are important human pathogens, listed number 9 on the list of 'global killer pathogens' with an estimated 500,000 deaths yearly [1]. It causes a variety of diseases ranging from mild uncomplicated skin and throat infections to severe invasive diseases and post-streptococcal sequelae. Two of the most severe invasive manifestations include streptococcal toxic shock syndrome and necrotizing fasciitis [1], which are hyper-inflammatory rapidly progressing conditions associated with high mortality and morbidity despite adequate antibiotic therapy in most cases. Superantigens and the M1 protein are recognized as central triggers of hyper-inflammatory responses and vascular leakage, which consequently results in the systemic toxicity associated with these infections [2–4]. In addition, *S. pyogenes* is equipped

with an impressive array of virulence factors that promote colonization and persistence through various immunomodulatory activities ranging from proteolytic cleavage of immune effector proteins to avoidance of phagocytic killing [reviewed in 5].

There have been reports that *S. pyogenes*, which is classically known as an extracellular pathogen, may invade and reside intracellularly in host cells. Intracellular *Streptococci* have been demonstrated in vitro in epithelial cells [6, 7] and in neutrophils [8, 9]. Furthermore, an intracellular reservoir of *S. pyogenes*, residing predominantly within macrophages, was demonstrated in patients with acute deep tissue infections in patients [10]. The fact that many of these patients had persistent massive bacterial load despite intravenous antibiotic therapy for a prolonged time underscored a major clinical problem with antibiotic eradication failure at the tissue site of infection.

Normally bacteria are killed by the conventional phagosomal-lysosomal degradation pathway following phagocytosis. However, intracellular pathogens have acquired specific mechanisms to avoid this default pathway and promote their survival in phagocytic cells [11, 12]. The 4 major evasion strategies involve (1) avoidance of fusion between endocytic vacuole and lysosomes, (2) modulation of phagosome biogenesis, (3) adaptation to the acidic environment in the phagolysosome and (4) escape from the phagolysosome into the cytoplasm. In macrophages, this may be achieved by bacteria-induced modulation of macrophages into the anti-inflammatory M2 phenotype, in contrast to the pro-inflammatory M1 phenotype usually associated with infection [13]. The two phenotypes may be characterized by their cytokine/chemokine receptor profiles, and their polarization was recently shown to be orchestrated by NF- κ B signalling [14]. In *S. pyogenes* infections, various intracellular survival strategies have been proposed including caveolae-mediated uptake in epithelial and endothelial cells [7], streptococcal M1 protein-mediated inhibition of granule fusion with the phagosomes in neutrophils [9] and streptolysin O-mediated avoidance of lysosomal degradation in epithelial cells [15].

In this study, we have explored the intracellular survival strategies of *S. pyogenes* in macrophages, as this is the principal reservoir during acute tissue infections [10]. Our findings demonstrate that intracellular survival is associated with an M1 protein-dependent intracellular trafficking in the phagosomal-lysosomal pathway, which results in impaired fusion with lysosomal vesicles. The phagocytic vacuoles harbouring M1 protein-expressing bacteria not only served as a safe haven for the bacteria,

but also as a replicating niche. Further studies emphasized the importance of this intracellular bacterial source as a reservoir for dissemination of infection and continued tissue injury.

Material and Methods

Ethics Statement

The Human Subjects Review Committee of Karolinska Institutet approved the studies using blood obtained from healthy volunteers. All blood donors were anonymous and therefore written informed consent was not required.

Primary Human Cell Preparation and Culture Conditions

Human monocyte-derived macrophages (hMDMs) were prepared essentially as previously described [10]. Briefly, for confocal and electron microscopy assays, peripheral blood mononuclear cells were isolated from whole blood of healthy donors through density gradient centrifugation using Lymphoprep (Axis-Shield). Monocytes were enriched through plastic adherence for 2 h in serum-free media in 12- or 24-well plates (Corning) with or without coverslips and allowed to differentiate into macrophages in RPMI 1640 supplemented with 10% FCS for 7 days. For flow cytometry, cytotoxicity and Western blot assays, monocytes were isolated using Rosette Sep (Stemcell Technologies) separation followed by a Lymphoprep (Axis-Shield) centrifugation step. Monocytes were then seeded in 6-well low-adherence plates (Corning) in RPMI containing 10% FCS and 50 ng/ml macrophage colony-stimulating factor (Immunotools), and cultured for 5–7 days to obtain hMDMs. Maturation of the cells was assessed morphologically using light microscopy and by flow cytometry studying MHC II, CD68 and CD14 markers.

Bacterial Strains

A clinical MIT1 isolate of *S. pyogenes* (strain 5448) [16] and its mutant lacking the M1 protein (5448 Δ M1) were grown at 37°C to stationary phase in Todd-Hewitt broth supplemented with 1.5% yeast extract and diluted to the required inoculum, 10–40 colony-forming units (CFU)/cell depending on experimental assay. A green fluorescent protein (GFP) expressing 5448 strain was created by use of the plasmid pGFP1 [17]. Streptococcal transformants were generated by electroporation essentially as described previously [18], and selected on blood agar plates containing erythromycin (100 μ g/ml). GFP-expressing 5448 strains were cultured as above using Todd-Hewitt supplemented with 150 μ g/ml erythromycin. Erythromycin-supplemented cell culture media was always used in infections with GFP-expressing strains. *Escherichia coli* DH5a containing the plasmid pGFP1 was grown at 37°C in Luria broth containing 85 μ g/ml chloramphenicol.

In vitro Infection Assay

S. pyogenes strains were co-cultured with hMDMs at 37°C and 5% CO₂ for different time points. A multiplicity of infection (MOI) of 20–30 CFU/cell was used for early time points 5–60 min, whereas an MOI of 10 CFU/cell was used for later time points 2–12 h, unless otherwise specified. At 2 h after infections, extracellular and adherent bacteria were killed by addition of 125 μ g/ml

gentamicin and 2.5 µg/ml penicillin G for 1 h, followed by washing in PBS twice, and addition of RPMI 1640 supplemented with 1 µg/ml penicillin G throughout the rest of the experiment. Egress of bacteria out of cells was assessed in culture media where the antibiotic had been removed after the initial killing of extracellular and adherent bacteria. CFU in cell culture media were enumerated through plating on blood agar plates at different time points post antibiotic removal.

Immunofluorescent Staining

hMDMs on coverslips were fixed in 1% freshly prepared paraformaldehyde for 30 min. Intracellular staining was done essentially as previously described [10]. Briefly, cells were blocked by 2% FCS, followed by avidin and biotin blocking (Vector Laboratories). Primary antibodies included polyclonal anti-serum against Lancefield group A carbohydrate (Difco), anti-EEA-1 (N-19) and anti-LAMP-1 (H5G11) (both from Santa Cruz Biotechnology). Further blocking was achieved by 1% normal goat serum or 1% normal rabbit serum depending on the species of the secondary antibody. This was followed by incubation with a secondary biotinylated antibody, whereafter a streptavidin-conjugated fluorophor (Alexa Fluor 594 or Alexa Fluor 488; Molecular Probes) was added. All dilutions and washing steps were done in 0.1% PBS-saponin. The coverslips were mounted with ProLong Gold mounting media with DAPI (Molecular Probes). Analysis was done using a Leica confocal scanner TCS SP II coupled to a Leica DMR microscope. For assessment of LysoTracker Red DND-99 (Molecular Probes), cells were incubated with 2.5 µM of the solution together with GFP-expressing bacteria for 2 h, after which the cells were washed and visualized using a Delta Vision spectris, wide-field microscope (Applied Precision) after 2–5 h of infection. Viability of intracellular bacteria was studied using the BacLight bacterial viability kit (Molecular Probes) as described by the manufacturer and analysed by a Leica DMR immunofluorescent microscope. Actin filaments were visualized by incubating the cells with Alexa Fluor 555 phalloidin (Molecular Probes) as described by the manufacturer and analysed in a Delta Vision spectris, wide-field microscope (Applied Precision).

Electron Microscopy

hMDMs were fixed in 2.5% glutaraldehyde in 0.15 M sodium cacodylate pH 7.4 over night at room temperature. They were subsequently washed with 0.15 M sodium cacodylate and post-fixed with 1% osmium tetroxide for 2 h at 4°C. This was followed by dehydration through a graded series of ethanol and embedding in Epon 812. The samples were sectioned using a LKB ultramicrotome and collected on grids followed by staining with uranyl acetate and lead citrate. Sections were analysed using a JEOL JEM 1230 electron microscope operated at 80 kV accelerating voltage, and images were recorded with a Gatan Multiscan 791 CCD camera.

Flow Cytometry

1×10^5 hMDMs were pre-incubated with or without Cytochalasin D (Sigma) 10 µg/ml for 30 min following addition of GFP expressing *S. pyogenes* at an MOI of 5 CFU/cell for 2 h. Then cells were fixed, processed with FACSCalibur (Becton Dickinson) and analysed with FlowJo software (TreeStar Inc.).

Western Blot Assay

1×10^6 hMDMs were infected with 5448 or 5448ΔM1 at an MOI of 5 CFU/cell or stimulated with LPS 100 ng/ml (Sigma) for 10 min. Cells were lysed in Laemmli sample buffer (Bio-Rad) and MilliQ water 1:1. Samples were resolved on 10–20% gradient Tris-glycine gels (Invitrogen) and then transferred to a polyvinylidene fluoride membrane (Invitrogen). Membranes were blocked with 5% BSA (Sigma) and probed with the following antibodies: anti-phospho-IκBα, anti-phospho-p65 (Cell Signalling Technologies) and anti-β-actin (Sigma). Secondary antibodies included peroxidase-conjugated anti-mouse and peroxidase-conjugated anti-rabbit (Jackson ImmunoResearch). The proteins were visualized by Extended Duration Substrate kit (ThermoScientific) and a Fujifilm LAS-4000 Mini instrument. Optical density was assessed by Image J software (NIH).

Real-Time PCR

hMDMs were infected in duplicate wells from 6 separate donors with either strains 5448 or 5448ΔM1, or mock-infected for 1 h, after which RNA was isolated using the RiboPure RNA Extraction Kit (Ambion). Concentration was measured using NanoDrop® spectrophotometer (NanoDrop Technologies). RNA was subsequently converted to cDNA with High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Fifty nanograms of reverse-transcribed RNA was used for qPCR analysis using primer-probe (FAM-MGB) gene expression assays from Applied Biosystems [18S (4352930), TNFα (Hs00174128_m1)]. qPCR reaction was performed in the 7500 Fast system using 2× Gene Expression Master Mix (both from Applied Biosystems) and in accordance with provided protocols. Results from well duplicates were pooled and analysed using the ΔΔCt method with mock-infected samples as calibrator and 18S as endogenous control in MS Excel. Statistical analysis (paired t test) was performed in Graphpad Prism (Graphpad Software Inc.).

Lactate Dehydrogenase Assay

One day prior to the assay, hMDMs were harvested from 6-well plates and 2.5×10^5 cells were seeded per well in a 48-well plate in cell culture media lacking phenol-red, antibiotics and containing 2% FCS. At the day of infection, bacteria were added to the plates at an MOI of 5 CFU/cell. After 2 h of infection, 1 µg/ml penicillin G was added to kill off extracellular bacteria. Cell culture supernatants were taken at 6, 9 and 12 h after infection. The amount of lactate dehydrogenase (LDH) in the cell culture media was measured using the CytoTox 96 cytotoxicity assay (Promega) according to the manufacturer's instructions. Briefly, 50 µl of supernatant was mixed with 50 µl of substrate reagent and incubated for 30 min at room temperature. Absorbance was measured at 490 nm. Cytotoxicity was calculated as: [(sample LDH release – spontaneous LDH release)/(maximum LDH release – spontaneous LDH release)] × 100. The spontaneous release equals the amount of LDH released by uninfected cells and the maximum released is the amount of LDH released by uninfected cells after incubation with 0.1% Triton X-100.

Re-Infection Assay

1×10^5 hMDMs were seeded per well in a 24-well plate. Cells were infected with GFP-expressing 5448 for 1 h with an MOI of 40 CFU/cell. Extracellular or adherent bacteria were killed off by antibiotics as described above, after which antibiotic-free media

was added. Cells stained with FarRed (Molecular Probes), according to the manufacturer's instructions, were added and co-incubated with the infected cells. At different time points, all cells were collected and the percentage of FarRed/GFP double-positive cells was measured by flow cytometry on FACSCalibur (Becton Dickinson) and analysed with FlowJo software (TreeStar Inc.). Microscopical analyses of the infected cells were performed to confirm intracellular localization of *S. pyogenes* in the FarRed-stained cells.

Statistical Evaluation

Data were analysed by GraphPad Prism version 4.0 for Windows (Graphpad Software). Comparisons between groups were performed using a Mann-Whitney test, Wilcoxon signed rank test or paired t test. Differences were considered significant when $p < 0.05$.

Results

S. pyogenes Uptake in Macrophages Is Actin Dependent and Involves Long Asymmetrical Pseudopod Loops

To gain insight into the uptake process, primary hMDMs were infected with a clinical MIT1 *S. pyogenes* isolate (5448) for defined time points, ranging from 5 min to several hours, and analysed at the ultrastructural level. Transmission electron microscopy analyses revealed bacterial uptake already after 5 min of infection. There were no apparent morphological differences with respect to membrane ruffles in infected or non-infected macrophages (fig. 1a–c). Ultrastructural data demonstrated spacious asymmetrical pseudopod loops surrounding the bacteria (fig. 1a) as well as bacteria tightly attached to the cell surface and encircled by membrane ruffle-like structures (fig. 1b). Taken together this resembles the process of triggered phagocytosis and coiling phagocytosis. In contrast, no evidence of involvement of phagocytic cups or caveolae invaginations was found at the *S. pyogenes* membrane attachment sites. Furthermore, the uptake process was not dependent on secreted bacterial factors, as evident by the identical uptake of paraformaldehyde-fixed bacteria (data not shown).

As both phagocytosis and macropinocytosis are actin-dependent processes, we assessed the effect of inhibiting actin-polymerization by pre-treatment of cells with cytochalasin D. This resulted in a significant reduction in *S. pyogenes* uptake as compared to untreated cells ($p = 0.0001$) (fig. 1d). This was also supported by immunofluorescent stainings of infected cells, which revealed actin surrounding the bacteria at the process of entry (fig. 1e). Although some bacteria may induce their uptake

by stimulating macropinocytosis, the long chains of *S. pyogenes* is likely a limitation for this uptake process. Furthermore, we did not observe any general stimulation of macropinocytic uptake of fluorescent dextran following *S. pyogenes* infection of macrophages (data not shown).

S. pyogenes Reside within Phagocytic Vacuoles, which Avoid Phagolysosomal Maturation

Transmission electron microscopy revealed that once inside the cells, *S. pyogenes* reside within spacious phagocytic vacuoles surrounded by an electron-dense membrane (fig. 2a). Throughout the experiments (up to 12 h), intracellular bacteria remained within these vacuoles and no free bacteria could be detected in the cytosol of the cells. Next we explored the intracellular trafficking by mapping bacterial localization within defined endosomal compartments. For this purpose, a GFP-expressing 5448 isolate was constructed and used in the infection model. Immunostaining of infected cells and analyses by confocal microscopy demonstrated that the bacteria transiently co-localized with the early endosomal marker EEA-1 up to 15 min after infection (fig. 2b), after which the association was lost (data not shown). In contrast, no association of bacteria and the late endosomes/lysosomes could be demonstrated using either the lysosome-associated membrane protein 1 (LAMP-1) or the acidotropic stain LysoTracker that targets lysosomes (fig. 2c, d and fig. 3f, respectively). To rule out that the lack of association was due to an inactivation of GFP expression resulting from the acidic environment, both phase-contrast images and, most importantly, the DAPI channels were carefully screened for coccal chains and assessed in relation to GFP positivity. The data of DAPI-stained coccal structures were fully congruent with the GFP-positive bacteria, thus, supporting a lack of co-localization between wild-type 5448 bacteria and the lysosomes. Fixed bacteria behaved in a similar manner and did not associate with lysosomal markers (data not shown).

Intracellular Survival of S. pyogenes in Macrophages Is Dependent on M1 Protein

Next we sought to identify bacterial factors that contribute to intracellular survival. Our principal candidate was the streptococcal M1 protein, as previous findings in neutrophils had implicated a role for this protein in impaired granule fusion [19]. For this purpose, a 5448 strain deficient in the M1 protein (5448 Δ M1) was used in the infection model. No evident morphological difference in the uptake process of the 5448 Δ M1 strain could be observed using electron-microscopic analysis of infected

Fig. 1. Uptake of *S. pyogenes* in macrophages involves long asymmetrical pseudopod loops. Transmission electron microscopy analysis of hMDMs infected with wild-type 5448 after 10 min (**a**) and 1 h (**b**), or uninfected cells (**c**). Higher magnifications of the boxed areas are shown in the lower panels. Representative images of 1 experiment out of 3 performed using different donors are shown. **d** Percentage of cells harbouring GFP-expressing wild-type 5448. Cells were pre-treated +/- 10 μ g/ml cytochalasin D for 30 min followed by infection with GFP-expressing 5448 for 4 h

and then analyzed by flow cytometry. The bars show mean $\pm$ SEM of 9 observations using cells from 3 different donors. Statistically significant differences were determined by the Mann-Whitney test. **e** Representative fluorescent overlay image of wild-type 5448 uptake in macrophages at 1 h after infection with actin filaments visualized by Alexa Fluor phalloidin 555 (red), 5448 stained for the Lancefield group A carbohydrate (green) and cell nuclei DAPI (blue). **Inset** Higher magnification of the boxed area with each staining/channel shown separately.

Fig. 2. Intracellular trafficking of *S. pyogenes* in macrophages. Transmission electron microscopy analyses revealed membrane-surrounded *S. pyogenes* in infected hMDMs at 1 h after infection (a). Co-localization of GFP-expressing 5448 (green) with endosomal/lysosomal markers (red, Alexa 594) was assessed by immunofluorescent stainings and confocal analyses. Representative images of infected cells stained for early endosomal antigen 1

(EEA-1) at 5 min after infection (b) or for lysosomal membrane-associated protein 1 (LAMP-1) at 1 h (c) and 4 h (d) after infection. The inset figure shows the magnification of the boxed area (b). Higher magnifications of the boxed area, alternatively phase contrast images, are shown in the lower panel. All images are representatives of 3 separate experiments.

cells (data not shown). Four hours after infection, the frequency of cells harbouring viable bacteria was assessed by a bacterial viability stain. On average, 20–30% of the cells harboured live bacteria following infection with the wild-type 5448 strain, whereas significantly lower frequencies of cells contained viable 5448ΔM1 bacteria ($p = 0.03$) (fig. 3a). Although the majority of intracellular 5448 wild-type bacteria were viable, it was also clear that a number of cells contained dead bacteria, likely resulting from an efficient phagocytic killing. We also performed experiments in which bacterial resurgence in the cell culture media was measured following removal of antibiotic after 4 h of infection. This demonstrated striking differences between the strains, as no viable bacteria were found in cell cultures infected with the mutant, as compared to 1×10^8 CFU for wild-type 5448 (fig. 3b). To determine whether this finding resulted from a more efficient intracellular killing of the mutant or alternatively that the mutant was unable to egress out of the cells, infected cells were visualized by transmission electron mi-

croscopy. At 6 h after infection, vesicle accumulation around, and fusion with, phagocytic vacuoles harbouring 5448ΔM1, but not 5448 wild-type bacteria, was observed (fig. 3c, d). Further support for a differential fusion with lysosomal compartments was obtained through live-cell imaging of cells co-incubated with the lysosomal-specific stain LysoTracker and GFP-expressing 5448 strains (fig. 3e, f; online supplementary fig. 1, www.karger.com/doi/10.1159/000317635). Cells were monitored for up to 5 h after infection, and whereas only a minute proportion of the 5448 wild-type bacteria were found to co-localize with LysoTracker, the opposite was true for 5448ΔM1 where bacterial vacuoles accumulated high amounts of this lysosomal-specific marker evident already 3 h after infection (online suppl. fig. 1).

M1 Protein-Dependent Macrophage Modulation Is Associated with a Skewed Inflammatory Response

We further investigated the inflammatory state of infected macrophages and found important differences be-

Fig. 3. Streptococcal M1 protein is crucial for intracellular bacterial survival. **a** Amount of macrophages harbouring viable bacteria after infection with *S. pyogenes* 5448 or 5448ΔM1. Cells were infected for 4 h, as described in Material and Methods, after which the cells were exposed to a bacterial viability stain and the frequency of cells containing viable bacteria was enumerated. The bars show mean $\pm$ SEM of a total of 5 experiments using cells from different donors. Statistically significant differences were determined by the Wilcoxon signed rank test. **b** Bacterial growth was measured in antibiotic-free cell culture media at different time points post infection of macrophages with *S. pyogenes* 5448 or 5448ΔM1 (for details see Material and Methods). Mean $\pm$ SD of triplicates from 1 experiment is shown. The experiment was repeated 3 times with reproducible results. Transmission electron micrographs of macrophages infected with *S. pyogenes* 5448 (**c**) or 5448ΔM1 (**d**) at 6 h after infection. Boxed areas are shown at higher magnification in the lower panel. Fusion of vesicles was evident in 5448ΔM1-infected cells as indicated by arrows. Live cell imaging of macrophages co-incubated for 5 h with the lysosomal-specific stain LysoTracker (red) during infection with GFP-5448 (green) (**e**) or GFP-5448ΔM1 (green) (**f**). The experiment was repeated 4 times with different donors (online suppl. fig. 1).

Fig. 4. M1 protein-dependent variation in NF- κ B signalling in infected macrophages. **a** Whole cell lysates from macrophages infected for 10 min with *S. pyogenes* 5448 or 5448 Δ M1. LPS-activated macrophages served as positive control. Proteins were transferred to polyvinylidene fluoride membranes that were stained with antibodies specific for p-I κ B α , p-p65 or β -actin. Cell extracts from 5 different donors were tested, and the image shows 1 representative donor. The lack of phosphorylation of I κ B α in

LPS-stimulated cells is due to a differential kinetics, inasmuch as a strong p-I κ B α was evident after 5 min of stimulation but not after 10 min. The bars show phosphorylation of p-65 (**b**) and I κ B α (**c**) in relation to β -actin in cells infected with either 5448 or 5448 Δ M1 strains, quantified using ImageJ software. Each symbol indicates a separate donor, 5 donors in total. Statistically significant differences were determined by the Wilcoxon signed rank test.

tween cells infected with 5448 wild type and 5448 Δ M1. First we analysed NF- κ B signalling responses in whole cell extracts of infected macrophages, which revealed a significantly higher level of phosphorylated p65 and phosphorylated I κ B α following infection with 5448 Δ M1, as compared to the wild-type strain ($p = 0.03$) (fig. 4). Based on the results recently published by Porta et al. [14], these data would translate into a suppressed inflammatory response in macrophages infected with M1 protein-expressing *S. pyogenes*, as compared to the mutant. This is supported by preliminary experiments assessing TNF α gene expression 1 h after infection, which demonstrated an almost 5-fold reduction in TNF α gene expression following infection with wild-type 5448 as compared to the M1-deficient mutant ($p = 0.03$; based on gene expression data obtained from 6 different donors). Taken together, the data imply that M1 protein-dependent macrophage modulation involves suppression of the inflammatory signals that activate macrophage defences required for killing of intracellular pathogens.

Intracellular Bacterial Replication, Egress and Re-Infection

Ultrastructural data revealed that once inside its phagocytic vacuole, a significant proportion of *S. pyogenes* remained intact and showed no signs of degradation even at 12 h after infection (fig. 5a). In fact, the images indicated that there is an ongoing intracellular

bacterial replication, as evident by cocci displaying the characteristic septum of dividing bacteria (fig. 5d). Further support for an intracellular replication was provided by the bacterial counts observed in cell cultures with antibiotic-free medium (fig. 3b), which were 10-fold higher than that obtained following growth of the same bacterial inoculum in culture medium without cells. The high extracellular bacterial counts also demonstrated the ability of the streptococci to leave their intracellular niche. This was further shown by electron micrographs that revealed bacteria egressing out of host cells already at 6 h, although the most impressive release occurred at 8–12 h after infection (fig. 5a). In contrast, no bacterial egress could be noted in cells infected with fixed wild-type 5448 or 5448 Δ M1 (fig. 5b, c).

Another striking observation was the dramatic morphological changes that could be observed in wild-type 5448-infected cells. After 8 h of infection, the vacuoles containing bacteria appeared looser and more spacious than shortly after uptake (fig. 5a and 2a, respectively), and at 12 h marked cell vacuolization and even cell burst was evident. Whether the cell burst is caused by the massive intracellular bacterial load or by enzymatic activity, such as that reported for streptolysin O [15, 20–22], remains to be addressed. However, the data clearly demonstrated that viable bacteria were required in order to induce cell burst, as cells cultured with paraformaldehyde-fixed 5448 showed an intact healthy morphology despite

a heavy intracellular bacterial load (fig. 5b). A similar unaffected cellular morphology was noted in cells infected with 5448ΔM1 (fig. 5c), which indicates that it is not the infection per se but rather specific host-bacterial interactions that affect this phenomenon. Cell death was also monitored in kinetic experiments by measuring release of the cytosolic enzyme LDH in the cell culture media after infection with wild-type 5448. In agreement with microscopy data, a time-dependent significant increase in LDH release was noted in infected, as compared to uninfected, cells (fig. 5e, f).

Finally, we addressed the clinically relevant question whether the egressed bacteria were capable of re-infecting new cells in their surrounding environment. For this purpose, FarRed-labelled macrophages were added to already infected cells and infection of new cells was evaluated by flow-cytometrical analyses assessing GFP/FarRed double positivity. The data demonstrated that FarRed-labelled cells became infected with GFP streptococci (fig. 5g), thus providing evidence that intracellular bacteria may re-infect new cells.

Discussion

In this study, we demonstrate that *S. pyogenes* is capable of modulating the human macrophage to promote bacterial persistence within phagocytic vacuoles. A major factor contributing to this event is the M1 protein, which was noted to contribute to impaired fusion with lysosomal vesicle. The bacteria not only persist, but also replicate intracellularly, which results in a significant

bacterial reservoir within the cell. The study demonstrates for the first time that *S. pyogenes* can exploit and remodel the macrophage to promote persistence and continued infection.

Defining the ultrastructure of the uptake process is an important step towards understanding entry and intracellular trafficking of pathogens. To this end, we employed transmission electron microscopy to visualize *S. pyogenes* uptake in macrophages, which revealed membrane ruffles and pseudopods encircling the bacteria, eventually resulting in bacterial spacious vacuoles. Since no evidence of phagocytic cup formation or caveolae involvement was found, the uptake is likely achieved either through a form of coiling phagocytosis or triggered phagocytosis. In addition, ultrastructural data revealed large asymmetrical pseudopod loops reaching out and encircling the bacteria, a profile highly similar to the looping phagocytosis previously only described for *Francisella tularensis* uptake into macrophages [23]. Thus, this study demonstrates that in macrophages, *S. pyogenes* exploit uptake processes distinct from the previously described phagocytic uptake of *S. pyogenes* in neutrophils [9] and zipper-like mechanisms or caveolae-mediated endocytosis in non-phagocytic cells [24–26]. In macrophages, bacterial uptake did not seem to be dependent on a secreted factor, since both viable and paraformaldehyde-fixed bacteria behaved in an identical manner.

Once inside the host cell, the pathogens employ different strategies to avoid the phagosomal-lysosomal degradation pathway. *Salmonella* reside in spacious late endocytic compartments but avoid fusion with lysosomes [reviewed in 27]. In contrast, *Brucella*- and *Legionella*-containing endosomes branch off early and convert into endoplasmic-reticulum organelles permissive of bacterial replication [28, 29]. On the other hand, intracellular survival of *Francisella* involves prevention of phagosomal maturation, which eventually results in bacterial escape into the host cytosol [30, 31]. In this paper, we show that *S. pyogenes* reside in phagocytic vacuoles that transiently associate with early endosomes, but do not acquire late endosomal or lysosomal markers, indicating an arrest in phagosome maturation. Importantly, lysosomal fusion with bacteria-containing phagocytic vacuoles that resulted in bacterial killing was evident when a *S. pyogenes* strain deficient in the M1 protein was used. This is in complete agreement with the previously reported M1 protein-dependent impairment in granule fusion in neutrophils [9, 19], and demonstrates a common immune evasion strategy employed by *S. pyogenes* to support intracellular persistence in phagocytic cells. Whether this

Fig. 5. Intracellular replication is followed by bacterial egress and cell death. Transmission electron microscopy images of macrophages infected with viable (a) or paraformaldehyde-fixed (b) *S. pyogenes* wild-type 5448, or viable 5448ΔM1 (c) for 6–8 or 12 h. d High-magnification electron micrograph of replicating bacteria showing the characteristic septum. e Cell death was quantified by calorimetrical measurement of LDH release from the cytosol of infected cells into the cell culture media at different time points after infection. f Mean ± SEM of LDH release at 10 h after infection of cells from 5 different donors. Statistically significant differences were determined by the paired t test. Ability of egressed bacteria to re-infect new cells was assessed by addition of FarRed-labelled cells to the cell cultures (for details see Material and Methods), and GFP-FarRed-double-positive cells were monitored by flow cytometry at different time points after infection. g Mean ± SD of results obtained from 2 separate experiments using cells from different donors.

is a unique property to M1 protein or whether other M proteins may exert the same suppressive effect on macrophages and neutrophils is a clinically relevant question that warrants further studies. It is well known that M1 strains are overrepresented among severe invasive streptococcal infections, but other types may also give rise to these severe life-threatening infections and intracellular bacterial persistence in phagocytes has been demonstrated in patients infected with diverging *S. pyogenes* types [10].

Further insight into M1 protein modulation of macrophages was sought by exploration of host responses, in particular inflammatory responses. Macrophage polarization towards an anti-inflammatory phenotype (that is M2 phenotype) is a strategy shared by several pathogens to promote intracellular survival [reviewed in 13]. In line with this, a higher level of phosphorylation of p65 and I κ B α was noted in cells infected with the M1 protein-deficient strain as compared to the wild-type strain. Thus, the results indicated that the inflammatory response was suppressed following infection with M1 protein-expressing *S. pyogenes*, a finding also supported by a reduction in TNF α gene expression. At first sight, these results may seem at odds with the well-described hyper-inflammatory responses characterizing severe invasive streptococcal infections [32–35]. However, as bacteria-mediated suppression of inflammation is likely to occur only at the single cell level, that is in cells harbouring viable bacteria, the overall response may well be pro-inflammatory. This is in agreement with a previous study, which demonstrated an atypical activation macrophage phenotype with both M1 (pro-inflammatory cytokines and chemokines) and M2 (IL-1ra, IL-10) markers following *S. pyogenes* infections in a murine model [36].

Another clinically important finding of this study is that of intracellular *S. pyogenes* replication in macrophages. In contrast, in non-phagocytic cells *S. pyogenes* survival over time is rather poor, a phenomenon attributed to autophagy [22, 37]. In macrophages, *S. pyogenes* remain intact within phagocytic vacuoles for a prolonged time, and bacterial replication is evident from approximately 6 h after infection based on viable counts measurements and electron micrographs. Simultaneously, marked morphological changes of the bacteria-containing vacuoles become apparent. As the infection and bacterial replication proceeds, extensive vacuolization, cellular disruption and cell death occur which coincide with bacterial egress out of their host cells. Ultrastructural images reveal a seemingly organized process of bacterial egress out of the cell, which makes it tempting to specu-

late that this precedes cell lysis rather than vice versa. The type of cell death and underlying mechanism is as yet unknown. Among the bacterial factors likely to be involved in this process, streptolysin O is a principal candidate as it is known to exert potent cytotoxic effects on a large variety of cells [22, 38], and has been implicated in different types of cell death, that is apoptosis and oncosis, of macrophages [21]. However, there are also other streptococcal factors with known cytotoxic and apoptotic activities, including among others streptolysin S and the cysteine protease SpeB, which may be involved in cell lysis [38, 39]. It is noteworthy that in this study, *S. pyogenes*-associated cell lysis is not evident until later time points (8–12 h after infection), in contrast to the rapid cell death occurring within hours as reported previously [20, 21]. One plausible explanation to this difference could be that while we based our studies on human primary monocyte-derived macrophages, the other reports focused on murine cells, a human monocytic cell line and/or human blood-derived monocytes. The fact that intracellular viable *S. pyogenes* can be detected in macrophages in infected tissue from patients for a prolonged time [10] supports our conclusion that bacterial modulation of the macrophage provides a hospitable niche for an extending time period during which bacterial replication occurs, resulting in a significant bacterial reservoir for continued infection and tissue injury.

The finding that M1 protein is involved in modulation of human macrophages adds yet another role to this multifunctional protein. Aside from its long-recognized anti-phagocytic activities in blood, it has recently been ascribed a central role in mediating shock and systemic toxicity associated with severe invasive streptococcal infections [3, 4, 40]. Taken together, this implicates M1 protein as a key factor and important target for novel therapeutic strategies in these severe infections.

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