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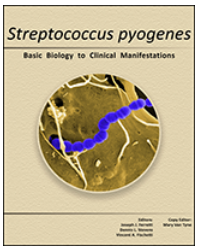

Adhesion and invasion of *Streptococcus pyogenes* into host cells and clinical relevance of intracellular streptococci

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Introduction

The heterogeneous genus of Streptococci plays an important role in human disease. Streptococci are estimated to cause 700 million human infections each year worldwide, with an estimated total of 500,000 deaths (Carapetis, McDonald, & Wilson, 2005). Louis Pasteur recognized streptococci as one of the first microorganisms to cause contagious disease in 1879. For family physicians, *Streptococcus pyogenes* has generally been associated with a sore throat (strep throat) and less often associated with complications, like rheumatic fever or glomerulonephritis. Since the late 1980s, a resurgence of severe infections by *S. pyogenes* have been reported, which involve expanding and invasive soft tissue infections, as well as necrotizing fasciitis, and which are often accompanied by streptococcal toxic shock syndrome (STSS) (Reglinski & Sriskandan, 2014). In 1998, a sudden onset of neuropsychiatric illness, pediatric autoimmune neuropsychiatric disorders associated with streptococcal infections (PANDAS) following pharyngitis, was described (Swedo, et al., 1998). During the last decade, it became clear that a related species, *S. dysgalactiae subsp. equisimilis,* can cause many of the same kinds of human infections with similar complications. Over the past 20 years, β-hemolytic species of streptococci were recognized as highly capable intracellular pathogens that are able to efficiently invade human cells in cell culture. (LaPenta, Rubens, Chi, & Cleary, 1994; Greco, et al., 1995; Rohde & Chhatwal, 2013). Evidence indicates that streptococci can survive and persist within human cells and remain impervious to antibiotic treatment and innate immune defenses.

A well-established assumption is that bacterial pathogens must first attain intimate contact with host extracellular matrix proteins (ECM) on host cells in order to establish successful infections. That initial contact with ECM proteins or cells is accomplished by highly specific adhesins (Courtney, Hasty, & Dale, 2002; Jenkinson & Lamont, 1997; Nobbs, Lamont, & Jenkinson, 2009). Adhesins and other macromolecules that trigger the uptake of bacteria or invasion of the host cells are named invasins. One hallmark of streptococci is the expression of a highly variable and extensive repertoire of adhesins and invasins. Those proteins are differentially

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regulated and expressed in response to signals from the different environments within the human host (Nobbs, Lamont, & Jenkinson, 2009). Streptococci sometimes use mechanisms similar to those of other intracellular bacterial species and viruses to invade host cells. Due to their variable repertoire of adhesins and invasins, streptococci have evolved numerous strategies to be internalised and survive in host cells for their own benefit, namely escaping antibiotic treatment and the host immune system (Cunningham, 2000; Courtney, Hasty, & Dale, 2002; Nitsche-Schmitz, Rohde, & Chhatwal, 2007; Nobbs, Lamont, & Jenkinson, 2009; Rohde & Chhatwal, 2013; Talay, Gram-positive adhesins, 2005). This chapter will focus on the extensive repertoire of adhesins and invasins that are expressed by β-hemolytic streptococci and will examine their molecular interactions with host human host cells, as well as address the clinical and epidemiologic relevance of intracellular streptococci.

Adhesion of Group A streptococci

The adhesion of streptococci to the extracellular matrix (ECM) is one of the initial and essential steps of streptococcal infections. In order to colonize their host, streptococci must overcome a number of obstacles: competition with other bacterial members of the normal flora, electrostatic and mechanical forces, and physiological responses that can dislodge or physically remove them from host tissues. The strategies for adhesion are multiple, complex and variable (Jenkinson & Lamont, 1997; Nobbs, Lamont, & Jenkinson, 2009). Moreover, the expression of specific adhesins is significantly influenced by their microenvironments (Rohde & Chhatwal, 2013). Therefore, interpretations of experimental data related to adhesion mechanisms are sometimes complicated by the use of different streptococcal strains, experimental settings, and the choice of cell cultures and animals models.

Aside from tight adhesion to specific tissues, it may at times be advantageous for a pathogen to detach from a surface in order reach a more favorable environment. Therefore, adherence should be considered a dynamic process. Due to the fact that most host surfaces are covered with extracellular matrix proteins, such as collagen, fibrinogen, laminin, vitronectin or fibronectin, many streptococcal adhesins specifically bind to these various components of the extracellular matrix (Cremer, Rosloniec, & Kang, 1998; Debelle & Tamburro, 1999; Dempfle & Mosesson, 2003; Pankov & Yamada, 2002; Schvartz, Seger, & Shaltiel, 1999). Once the first step of colonization is established, streptococci may multiply extracellularly to form small colonies and/or develop biofilm-like structures that sequester them from host defenses; however, on mucosal surfaces, adhesion often leads to internalization by host cells.

Cell wall anchored and anchorless adhesins

Recognizing and binding to various ECM proteins is an important precursor for streptococcal colonization of human tissue. The ECM supports cells and tissue, maintains strength and elasticity of the body and is, therefore, ubiquitous. However, the ECM is frequently exposed by trauma and injury, which creates a prime target for streptococcal adhesion. Streptococcal adhesins can be grouped into four different families, depending on their association with their surfaces: i) those that are covalently linked by their C-terminus to the cell wall peptidoglycan through a LPxTz motif; ii) those that are tethered to the bacterial cell membrane through N-terminal modifications of lipids to form lipoproteins; iii) those that are bound to the bacterial surface by non-covalent interactions; or iv) those that are expressed and retained on the surface by an as-yet unknown mechanism. The most prominent adhesins belong to the family of cell wall-anchored proteins that are covalently linked to the peptidoglycan by membrane-associated transpeptidases, called sortase A (Marraffini, Dedent, & Schneewind, 2006).

Hyaluronic acid capsule and lipoteichoic acid

β-hemolytic streptococci are known to produce polysaccharide capsules composed of hyaluronic acid (HA), a glycosaminoglycan that is a linear polymer of alternating monosaccharide-units of N-acetylglucosamine and glucuronic acid. Expression of the HA capsule provides protection against phagocytosis by cells of the immune

system. For example, the HA capsule was reported to decrease the association with PMNs, thereby counteracting phagocytosis (Dale, Washburn, Marques, & Wessels, 1996). Wessels et al. were the first to observe that encapsulated strains were better able to colonize the nasopharynx than non-encapsulated strains (Wessels, Moses, Goldberg, & DiCesare, 1991). Further studies revealed that the HA capsule can act as a non-protein adhesin by binding to the hyaluronic acid receptor CD44 on skin keratinocytes and murine epithelial keratinocytes (Cywes, Stamenkovic, & Wessels, 2000; Schrager, Rheinwald, & Wessels, 1996; Schrager, Albertí, Cywes, Dougherty, & Wessels, 1998). Moreover, highly encapsulated strains exhibit the ability to breach epithelial barriers and allow dissemination into deeper soft tissue of the human body. The interaction of HA with CD44 receptors leads to cytoskeletal rearrangements in human epithelial cells. These rearrangements can cause disruption of intracellular junctions and allow the dissemination of streptococci into deeper, underlying sterile tissue (Cywes & Wessels, 2001).

Chain-like, glycerol phosphate polymers constitute the backbone of lipoteichoic acid (LTA). The polymers are covalently anchored to glycolipids and represent a component of the streptococcal cell wall. LTA is thought to mediate the first-step of adhesion and have little cellular specificity (Beachey & Ofek, 1976; Courtney & Hasty, 1991; Courtney, et al., 1992; Leon & Panos, 1990; Simpson & Beachey, 1983). To establish tighter links to host cells, a second, high-avidity, cellular-specific step must follow (Courtney, et al., 1992; Hasty, Ofek, Courtney, & Doyle, 1992). As a result, the latter is thought to significantly influence tissue tropisms of infections.

Fimbrious structures or pili

It is widely accepted that pili (which are sometimes referred to as fimbriae) are important mediators of Gram-negative pathogen adherence to host cells, but their role in overall pathogenesis is less studied. The expression of fibrils was associated with growth, colonization and survival of oral streptococci in the oral cavity (Handley, Carter, & Fielding, 1984; Handley, et al., 1987). Negative staining with 2% aqueous uranyl acetate is the method of choice for detecting appendages on bacterial surfaces by electron microscopic imaging. By applying this method, fibrils were detected on the cell wall of oral streptococci. Pili are long hair-like extensions on the cell surface, and were first described in Gram-positive Corynebacteria in 1968 and then in streptococci during the 1990s (Wu & Fives-Taylor, 1999; Yanagawa, Otsuki, & Tokui, 1968; Yanagawa & Honda, 1976). Streptococcal pili have recently attracted attention as promising candidates for vaccine development (Gianfaldoni, et al., 2007). All three of the invasive streptococcal pathogens (*S. pyogenes, S. dysgalactiae subsp equisimilis,* and *S. pneumonia*) were shown to have pili on their surface (Barocchi, et al., 2006; Lauer, et al., 2005; Mora, et al., 2005; Rosini, et al., 2006).

Contrary to their fragile appearance, the mechanical strength of pili is achieved by novel chemical modifications. These and other cell surface proteins contain isopeptide and thioester bonds—highly unusual intramolecular covalent linkages between amino acid side chains within the shaft—while the adhesion subunit is located at the pilus tip. These findings were very surprising, since the thioester binding domain was only known to exist in the C3 and C4 complement proteins and in alpha-macroglobulin. Similar thioester domains were identified in cell wall proteins of other Gram-positive pathogens, such as *Clostridium diphtheriae*, *C. perfringens*, and *Bacillus cereus* (Kang, Coulibaly, Clow, Proft, & Baker, 2007; Kang & Baker, 2012; Kang & Baker, 2011; Kang & Baker, 2009; Linke-Winnebeck, et al., 2014; Pointon, et al., 2010). The discovery of thioester bonds in *S. pyogenes* invasin, FbaB, is an interesting finding (Hagan, et al., 2010). Studies have shown that internal isopeptide bonds form spontaneously during the assembly process of streptococcal pili, and that isopeptide bonds are responsible for both the proteolytic and thermal stability, as well as for mechanical resistance of streptococcal pili. Therefore, pili can withstand tensile forces during the first steps of adhesion to the host cells. Although there is little evidence for its involvement in adhesion, the thioester domain was postulated to form covalent interactions with host cells (Walden, Crow, Nelson, & Banfield, 2014).

Genes that encode pilus proteins are located on pathogenicity islands (PI) in the streptococcal genome and are clustered in operons within close proximity to those that encode sortases (Scott & Zähner, 2006). Remarkably,

genes that code for the fibronectin-binding, collagen-binding, and T-antigen region (or the FCT-region) are located in the same vicinity on the chromosome of *S. pyogenes.* The Lancefield T-antigen was used for years to serotype *S. pyogenes* (Cunningham, 2000). Mora et al. (Mora, et al., 2005) first showed that a T-antigen is the shaft of pili. On these pili, the adhesin molecule is on the tip, but a third component, the AP1 (ancillary protein 1), was identified that is added at intervals along the shaft of the pilus and that has the capacity to bind collagen (Falugi, et al., 2008). This observation suggests that pili mediate the first contact with the ECM that leads to adhesion to host cells. Cell culture models of infection demonstrate that pili are involved in the adhesion process for a broad range of host epithelial cells, including cells from the nasopharynx, tonsils, lung, cervix, and intestine (Abbot, et al., 2007; Crotty Alexander, et al., 2010). Remarkably, *S. agalactiae's* pili can trigger the paracellular passage through an epithelial cell barrier and promote uptake by brain microvascular endothelial cells (Maisey, Hensler, Nizet, & Doran, 2007; Pezzicoli, et al., 2008). The so-called minor pili, which are not as long as the other pili, were first identified in both *Corynebacterium* and streptococci. These pili may also be involved in streptococcal tissue tropisms, since they mediate adhesion to human tonsil epithelium and primary keratinocytes, which are some of the prime colonization targets of streptococci (Abbot, et al., 2007). The minor pilin protein, Cpa, of *S. pyogenes* can bind collagen (Kreikemeyer, et al., 2005).

In conclusion, a putative model of cell adhesion through pili was postulated by Telford et al. (Abbot, et al., 2007; Telford, Barocchi, Margarit, Rappuoli, & Grandi, 2006). The first contact with ECM proteins of the host is mediated by extended pili via the AP2-protein (ancillary protein 2) on their tips. This interaction may be a non-covalent, reversible reaction that allows streptococci to find the specific tissue. The next step may involve the interaction of AP1-protein (ancillary protein 2, interdispersed in the pilus backbone or forming branches in the pilus backbone) with collagen (Talay, 2005), which more firmly attaches the bacterium to host cells. Consequently, streptococci come into closer contact with the cellular surface, which allows the bacterial cell surface anchored adhesins to establish intimate contact. Although the current hypothesis of bacterial adherence to host cell receptors favors strong non-covalent interactions, the finding of thioester bonds in pili and other surface proteins (invasins, adhesins) suggests an alternative new hypothesis; namely, the adhesion through a covalent bond to the eukaryotic cell surface. Future studies will test this possibility and further refine the role of pili in adherence of *S. pyogenes* to different tissues.

Anchorless adhesins

Several streptococcal proteins are localized at the cell surface, but lack the LPxTz peptidoglycan anchor motif. In addition, these so-called anchorless proteins or "moonlighting" proteins lack N-terminal signal sequences. How they are exported from the cytoplasm to the cell surface and then remain associated with the Gram-positive cell wall is still a mystery. The anchorless adhesins represent a group of very diverse proteins, both in their structures and in their functions. In addition, they bind to different ligands. Many represent members of the glycolytic cycle, such as triosephosphate isomerase, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 3-phosphoglycerate kinase, 3-phosphoglycerate mutase, and alpha-enolase, with a cytoplasmic localization in the bacterial cell. All five of these proteins were found to be associated with the cell wall surface (Kinnby, Booth, & Svensäter, 2008; Pancholi & Fischetti, 1992; Pancholi & Fischetti, 1998). Moonlighting proteins interact with multiple ECM proteins, including fibronectin, fibrinogen, plasmin, and plasminogen, and, therefore, are likely contribute to streptococcal colonization (Esgleas, et al., 2008; Henderson & Martin, 2011; Siemens, Patenge, Otto, Fiedler, & Kreikemeyer, 2011). Surprisingly, experiments suggest that GAPDH also interacts with the cytoskeletal proteins actin and myosin, and with the urokinase plasminogen activator receptor (Jin, Song, Boel, Kochar, & Pancholi, 2005; Pancholi & Fischetti, 1992; Seifert, McArthur, Bleiweis, & Brady, 2003). Alpha-enolase is distributed among different streptococci and is bound in significant amounts to the bacterial surface. Alpha-enolase serves as a major plasminogen-binding protein for streptococci. Similar to fibronectin, plasminogen mediates adherence and subsequent internalization into host cells of *S. pyogenes* (Siemens, Patenge, Otto, Fiedler, & Kreikemeyer, 2011). In addition, after plasminogen is bound, it can be converted to plasmin by *S. pyogenes* streptokinases. Plasmin represents a very potent serine protease that degrades ECM matrix proteins to allow

streptococci to come into closer contact with the host cell surface, or to gain access into deeper soft tissue after breaching barriers in the human body. Recently, there have been increasing discussions on the involvement of plasmin in facilitating streptococcal dissemination through epithelial or endothelial barriers (Bergmann, Schoenen, & Hammerschmidt, 2013; Fulde, Steinert, & Bergmann, 2013).

M proteins

M proteins are multifunctional virulence factors on the streptococcal surface, which were the first reported adhesins of *S. pyogenes* (Ellen & Gibbons, 1972). Although they are structurally related, M proteins are a heterogeneous group of adhesins that engage a variety of target cells. These surface proteins bind to a wide range of different plasma and ECM proteins, including plasminogen, IgA, IgG, factor H, and C4b-binding protein (C4BP) (André, et al., 2006; McArthur & Walker, 2006; Oehmcke, Shannon, Mörgelin, & Herwald, 2010). The B repeats of M proteins bind to fibrinogen, human serum albumin, and IgG; but the capacity to bind these host proteins is highly variable among different M proteins. M proteins are also known to interact with glycosaminoglycanes. These interactions are mediated by their conserved C-terminal domains (Berkower, Ravins, Moses, & Hanski, 1999; Frick, Schmidtchen, & Sjöbring, 2003). The N-terminal sequences of M1 proteins are thought to be responsible for bacterial aggregation, which may also be crucial for colonization, resistance to phagocytosis, and the subsequent invasion of cells (Cue, Lam, & Cleary, 2001; Frick, Mörgelin, & Björck, 2000).

The M6 protein was shown to interact with the membrane-bound cofactor CD46 on keratinocytes. Biochemical data demonstrated that the C-terminal region of this protein and the short consensus domains 3 and 4 of CD46 must interact to establish attachment to keratinocytes (Giannakis, et al., 2002; Okada, Liszewski, Atkinson, & Caparon, 1995). M1 and M24 proteins were shown to be necessary for efficient adhesion to epithelial HEp-2 or and HeLa cells; mutant strains deficient in these M proteins lacked the capacity to adhere to these cell lines (Courtney, Bronze, Dale, & Hasty, 1994; Cue, Dombek, Lam, & Cleary, 1998). On the other hand, M24- and M6-deficient mutants are able to bind to buccal cells, but are unable to adhere to HEp-2 cells, which suggests that another adhesin (and not M protein) was responsible for adhesion to buccal cells. This agreed with another study, which demonstrated that the M6 protein does not contribute to adherence to buccal and tonsillar epithelial cells (Caparon, Stephens, Olsén, & Scott, 1991). M protein also binds to factor H, which may contribute to complementary inhibition by streptococci (Horstmann, Sievertsen, Knobloch, & Fischetti, 1988).

The M3 protein is unique in that it serves as an adhesin to soluble type I and type IV collagen and to native collagen matrices of the host. The highly-specific collagen binding region is located in the N-terminal variable region of this M protein. The M18 protein is the only other M protein with this binding specificity (Dinkla, et al., 2003a). Notably, the collagen binding protein, Cpa, is the only other collagen binding protein expressed by *S. pyogenes* and, to date is limited to M49 isolates (Podbielski, Woischnik, Leonard, & Schmidt, 1999). The role of collagen binding in streptococcal pathogenesis is an important area for future studies.

In summary, the evidence that M proteins mediate adhesion to human cells and tissue is strong, but differences in specificities that reflect the heterogeneity of this protein and expression of other surface adhesins create a complex picture. However, it is clear that streptococci generally do not bind directly to the host cell surfaces, but instead interact with extracellular matrix proteins that form bridges to host cells. It should be noted that some mucin binding cannot be ruled out, which might also contribute to direct binding to host cells.

Fibronectin-binding proteins

Fibronectin is a primary target of the streptococcal adhesion that connects bacterial adhesins to integrin receptors on the surface of eukaryotic cells. Fibronectin is a large glycoprotein that exists both as a soluble protein in plasma and as a fibrillar polymer in the ECM. It is a dimer of two 250 kDa subunits, linked by disulfide bonds at the C-terminal end. Each subunit has three distinct modules, which are termed type I, II and

III. The classical fibronectin-binding partner is the $\alpha_5\beta_1$ integrin. Integrin binding is mediated through the RGD sequence within the fibronectin subunits (Pankov & Yamada, 2002).

All streptococci express a complex repertoire of proteins that have different fibronectin-binding capacities. Some strains bind soluble fibronectin with high affinities (in the nanomolar range), whereas others can only bind to immobilized fibronectin. Overall, *S. pyogenes* strains are known to express at least 11 distinct fibronectin-binding adhesins. These include SfbI/F1, protein F2, serum opacity factor (SOF), FbaA, FbaB, FBP54, and several M proteins. SfbI and FBP54 proteins are produced by several *emm* types, while expressions of M1and M24 proteins are restricted to those serotypes (Caparon, Stephens, Olsén, & Scott, 1991; Cue, et al., 2000; Hanski & Caparon, 1992; Kreikemeyer, Oehmcke, Nakata, Hoffrogge, & Podbielski, 2004a; Natanson, et al., 1995; Neeman, Keller, Barzilai, Korenman, & Sela, 1998).

As expected, the environmental niche influences expression of these fibronectin-binding proteins. For example, at high partial pressures of O_2, the expression of SfbI is increased. On the other hand, M protein is up-regulated in a CO_2-rich environment in some strains of *S. pyogenes*. Therefore, SfbI-protein is presumed to serve as the primary adhesion when colonizing the respiratory tract or skin, but may be less important in deeper tissue infections where higher CO_2 concentrations up-regulate the M protein (Gibson, et al., 1995; Gibson & Caparon, 1996; Kreikemeyer, McIver, & Podbielski, 2003; Kreikemeyer, Klenk, & Podbielski, 2004b).

The SfbI protein and its allelic variant F1 have been extensively studied. Identified in 1992, it became immediately clear that SfbI is an important adhesin due to its wide distribution among 75% of *emm* types and clinical isolates (Hanski & Caparon, 1992; Talay, 2005).

SfbI protein has a modular architecture with a domain rich in aromatic amino acids (ARO) at the N-terminus, a proline rich repeat region (PRR) in the middle of the molecule, and a fibronectin-binding repeat region (FnBR) at the C-terminus (Talay, Valentin-Weigand, Timmis, & Chhatwal, 1994). Sequences of different clinical isolates are variable in the number of repeats in PRR and FnBR regions. Thirty-four distinct alleles of SfbI proteins were described among 54 *S. pyogenes* strains (Towers, et al., 2003). The ARO region also has a high degree of sequence variability and the number of repeat units in PRR (1-11 repeats) and FnBR (1-5 repeats) varies, as well—an apparent consequence of sequence duplication and deletion. The impact of sequence variability on the molecular interactions of SfbI with ECM and on virulence remains is unknown.

Binding to fibronectin is mediated by two distinct domains: the C-terminal fibronectin-binding repeat region and the adjacent non-repetitive domain, which is termed spacer 2 or UR. Both regions bind synergistically to two distinct regions on the fibronectin molecule: the N-terminal fibrin-binding fragment that harbors fibronectin F1 modules 1-5; and the gelatine/collagen binding fragment that harbors F1 modules 6-9 and the two F2 modules. Due to this cooperative binding, the quaternary structure of fibronectin is changed in such a way that the RGD-region in fibronectin is exposed on the outer side of the molecule. Subsequently, the RGD region can bind to $\alpha_5\beta_1$ integrin receptors on the host cell surface (Ozeri, et al., 1996; Sela, et al., 1993; Talay, et al., 2000). These interactions between SfbI and fibronectin were studied in detail through biochemical methods (Marjenberg, et al., 2011; Schwarz-Linek, et al., 2003; Schwarz-Linek, Höök, & Potts, 2004a; Schwarz-Linek, et al., 2004b).

The existing structural model for fibronectin-binding proteins has been comprehensively reviewed (Schwarz-Linek, Höök, & Potts, 2004a). Briefly, SfbI and fibronectin bind to each other in an antiparallel fashion. The C-terminal FnBR in SfbI recognizes the N-terminal domain of fibronectin with high specificity and high affinity (in the nanomolar range) by forming a novel protein-protein interaction mechanism, which is termed the tandem ß-zipper. According to the tandem ß-zipper model, FnBRs in SfbI can bind multiple copies of fibronectin, depending on the number of repeats in the FnBR region. For SfbI from a *S. pyogenes* strain, it was demonstrated that a single SfbI molecule is able to bind up to five fibronectin molecules. For the following adhesion process SfbI-expressing streptococci are covered in a cloud of fibronectin that allows the binding of the $\alpha_5\beta_1$ integrin

receptors more easily on the host cell surface. Notably, the observed high affinity is of great importance: high affinity binding is a prerequisite for firm bacterial attachment, because adherent streptococci have to withstand shear forces that occur on the mucosal surfaces, in the blood stream, or during the internalization process itself. Several studies demonstrated that SfbI mediates attachment to epithelial cells of the oral mucosa and the lung, but also to endothelial cells (Molinari, Rohde, Guzmán, & Chhatwal, 2000; Rohde, Müller, Chhatwal, & Talay, 2003). Besides its potential to bind to cellular integrin receptors, SfbI has the ability to recruit collagen to the bacterial surface via pre-bound fibronectin. This allows *S. pyogenes* to form aggregates that adhere to collagen matrices within the body (Dinkla, et al., 2003b). Talay et al. (Talay, et al., 2000) were the first study to define the adhesion protein domain (the FnBR region) within SfbI that specifically interacts with an ECM protein.

Most isolates of *S. pyogenes* that lack the *sfbI* gene express other similar (but distinct) fibronectin-binding proteins. Some examples of these are protein F2 or PFBP (Jaffe, Natanson-Yaron, Caparon, & Hanski, 1996; Kreikemeyer, Oehmcke, Nakata, Hoffrogge, & Podbielski, 2004a; Rocha & Fischetti, 1999). In contrast to SfbI protein, F2 has two binding domains that exclusively interact with fibronectin by targeting the 30-kDa N-terminal fibronectin fragment. Surprisingly, the most common fibronectin-binding protein found in all *S. pyogenes* isolates, FBP54, lacks the LPxTG motif for a membrane anchor. Nevertheless, FBP54 seems to be localized on the streptococcal surface and functions as an adhesin for buccal epithelial, but not HEp2 cells (Chhatwal, 2002; Courtney, Dale, & Hasty, 1996). Some *S. pyogenes* isolates express two other fibronectin-binding proteins, named Fba and FbaB. The *fba* gene was found only in five serotypes of *S. pyogenes*. A mutant that lacked the Fba protein showed reduced adhesion to HEp2 cells, which suggests that Fba is also involved in the adhesion process. The unique fibronectin-binding protein, FbaB, appears to be genetically related to protein F2 and has only been detected in serotype M3 and M18 *S. pyogenes* isolates (Terao, et al., 2001; Terao, Kawabata, Nakata, Nakagawa, & Hamada, 2002). FbaB protein from serotype M3 *S. pyogenes* was shown to be an important invasin for this serotype, and uniquely mediates adherence only to endothelial (HUVEC cells), not to cultured epithelial cells (Amelung, et al., 2011).

Protein H is a fibronectin-binding protein and a member of the M protein family. In contrast to those that bind to type I or type II modules of fibronectin, protein H binds to the type III modules. In addition, protein H was shown to mediate streptococcal aggregation through a so-called AHP sequence that also promoted adhesion to epithelial cells (Frick, Crossin, Edelman, & Björck, 1995; Frick, Mörgelin, & Björck, 2000). M1 protein also binds to fibronectin, which subsequently engages the $\alpha_5\beta_1$ integrin receptors. M1-specific polyclonal antibodies efficiently block adherence to HeLa cells and as anticipated, an M1 protein-deficient mutant was less able to adhere to Hela cells, which confirms its contribution to adhesion for some M1 strains (Cue, Dombek, Lam, & Cleary, 1998; Cue, et al., 2000; Dombek, et al., 1999).

Uptake of streptococci by eukaryotic cells

For decades, streptococci were regarded as extracellular pathogens; however, over the last two decades, considerable experimental evidence from several laboratories has otherwise demonstrated that *S. pyogenes* is a capable intracellular bacterium, and that this intracellular state likely accounts for the commonly observed persistence of streptococcal in the oral cavity following antibiotic therapy. Efficient intracellular invasion was first suggested in 1994, when LaPenta and colleagues used a cell culture infection model to demonstrate that *S. pyogenes* enter non-phagocytic human epithelial cells at frequencies equal to or greater than classical intracellular pathogens, such as *Listeria* or *Salmonella* (Greco, et al., 1995; LaPenta, Rubens, Chi, & Cleary, 1994). Shortly thereafter, immunohistological methods were used to visualize intracellular streptococci in surgically removed tonsils from patients with recurrent infections (Österlund & Engstrand, 1995; Österlund & Engstrand, 1997; Österlund, Popa, Nikkilä, Scheynius, & Engstrand, 1997). These findings were confirmed and extended by a demonstration that showed that excised tonsils from patients with recurrent tonsillitis contain viable streptococci (Podbielski, et al., 2003). Overwhelming evidence demonstrated that other streptococcal species can efficiently invade a variety of epithelial cells, like *S. dysgalactiae subsp equisimilis* and *S. suis* (Benga, Goethe,

Rohde, & Valentin-Weigand, 2004; Haidan, et al., 2000; Norton, Rolph, Ward, Bentley, & Leigh, 1999) and that some are efficiently ingested by endothelial cells (Amelung, et al., 2011; Nerlich, et al., 2009; Ochel, Rohde, Chhatwal, & Talay, 2014).

With the advent of field emission scanning electron microscopes (FESEM), pathogen-host interactions can now be imaged at much higher magnifications and resolutions. FESEM has revealed that streptococci use multiple morphologically distinct mechanisms. One invasion mechanism involves the formation of invaginations in the host cell membrane through which bacteria invade (Figure 1, A). Although an overlap exists, streptococcal uptake mechanisms are more varied than those reported for *Listeria* and *Shigella*. Some strains of streptococci exhibit a classical membrane-ruffling pattern (triggering mechanism, Figure 1, B) as described in Salmonella, while others invade via a well-defined zipper-like mechanism (Figure 1, C), as described in Listeria. Streptococci are not restricted to these two mechanisms; isolates that express the SfbI protein induced a third, previously unknown invasion pathway with morphological features seen only by FESEM. These streptococci induce large invaginations during the internalization process, which sometimes resemble a "hole" in the host cell membrane (Molinari, Rohde, Guzmán, & Chhatwal, 2000; Rohde, Müller, Chhatwal, & Talay, 2003). A variety of invasion mechanisms were also reported for *Streptococcus dysgalactiae subsp equisimilis* (Group C and Group G streptococci) (Haidan, et al., 2000) and non-encapsulated strains of *S. suis* (Benga, Goethe, Rohde, & Valentin-Weigand, 2004) and *Staphylococcus aureus* (Agerer, et al., 2005). Long chains of streptococci invade host cells by yet another mechanism in which engulfment begins at the middle of the streptococcal chain. Host cell microvilli on both sides of the chain start to grow over the adherent chain. The bacteria are physically ingested by a "flap-like" mechanism. Thus, the streptococcal chain is engulfed from the middle when microvilli have fused with each other. In some cases, the remainder of the chain protrudes from both sides of the fused microvilli (see Figure 1, D). Representative scanning images of the different streptococcal invasion mechanisms are shown in Figure 1.

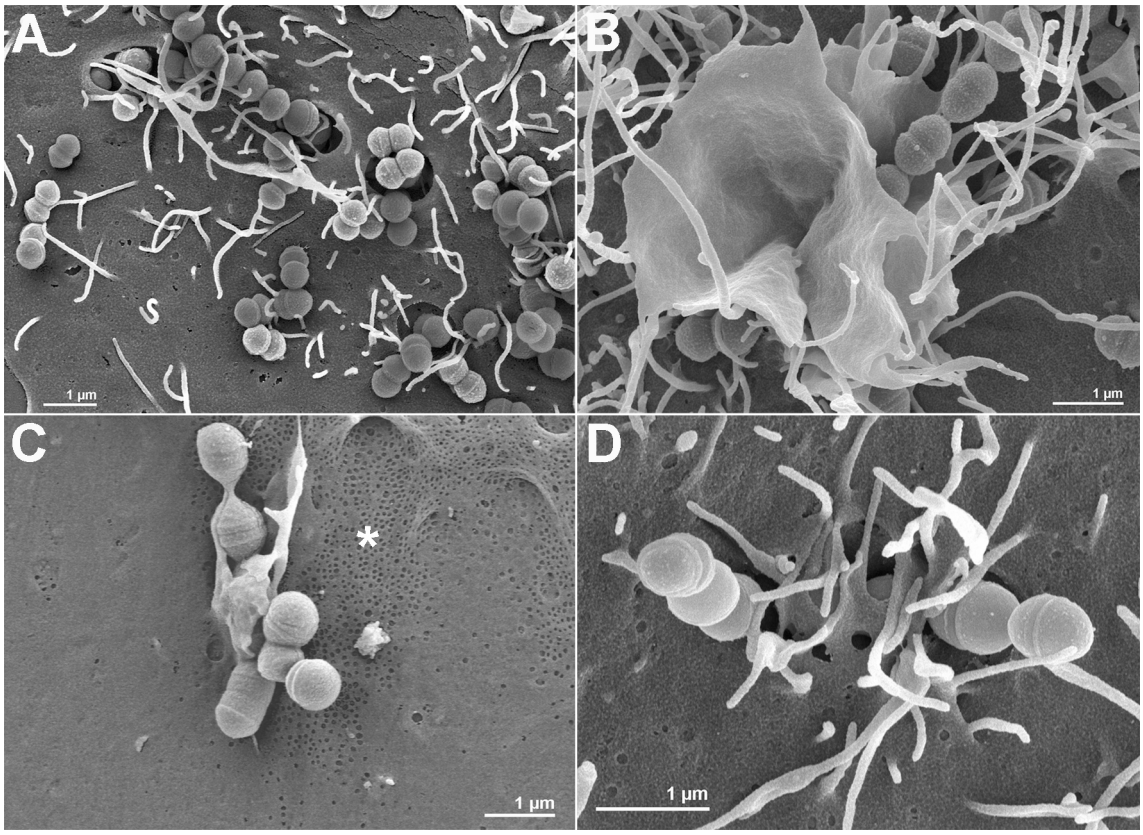

Figure 1. Different invasion mechanisms of *S. pyogenes* into epithelial cells. Field emission scanning electron microscopy (FESEM) depicts different invasion mechanisms of *S. pyogenes* isolates into host epithelial cells. A) Invasion via SfbI-mediated fibronectin

binding and integrin clustering leads to the formation of large invaginations in the host epithelial cell (HEp-2) membrane, through which streptococci invade; B) Non-SfbI-expressing isolates induce the formation of large cytoskeletal rearrangements (membrane ruffling); C) Even in the presence of numerous caveolae at the adhesion site (star) non-SfbI expressing isolates trigger signaling events that lead to cytoskeletal rearrangements with subsequent engulfment; D) Chains of non-SfbI-expressing isolates are sometimes taken up by a "flap-like" mechanism, in which the uptake process often starts at the middle of the chain with both ends of the chain still outside the host cell.

Streptococcal invasins are surface exposed and/or diffusible proteins that can promote actin rearrangement of the host cytoskeleton to produce membrane ruffles (Dombek, et al., 1999), or can co-opt host cell endocytic pathways, called caveolae (Rohde, Müller, Chhatwal, & Talay, 2003), which ultimately lead to the internalization of the bacteria. Interactions with specific host cell receptors trigger cellular signalling events that instigate these physical changes in host cells (Ozeri, et al., 2001; Purushothaman, Wang, & Cleary, 2003; Wang B. , Li, Southern, & Cleary, 2006a; Wang B. , Li, Dedhar, & Cleary, 2007). The most studied *S. pyogenes* invasins are the multifunctional SfbI and M proteins. However, several investigators have linked streptococcal dehydrogenase (Pancholi & Fischetti, 1997), SpeB (extracellular cysteine protease) (Tsai, et al., 1998), superantigen Spe A (Cleary, LaPenta, Vessela, Lam, & Cue, 1998a), and the C5a peptidase (Purushothaman, Wang, & Cleary, 2003) of *S. pyogenes* and/or *S. dysgalactiae subsp equsimilis* with the invasion of host cells. It is unclear whether these proteins contribute directly or indirectly to the invasion process; therefore, this review will focus on the functions of SfbI and the M protein.

SfbI/F1 invasion of epithelial and endothelial cells

The molecular basis for the interaction of SfbI with fibronectin was described by Talay et al. (Talay, et al., 2000). They were the first to separate adherence from the subsequent ingestion process. Co-operative protein interactions were further described on a structural basis to involve a tandem ß-zipper mechanism by Schwarz-Linek et al. (Schwarz-Linek, et al., 2003). The binding of SfbI to fibronectin changes the quaternary structure of fibronectin, which leads to exposure of the RGD-region within the molecule. This permits the RGD sequence to engage $\alpha_5\beta_1$ integrins on host cell surfaces, and the interaction between the RGD-region of fibronectin and integrins can be blocked by antibodies against the ß-subunit of the integrin, or by competitive RGD peptides, which results in significantly reduced invasion frequencies by streptococci (Jadoun, et al., 1998; Molinari, Rohde, Guzmán, & Chhatwal, 2000; Ozeri, Rosenshine, Mosher, Fässler, & Hanski, 1998). Ozeri et al. were the first to suggest that the amount of bound fibronectin on the bacterial surface influences uptake efficiency by host cells (Ozeri, Rosenshine, Mosher, Fässler, & Hanski, 1998). It is reasonable to postulate the existence of a threshold of integrin-bound fibronectin before signalling is initiated. Engagement and clustering of integrin receptors were shown to be a prerequisite for integrin signalling and subsequent invasion of host cells by other bacterial pathogens (Isberg & Leong, 1990; Isberg, 1991; Isberg & Barnes, 2001). The involvement of integrin-clustering during invasion due to multiple binding sites of RGD regions of fibronectin to integrins is strongly supported by the proposed tandem ß-zipper mechanism (Schwarz-Linek, et al., 2003). In this model, a single SfbI molecule is able to bind up to five fibronectin molecules, which results in dense coverage of the streptococcal surface with fibronectin and formation of integrin clusters with a subsequent outside-inside signalling upon contact with host cells. Clustering of integrins underneath attached streptococci was confirmed by staining the ß-subunits of integrins with specific antibodies. The application of high-resolution FESEM to visualize integrin clustering with recombinant SfbI protein coated 15 nm colloidal gold nanoparticles defined the distribution of SfbI on the surface of endothelial HUVEC cells (Rohde, Müller, Chhatwal, & Talay, 2003). Other bacterial pathogens, *Neisseria gonorrhoeae* (van Putten, Duensing, & Cole, 1998), *Staphylococcus aureus* (Fowler, et al., 2000) and *Yersiniae* species (Isberg & Barnes, 2001) specifically bind fibronectin that can engage integrins and lead to intracellular uptake by host cells.

The induction of large invaginations in epithelial and endothelial cells by SfbI-expressing streptococci was a mystery for a long time (see Figure 1, A). However, when FESEM was applied to image the surrounding cellular

architecture, aggregates of approximately 80 nm wide depressions in the host cell surface were often observed. Ultrathin sections revealed omega-like structures underneath adherent bacteria, and were concluded to be caveolae from examining their shape (Rezcallah, et al., 2005). Further studies demonstrated that recombinant SfbI protein triggered caveolae aggregation and more completely defined the large invaginations associated with streptococci. Caveolae fuse with each other to form the large invaginations that were observed in FESEM studies. Once inside the host cell, SfbI-expressing streptococci traffic into a new compartment, called the "caveosome." The intriguing aspect of this type of streptococcal invasion is the fact that caveosomes do not fuse with lysosomes. By co-opting the caveolae-mediated cellular pathway, SfbI-expressing streptococci bypass the lysosomal degradation machinery of the host cells (Rohde, Müller, Chhatwal, & Talay, 2003). An identical mechanism for invasion and intracellular trafficking was reported for the simian virus 40 (SV40) (Pelkmans, Püntener, & Helenius, 2002).

S. dysgalactiae supsp equisimilis expresses Group G fibronectin binding protein A (GfbA protein), which also functions as an adhesin and invasin (Kline, Xu, Bisno, & Collins, 1996). GfbA with bound fibronectin interacts with $\alpha_5\beta_1$ integrins, with subsequent formation of membrane ruffles and rearrangements of the host cell cytoskeleton. Large invaginations were only very rarely observed. Immune fluorescence studies that labelled the lysosomal marker enzyme LAMP-1 demonstrated that GfbA-expressing streptococci follow the classical endocytic pathway, with subsequent fusion with lysosomes to form phagolysosomes. This is in contrast to the SfbI-mediated invasion process and was surprising, since both isolates bind similar amounts of fibronectin on their surface (Rohde, et al., 2011).

A definitive method for investigation of the impact of a specific protein on invasion of human cells is to express that protein in Gram-positive non-invasive bacteria, such as *S. gordonii* or *Lactococcus lactis*. Figure 2 shows *Lactococcus lactis* with surface-expressed, SfbI protein-associated caveolae during an invasion event.

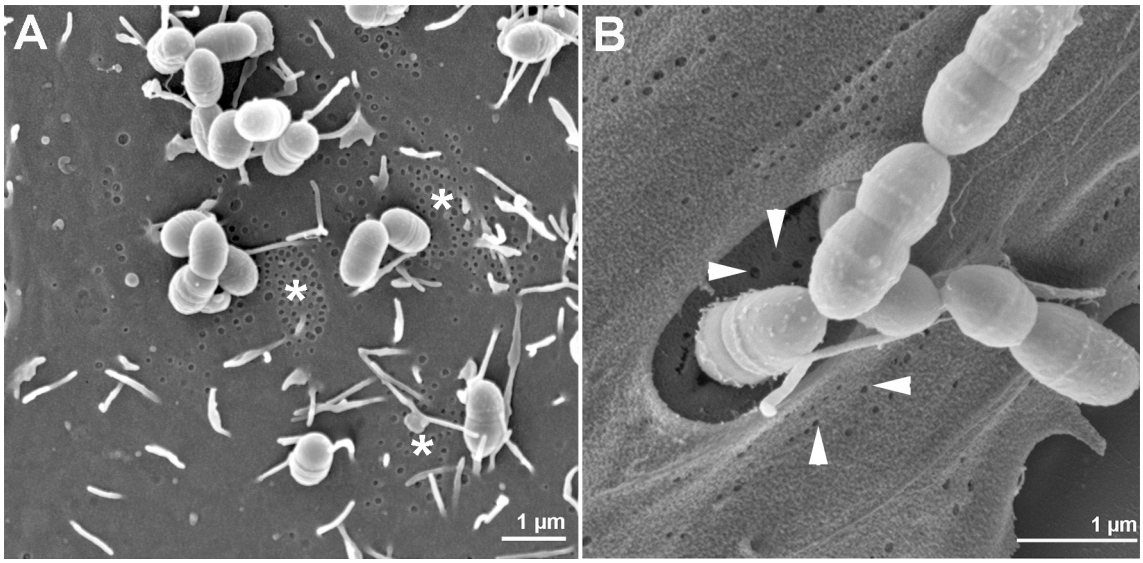

Figure 2. Model for investigation of the role of pathogenicity factors on invasion. A definitive model expresses those factors on the surface of *Lactococcus lactis* or *S. gordonii*. Depicted is the invasion mechanism of surface expressed SfbI in *L. lactis*. As is evident in (A), caveolae (stars) aggregate around adherent SfbI-expressing cells, followed by the formation of large invaginations in the host cell membrane, (B) in which some caveolae can still be detected (shown with arrows) that fuse with the membrane to further enlarge the invagination. The invasion mechanism is identical to that of the SfbI-expressing *S. pyogenes* wild-type isolate, which demonstrates that SfbI alone represents the adhesin and invasin triggering uptake through invaginations into the host cell of the isolate.

The differential impact of Gfb and SfbI on the physical uptake mechanism was investigated in more depth. Sequencing of the GfbA gene revealed that the PRR and FnBR region in the C-terminal part is very similar to the SfbI gene; however, the N-terminal sequence, including the aromatic domain (ARO) of GfbA and SfbI, is significantly different. Therefore, it was postulated that the ARO region was responsible for the morphological

differences in the invasion mechanism. This hypothesis was tested by constructing a GfbA mutant protein that lacked the aromatic domain. In addition, the ARO region of the GfbA protein replaced the aromatic domain in SfbI protein. FESEM studies revealed that GfbA protein with a deleted ARO region invades with the formation of large invaginations on the host cell surface, which are structures comparable to those induced SfbI-expressing strains. In addition, intracellular *S. gordonii* that expresses GfbA without the ARO region failed to fuse with lysosomes and instead resided in caveosomes. Moreover, the strain that expressed the SfbI containing the ARO region of GfbA was now able to induce membrane ruffles, and intracellular trafficking resulted in the fusion with lysosomes. Thus, these invasins were altering to trigger an absolutely different invasion mechanism by replacing the ARO region in the protein. In addition, the invasion mechanism clearly influences subsequent intracellular trafficking. FESEM showed that only GfbA without the aromatic domain induced integrin clustering and signalling, while SfbI with the ARO region of GfbA was unable to induce integrin-clustering and signalling, like wild-type GfbA (Rohde, et al., 2011).

In summary, heterologous surface expression of GfbA in the non-pathogenic *S. gordonii* demonstrated that the ARO region of GfbA alone is responsible for the morphologically distinct invasion mechanism. The invasion mechanism subsequently leads to different intracellular trafficking, as compared to the SfbI-mediated caveolae-dependent invasion mechanism. Thus, these studies have revealed a biological function of the ARO region in a fibronectin-binding protein for the first time (Rohde, et al., 2011).

M protein mediates invasion of human cells

The importance of intracellular invasion to *S. pyogenes* virulence and pathogenicity can be realized from the fact that most (if not all) strains produce one or more invasins. Those strains that lack the potential to produce high-affinity fibronectin-binding proteins, such SfbI, express M proteins that can take over the job and efficiently promote uptake of streptococci by both human endothelial and epithelial cells. To date, M1, M3, M5, M6, M12, M18, and M49 strains have been studied and are shown to invade cultured endothelial and/or epithelial cell lines (Amelung, et al., 2011; Berkower, Ravins, Moses, & Hanski, 1999; Dombek, et al., 1999; Molinari, Rohde, Guzmán, & Chhatwal, 2000; Nerlich, et al., 2009; Rohde, Müller, Chhatwal, & Talay, 2003). The efficiency or frequency of ingestion of different strains can be very different even within an individual serotype, which may reflect minor *emm* gene sequence differences or the acquisition of unidentified invasins encoded by prophages (Cleary, LaPenta, Vessela, Lam, & Cue, 1998a). Only the M18 serotype was found to be less invasive, due to its enormous hyaluronic acid capsule that interferes with initial adherence to cells. The efficiency of ingestion is also influenced by the quality and concentration of fibronectin and glucose in culture medium (Cleary, unpublished data), and by the number of passages of the cell line. Moreover, the genetic stability of the expression of M^+ streptococci following laboratory passage affects the invasion frequency (Cleary, et al., 1998b).

High-frequency invasion of A549 cells and primary cultures of tonsil tissue by the highly virulent M1 clone is dependent on the streptococcal bound fibronectin engagement of $\alpha_5\beta_1$-integrins on those human cells (Cue, Dombek, Lam, & Cleary, 1998; Wang, Li, Southern, & Cleary, 2006a). TGF-ß1 is known to regulate the expression of fibronectin and $\alpha_5\beta_1$-integrins by human cells. Infection of HEp2 cells and intranasal infection of mice with the above M1 strain induced robust TGF-ß1 responses in both. Moreover, the co-incubation of cells with TGF-ß1 increased the frequency of streptococcal ingestion by HEp2 cells. This was attributed to display of more integrins or integrin-binding sites on those cells (Wang, Li, Southern, & Cleary, 2006a).

M1 protein is the primary invasin, as this highly virulent clone lacks other known fibronectin-binding proteins, and in-frame deletion of the *emm1* gene significantly reduced invasion ability. This M1 protein binds both fibronectin and laminin with a much lower affinity than SfbI (Cue, Dombek, Lam, & Cleary, 1998); yet these interactions still promote efficient uptake of these streptococci by epithelial cells. Scanning EM revealed that the invasion of HeLa cells was accompanied by membrane ruffling, which suggests a zipper-like uptake mechanism (Dombek, et al., 1999). Phalloidin labeling showed the rich accumulation of actin around adherent M1 bacteria. Actin polymerisation underneath the port of entry was also observed for an M5 strain (Molinari, Rohde,

Guzmán, & Chhatwal, 2000), which suggests a similar uptake mechanism. At later time points, M1 bacteria were found inside membrane-bound vacuoles marked by the lysosome LAMP-1 protein, which strongly suggests that vacuoles that contain this M1 streptococcus fuse with lysosomes and ultimately end up in phagolysosomes (Dombek, et al., 1999). Intracellular trafficking to phagolysosomes accounts for the fact that most internalized M1 streptococci are ultimately killed within seven days. Nevertheless, a few streptococci escape and survive, and thereby provide a reservoir in the human body for recurrent infections. A recent report highlighted that an M1 streptococcus can invade endothelial cells, the cellular barriers that surround blood vessels, and showed that the M1 protein was the primary invasin (Ochel, Rohde, Chhatwal, & Talay, 2014).

Invasive serotype M3 *Streptococcus pyogenes* are among the most frequently isolated streptococci from patients who suffer from invasive streptococcal disease (Sumby, Whitney, Graviss, DeLeo, & Musser, 2006; Terao, Kawabata, Nakata, Nakagawa, & Hamada, 2002). These streptococci also lack genes that encode the high-affinity fibronectin-binding protein, SfbI. Instead, M3 streptococci harbor the *fbaB* gene, which is located within the fibronectin-collagen-T antigen (FCT) locus that contains genes for pilus proteins (Terao, et al., 2001; Terao, Kawabata, Nakata, Nakagawa, & Hamada, 2002). The FbaB fibronectin-binding protein is highly conserved among M3 clinical isolates and was demonstrated to promote efficient invasion of endothelial cells by Nerlich et al. (Nerlich, et al., 2009). A study by Amelung et al. (Amelung, et al., 2011) confirmed the importance of FbaB in the uptake of M3 streptococci by endothelial cells. The uptake of streptococcus was accompanied by F-actin accumulation around adherent streptococci and formation of cellular membrane protrusions adjacent to entering bacteria. In addition, the actin branching protein, Arp2/3, was shown to accumulate near the point of entry. FbaB triggered a phagocytosis-like uptake mechanism, and intracellular streptococci travel the classical endocytic pathway. FbaB was also demonstrated to bind only to endothelial cell lines and not to epithelial cells—a possible FbaB explanation for the highly invasive nature of human M3 streptococci infections.

Dissemination of streptococci from blood vessels into tissue requires them to engage the basal side of the endothelial barrier. Indeed, passage through the endothelial cell barrier of umbilical cords in *ex vivo* experiments by M3/M18 streptococci was demonstrated. A phagocytosis-like uptake delivers streptococci into endocytic vacuoles. Those compartments fuse with lysosomes to form phagolysosomes, but some strains can trigger their own exocytosis by an unknown mechanism that involves the GTPase Rab27 (Talay, Nerlich, Dinkla, Rohde, & Chhatwal, 2008) Talay, personal communication). Figure 3 shows the dissemination of *S. pyogenes* after its intravenous administration to a mouse. Red streptococci have transmigrated through the endothelial cell barrier of the blood vessel, while green streptococci are still attached to the apical side of the endothelial barrier (facing into the blood stream). The blue streptococci may have been just released through that barrier by exocytosis.

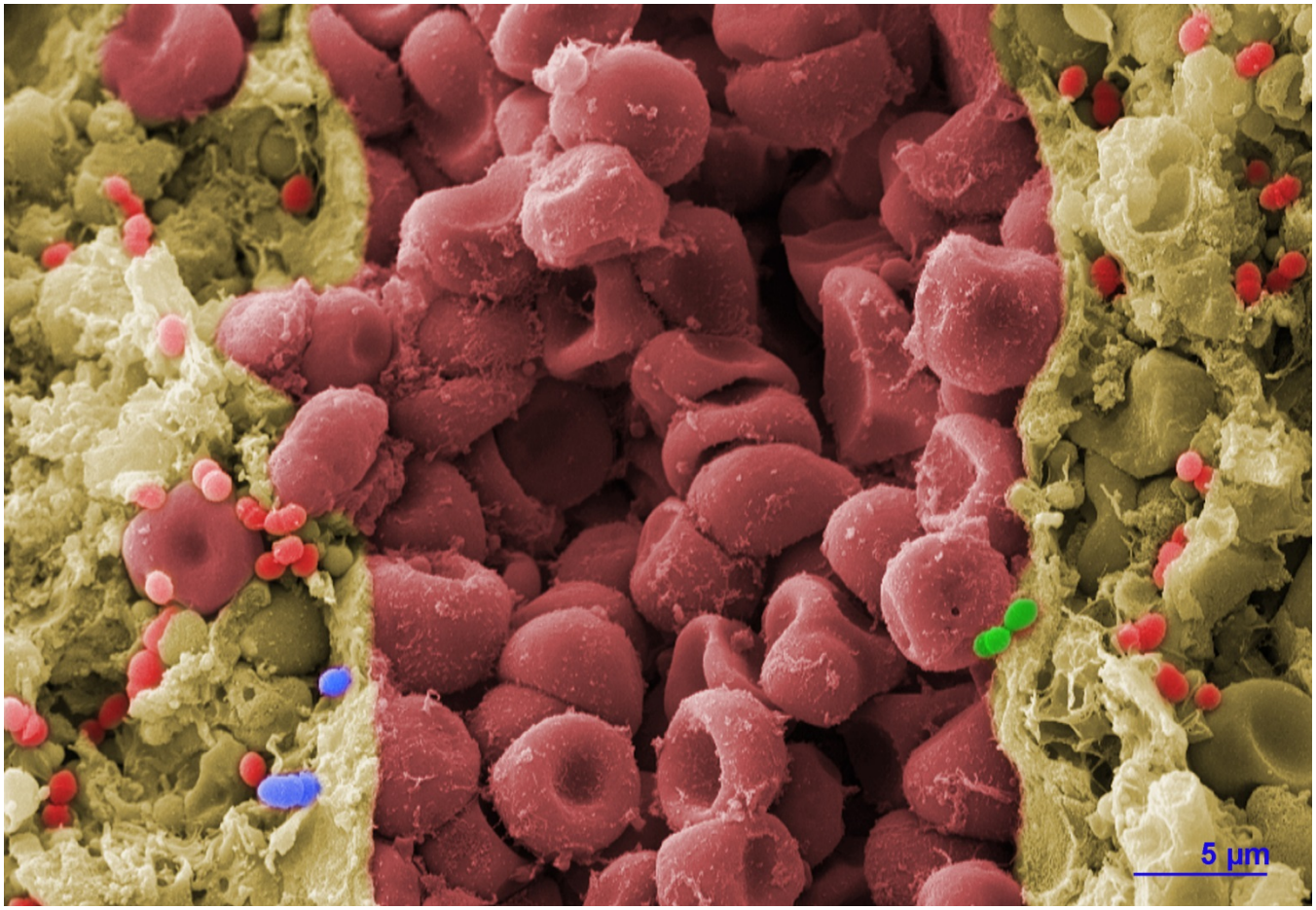

Figure 3. FESEM image of dissemination of *S. pyogenes* in the mouse model. *S. pyogenes* were intravenously administered into a mouse. From the blood stream, streptococci passed through the endothelial barrier layer of the blood vessels by an exocytosis process into deeper tissue (red blood cells are shown in red, streptococci shown in pink are residing in the adjacent tissue, green streptococci are attached to the endothelial barrier layer inside the blood vessel, and blue streptococci are exocytosed after passage through the endothelial layer). Samples are fractured after critical-point drying.

In summary, growing evidence suggests the following scenario for streptococcal invasion into epithelial and endothelial cells. SfbI and M proteins initiate the process by binding fibronectin, which then interacts with integrin receptors. Extensive integrin-clustering triggered caveolae aggregation to form large invaginations that ingest streptococci. Alternately, some streptococci that engage fibronectin with the M protein induce focal adhesion complexes, which are ingested through a zipper-like or membrane ruffling mechanism. The first mechanism ultimately directs streptococci to the safer caveosomal compartment within a cell, while the second mechanism deposits the bacteria into phagolysosomes, where most are destroyed. Figure 4 depicts these two different invasion mechanisms and intracellular trafficking routes.

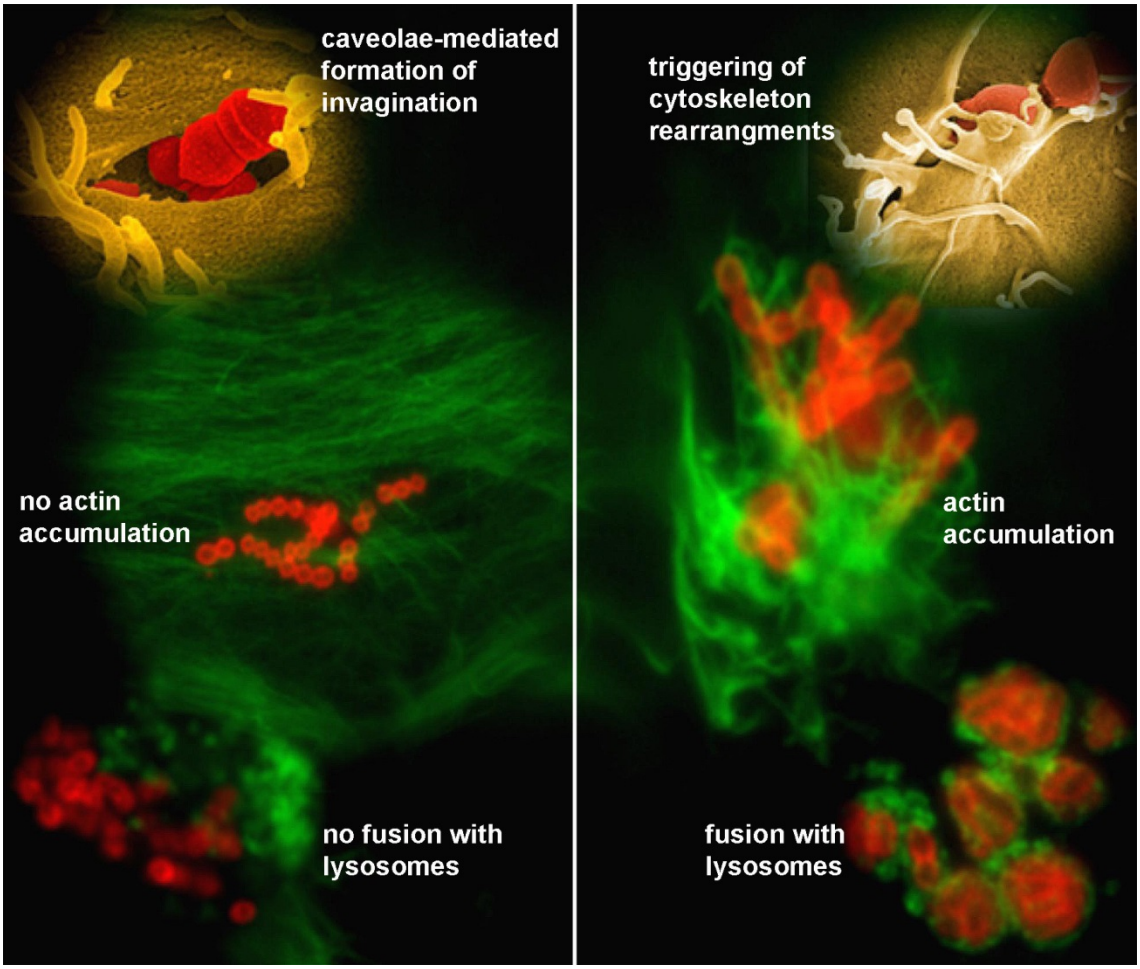

Figure 4. Summary of invasion mechanisms and subsequent intracellular trafficking of *S. pyogenes*. SfbI-expressing isolates co-opt caveolae for invasion into epithelial cells without detectable actin accumulation around invading streptococci. Intracellular trafficking bypasses fusion with lysosomes and intracellular streptococci reside in a safe niche within caveosomes. Non-SfbI-expressing isolates trigger cytoskeletal rearrangements with visible actin accumulation around invading streptococci and traffic inside the host cell through early and late endosomes, and fuse with lysosomes to form phagolysosomes. So far, all *S. pyogenes* invading through cytoskeletal rearrangements (zipper mechanism, membrane ruffling, or "flap-like") form phagolysosomes after fusing with lysosomes.

Streptococcal adhesion complexes, caveolae, and signal transduction

The fact that different strains of *S. pyogenes* express a variety of adhesins and invasins that interact with different host cellular receptors likely accounts for the diversity of tissue tropisms and a wide spectrum of human infections. Uptake by host cells is generated from α5β1 cellular receptors after interaction with fibronectin bound to the streptococci. These interactions trigger a cascade of signals that cause cytoskeleton rearrangements, and can lead to the ingestion of streptococci by non-phagocytic epithelial and endothelial cells. Investigations of signaling pathways generated by these events was motivated by the possibility of discovering targets for drugs that would prevent ingestion of streptococci into antibiotic impermeable cellular compartments, and that could reduce the incidence of antibiotic treatment failures that result in persistent carriage *of S. pyogenes*. Most studies of downstream signaling have focused on SfbI and M1. Although SfbI binds fibronectin with a much higher affinity than the M1 protein, both effectively link streptococci to α5β1 receptors. As described above, SfbI-Fn engagement of the receptor results in integrin clustering that leads to internalization of streptococci into caveosomes. In contrast, M1-Fn engagement of the same receptor results in actin rearrangement to form focal adhesion complexes and internalization of streptococci into phagolysosomes. Figure 5 depicts what is known about the signaling pathways generated by these streptococcal invasins, which lead to these distinct outcomes.

Differential signaling models for SfbI-Fn and M1-Fn mediated *S. pyogenes* invasion of epithelial cells, based on earlier work (Ozeri, et al., 2001) and the more recent work of Wang et al. (Wang, Yurecko, Dedhar, & Cleary, 2006b; Wang, Li, Dedhar, & Cleary, 2007) are proposed in Figure 5. Strain JSR4, an $M6^+$ $SfbI^+$ streptococcus, induced integrin complexes with phosphorylated focal adhesion kinase (FAK) and phosphorylated paxillin, small GTPases Rac, and Cdc42 (Ozeri, et al., 2001; Wang B. , Li, Dedhar, & Cleary, 2007). The activation of PI3K catalyzes phosphorylation of membrane-associated phosphatidylinositol, which binds to downstream targets, including integrin-linked kinase (ILK) (Persad, et al., 2001). Purushothaman et al. and others showed that both SfbI-Fn and M1-Fn mediate invasion of epithelial cells that requires phosphatidylinositol 3-kinase (PI3K) (Purushothaman, Wang, & Cleary, 2003) and ILK (Wang, Yurecko, Dedhar, & Cleary, 2006b). The latter is capable of phosphorylating the β_1 integrin cytoplasmic domain and is known to be a crucial link between integrins and the cytoskeleton. The invasion of epithelial cells by streptococci that expressed either M1 or SfbI proteins was impaired by a specific chemical inhibitor of ILK and by the expression of ILK-specific siRNA in target cells. Control assays using non-integrin dependent invasion of epithelial cells by *Salmonella* confirmed that the chemical inhibition of PI3K or ILK did not have a generally negative impact on the host cell's ability to ingest bacteria, but instead was specific to the Fn mediated uptake of *S. pyogenes*. ILK can indirectly activate Rac and Cdc42, which in turn can regulate actin cytoskeleton rearrangement (Hall, 1998), and can lead to various forms of cell adhesion complexes and stress fibers formation. Therefore, ILK may be a key intermediate protein between integrins and Rac and/or Cdc42—and it's clear that M1-Fn and SfbI-Fn complexes share a common signaling network.

How do these different fibronectin complexes finally direct streptococci into different intracellular compartments? M1-mediated invasion was shown to be significantly less sensitive to genistein than that promoted by SfbI, which suggests a branch in their respective pathways (Wang B. , Li, Southern, & Cleary, 2006a). The common signaling pathway activated by these distinct Fn-binding proteins was reported to diverge at Paxillin (Wang, Li, Dedhar, & Cleary, 2007) (Figure 5). Paxillin phosphorylation was induced by $M6^+$ $SfbI^+$ streptococci (Ozeri, et al., 2001), but the bacterial molecules leading to Paxillin phosphorylation were not defined, nor was the phosphorylated form of Paxillin shown to be required for invasion of epithelial cells in that study. Streptococci and Lactococci that express either surface protein invaded HeLa cells and promoted phosphorylation of Paxillin; however, the inhibition of Paxillin phosphorylation by PP2, a specific tyrosine kinase inhibitor, significantly prevented uptake of $M1^+$ streptococci by HeLa cells, but did not inhibit internalization of streptococci with SfbI on their surface. Still, Paxillin was observed underneath the attached $SfbI^+$ streptococci (Ozeri, et al., 2001) and, therefore, it likely participates in directing signals that lead to caveolae formation. In this case, Paxillin may serve as a scaffold for other components of the signaling complex.

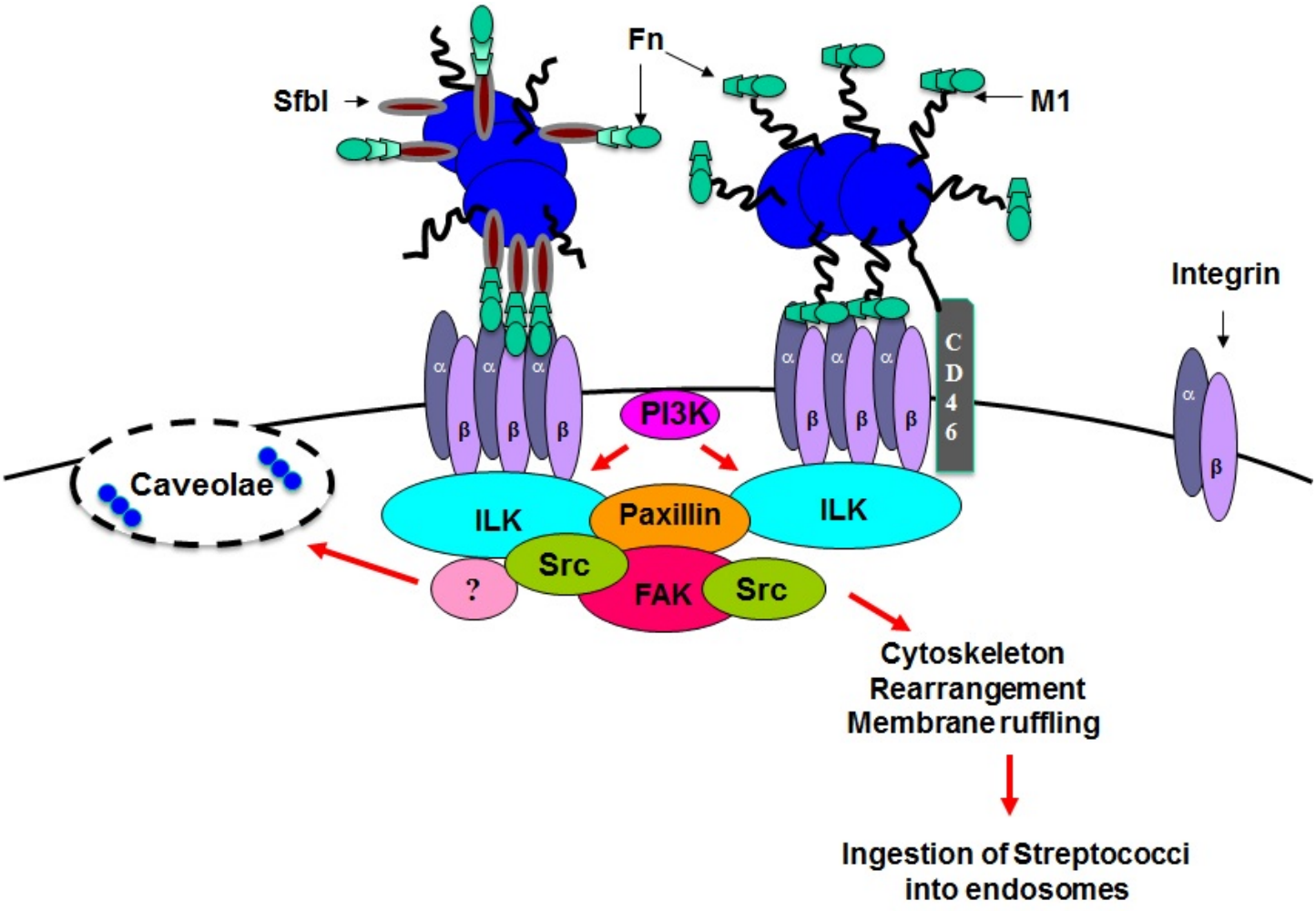

Figure 5. Model for differential ingestion of *S. pyogenes* mediated by Sfb1 and M1 proteins. Interactions of fibronectin bound to Sfb1 and M1 protein with epithelial cells lead to different ingestion pathways. Sfb1-Fn complexes promote uptake of streptococci via caveolae, while M1-Fn complexes foster receptor-mediated endocytosis. The model proposes that these two different streptococcal fibronectin binding proteins direct distinct changes in the quaternary structure of fibronectin, which in turn generate divergent signals when fibronectin engages α5β1 receptors at the surface of epithelial cells. In the end, streptococci are either deposited into relatively safe caveosomes or into bactericidal phagolysosomes. Common components of the signal cascade include PI3K, ILK, and Paxillin; however, data suggest that the signals diverge at the point of Paxillin phosphorylation.

The quaternary structure of fibronectin is partially influenced by the ligands it binds, including bacterial surface proteins. It is reasonable to postulate that the M1 protein and SfbI, which are structurally very different proteins, both induce different fibronectin quaternary conformations that inform the composition of focal adhesion complexes to generate different signals, which ultimately direct streptococci to different intracellular compartments. The impact of the ARO domain of SfbI on invasion supports this idea. As described above, the N-terminal ARO domain of SfbI is critical for ingestion of streptococci into caveosomes, and when removed or replaced by the ARO domain of GfbA, the trafficking route changed (Rohde, et al., 2011). It would be interesting to compare compositions and phosphorylation states of proteins in adhesion complexes generated by these different forms of SfbI.

The M1 protein is able to interact with multiple cellular receptors, either directly or indirectly, including CD46, a cofactor in Factor I-mediated inactivation of complement proteins C3b and C46 (Liszewski, Post, & Atkinson, 1991). CD46 is expressed on the surfaces of most human cells and was shown to enhance invasion of epithelial cells by $M3^+$ and $M1^+$ streptococci (Berkower, Ravins, Moses, & Hanski, 1999; Rezcallah, et al., 2005). Rezcallah et al. demonstrated that over-expression of CD46 with a deletion of the cytoplasmic domain reduced invasion of

epithelial cells. Their results suggested that optimal M1 protein mediated ingestion of *S. pyogenes* requires co-stimulation from both CD46 and α5β1 cellular receptors (Rezcallah, et al., 2005). There are still many important unanswered questions that remain before a clear understanding of the molecular cross-talk between *S. pyogenes* and their host cells can be obtained. The potential to apply this information to the development of drugs, such as kinase inhibitors or peptide mimics that block the bacteria-bound fibronectin engagement with integrins, also remains as an important challenge.

Invasion of professional phagocytes

The classical intracellular pathogens not only efficiently invade epithelial cells, but also survive ingestion by macrophages by blocking intracellular armaments in various ways. Some escape phagosomes and replicate in the cytoplasm of these and other cells. Molinari et al. were the first to identify a strain of *S. pyogenes*, A8, which actually escaped the phagosome and multiplied extensively in the cytoplasm of HEp2 cells (Molinari & Chhatwal, 1998). Resident macrophages and polymorphonuclear neutrophils (PMNs) constitute the first line of cellular defense against invading streptococci. Contrary to the long-held dogma that virulent streptococci are resistant to phagocytosis in blood, some strains of *S. pyogenes* are able to survive the intracellular killing mechanisms of PMNs (Medina, Rohde, & Chhatwal, 2003a). Details of these molecular processes still have to be elucidated, but streptococci clearly have the ability to escape from the phagocytic vacuole into the cytoplasma of PMNs and replicate (Medina, Goldmann, Toppel, & Chhatwal, 2003b). Furthermore, surviving streptococci exhibited increased virulence that was associated with the production of enlarged, hyaluronic acid capsules. Consistent with findings from Medina et al. (Medina, Rohde, & Chhatwal, 2003a), Staali and colleagues reported that M1 protein and the M-like protein H were crucial for the intracellular survival of *S. pyogenes* in PMNs (Staali, Mörgelin, Björck, & Tapper, 2003). Their subsequent work suggested that both M protein and protein H impair the fusion of azurophilic granules with the phagosome, thereby preventing a step that leads to the maturation of bactericidal phagosomes (Staali, Bauer, Mörgelin, Björck, & Tapper, 2006). Moreover, streptococci can accelerate neutrophil apoptosis, which facilitate their escape from host cells (Kobayashi, et al., 2003). The above results support the intriguing hypothesis that PMNs may function as a vehicle for their dissemination through the blood stream; namely, that they serve as a Trojan horse.

Surprisingly, and also in contrast to the assumption that macrophages are responsible for the clearance of streptococci from infected tissue, live streptococci were conclusively found within macrophages in biopsies of patients with soft tissue infections (Thulin, et al., 2006). Small numbers of intracellular streptococci were predominantly found in non-inflamed tissue, while large bacterial loads were observed in inflamed tissue, even after prolonged intravenous treatment with antibiotics. The implication of this study is that intracellular streptococci spread locally to uninfected tissue in low numbers.

Clinical relevance of intracellular streptococci

As described above, the capacity to invade endothelial cells and to survive ingestion by professional phagocytes is consistent with the potential of *S. pyogenes* to cause invasive disease. The epidemiology of *S. pyogenes* infections over the past 20 years confirms that *S. pyogenes* strains are not all equally able to cause serious systemic illness. The high frequency of intracellular invasions of cell lines by multiple strains of the globally disseminated M1 clone (Cleary, et al., 1992; LaPenta, Rubens, Chi, & Cleary, 1994) suggested a positive relationship between that phenotype and the world wide increase in systemic infections in the early 1990s (Musser, et al., 1995). Other M1 subclones isolated from cases of uncomplicated pharyngitis were significantly less able to enter HEp-2 cells (Cleary, LaPenta, Vessela, Lam, & Cue, 1998a). Strains isolated from cases of invasive disease in Spain between 1998 and 2009 were also dominated by the *emm1*/ST28 subclone, which again suggests that the M1 strains represent a hypervirulent lineage of *S. pyogenes* (Montes, et al., 2011). The capacity of M1 and M3 streptococci to be ingested by endothelial cells, which constitute the barriers that line blood vessels (Amelung, et al., 2011; Ochel, Rohde, Chhatwal, & Talay, 2014), also supports the possibility that

intracellular invasion of these cells leads to rapid dissemination into underlying soft tissue after exposure to streptococci. This may explain why cases of invasive infections with these M types have been reported in previously healthy persons without an apparent wound or other port of entry into soft tissue (Stevens, et al., 1989). Perhaps streptococci that hide in the endothelial barrier are also a source of transient bacteremia.

Several more recent surveys that compared strains isolated from sterile tissue to those from non-sterile tissues for the potential to express known invasins or to invade cell lines question the link between high-frequency invasions and disseminated, systemic diseases. Molinari et al. were the first to report that *S. pyogenes* isolated from throats and skin were engulfed by epithelial cells at a much higher frequency than those isolated from blood (Molinari & Chhatwal, 1998; Molinari, Rohde, Guzmán, & Chhatwal, 2000). Analysis of *S. pyogenes* isolates from patients with invasive or uncomplicated infections from Italian and Australian populations found no significant difference in the frequency of genes that encode fibronectin-binding proteins, SfbI, Sfb2, or fba54 (Baldassarri, et al., 2007; Delvecchio, Currie, McArthur, Walker, & Sriprakash, 2002; Musumeci, et al., 2003). The reasons for such systemic dissemination are likely to be complex and to depend on unique expression of an array of multiple virulence genes by highly virulent clones; a variety of host factors must also surely play a role. The capacity to multiply and survive in blood due to mutations in the CovR/S locus that up-regulate the expression of many genes (up to 10% of transcribed genes, including those responsible for hyaluronic acid capsule and a variety of other known determinants of virulence) is one explanation for such hypervirulent clones (Cao, et al., 2014; Sumby, et al., 2005; Sumby, Whitney, Graviss, DeLeo, & Musser, 2006). Although hypervirulent clones of *S. pyogenes* clearly exist, the exact relationship of intracellular invasion to systemic disease is still uncertain.

Several studies suggest that the capacity to hide inside cells is an important characteristic of streptococcal strains that persist in the throats and/or tonsils of asymptomatic carriers or that subsequently resist antibiotic therapy. The intracellular state may significantly increase the capacity of this bacterium to disseminate and persist in human populations.

When strains from carriers and patients with uncomplicated pharyngitis and sepsis were compared, respectively, those from carriers were observed to adhere to and be internalized by HEp-2 cell at higher frequency than strains that were successfully eradicated by antibiotic treatments (Neeman, Keller, Barzilai, Korenman, & Sela, 1998; Sela, Neeman, Keller, & Barzilai, 2000). The former also more often carried the SfbI gene. Contrary to these results a smaller study conducted by Brandt et al. (Brandt, et al., 2001) concluded that the expression of SfbI does not contribute to treatment failure and to the subsequent asymptomatic carriage of streptococci. They reported that only 11 isolates from 4 of 18 patients harbored SfbI. Unfortunately, these authors did not compare strains from treatment failure to those successfully eradicated by antibiotics for their capacity to invade epithelial cells; an essential comparison, since surface proteins other than SfbI can also promote high-frequency invasion (Brandt, et al., 2001). In-vitro serial passage of highly variable M1 cultures through human epithelial cells could enrich more invasive streptococci, presumably concentrating the number of M^+ streptococci in the culture (Cleary, et al., 1998b); therefore, *in vivo* cycling of streptococci between the interior and exterior of the mucosal epithelium may select for variants that are more efficiently internalized.

The earliest, most direct evidence that intracellular streptococci are an important source for the dissemination of streptococci and recurrent tonsillitis is based on microscopic studies of surgically excised tonsils (Österlund & Engstrand, 1995; Österlund & Engstrand, 1997; Österlund, Popa, Nikkilä, Scheynius, & Engstrand, 1997). Most tonsil specimens from children undergoing surgery to stop recurrent tonsillitis harbored viable streptococci within epithelial cells along the tonsillar crypts. *S. pyogenes* was also observed in macrophage-like cells at high frequency in these specimens. Tonsils from control subjects who had their tonsils removed for other reasons did not contain streptococci. More recently, viable intracellular *S. pyogenes* and *Staphylococcus aureus* were found in tonsil specimens of patients plagued by recurrent tonsillitis (Podbielski, et al., 2003; Zautner, et al., 2010). The hyper-invasive M1 subclone was confirmed to efficiently invade primary keratinized tonsillar epithelial cells *in vitro* (Cue, et al., 2000). A tropism for nasal associated lymphoid tissue, tissue known to be functionally

homologous to human tonsils, was discovered using a murine intranasal infection model. M1 streptococci were observed inside M-like cells, sporadically located along the base of the nasal epithelium, within 6 hours after intranasal inoculation. By 24 hours post-inoculation, micro-colonies were observed throughout this lymphoid organ (Park, Francis, Yu, & Cleary, 2003). From 1–10% of streptococci present in single-cell suspensions of nasal-associated lymphoid tissue after intranasal challenge of mice were resistant to penicillin, which confirms their intracellular location.

Surprisingly, *S. pyogenes* has never acquired beta-lactamase genes or penicillin binding protein based resistance to penicillin, even though that antibiotic has been the primary treatment for pharyngitis for decades (Horn, et al., 1998). However, 30 to 40% of children continue to shed streptococci after treatment with penicillin. Brandt et al. reported an 80% relapse by the same initial strain following vigorous penicillin therapy (Brandt, et al., 2001). In vitro, intracellular *S. pyogenes* can resist at least 100 µg/ml of penicillin (unpublished data). Kaplan et al. observed no degradation of intracellular streptococci after exposure to bactericidal levels of penicillin; however, erythromycin or azithromycin that penetrate mammalian cells efficiently can kill intracellular streptococci (Kaplan, Chhatwal, & Rohde, 2006). Those findings support the clinical decision to use these macrolides for a complete elimination of *S. pyogenes* from patients plagued by recurrent tonsillitis. The over-use of penicillin for treatment of pharyngitis may further select for strains that can be efficiently internalized by epithelial cells. High-frequency intracellular invasion of the mucosal epithelium may increase the rate of antibiotic therapy failure, and therefore, increase the size of the human reservoir that can disseminate the organism to others in the population. As this reservoir enlarges, the probability of serious, systemic infection will also increase, and as a result, the strains or serotypes that are less able to acquire an antibiotic-free niche may be less often associated with severe disease. Facinelli and colleagues (Facinelli, Spinaci, Magi, Giovanetti, & Varaldo, 2001) observed an association between genetic resistance to erythromycin (ErmR) and the more efficient uptake of *S. pyogenes* by epithelial cells, which suggests that erythromycin resistance may have been genetically linked to efficient invasion of human cells and thus was co-selected by antibiotic therapy (Facinelli, Spinaci, Magi, Giovanetti, & Varaldo, 2001). SfbI and ErmR genes were commonly present and chromosomally linked in these clinical isolates, which were otherwise genetically diverse.

Considerable evidence leads to the conclusion that intracellular invasion provides a safe haven for pathogens from antibiotics and immune-system defenses, and therefore increases the incidence of asymptomatic carriage of these potentially very dangerous pathogens. Immune carriage of *S. pyogene*s is in some ways analogous to a benign, non-metastatic cancer cell, which is relatively dormant until a mutation converts it to an aggressive phenotype that is able to spread systemically. As reported by Treviño et al. (Treviño, et al., 2009), *S. pyogenes* isolated from the oral mucosa were able to grow in saliva, but grew poorly in blood. On the other hand, these streptococci readily accumulated mutations in the CovR/S master regulator of transcription with striking changes in transcriptome profiles. These changes led to better growth in blood and an inability to compete with wild-type bacteria in saliva (Treviño, et al., 2009). The intracellular persistence and carriage of *S. pyogenes* can also indirectly impact the incidence of systemic, deadly disease. A cluster of toxic shock cases in elderly individuals from southern Minnesota was caused by a serotype M3 clone. That clone was carried in the throats of nearly 40% of school children in nearby communities, which suggests that school children were the reservoir for *S. pyogenes* responsible for that outbreak of toxic shock in older disabled adults (Cockerill, et al., 1997).

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