The Natural Variants of the Streptococcus NAD+-glycohydrolase SPN Modulate Divergent Host Cell Signaling Pathways and Death

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The Natural Variants of the Streptococcus NAD\(^+\)-glycohydrolase SPN Modulate Divergent Host Cell Signaling Pathways and Death

by

Sukantha Chandrasekaran

A dissertation presented to the Graduate School of Arts and Sciences of Washington University in partial fulfillment of the requirements for the degree of Doctor of Philosophy

August 2013

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ABSTRACT OF THE DISSERTATION

The Natural Variants of the Streptococcus NAD\(^+\)-glycohydrolase SPN Modulate Divergent Host Cell Signaling Pathways and Death

by

Sukantha Chandrasekaran

Doctor of Philosophy in Molecular Microbiology and Microbial Pathogenesis

Washington University in St. Louis, 2013

Professor Michael G. Caparon, Chairperson

*Streptococcus pyogenes* has an arsenal of virulence factors that promote its ability to cause a broad range of diseases and fight host defenses. One of these factors is the *S. pyogenes* NAD\(^+\)-glycohydrolase (SPN), which is secreted from the bacterial cell and translocated into the host cell cytosol where it contributes to cell death. Every strain examined to date has SPN and recent studies suggest that SPN is continuing to evolve diverging into two variants that correlate with tissue tropism, one of which lacks the signature NADase activity. SPN’s roles in both cytotoxicity and niche selection are unknown. To gain insight into the forces driving the adaptation of SPN, a detailed comparison of representative glycohydrolase activity-proficient and -deficient variants was conducted.

Out of a total 454 amino acids, the glycohydrolase activity-deficient variants differed at only 9 highly conserved positions. A mutational approach to exchange these residues between variants revealed that reciprocal changes at 3 specific residues were required to both abolish activity of the proficient version and restore full activity to the deficient variant. Remarkably, all versions of SPN were equally cytotoxic to cultured epithelial cells. However, differences in
glycohydrolase activity had a significant influence in cell signaling resulting in cellular necrosis and the subsequent inflammatory response.

Necrosis requires SLO, which activates JNK and PARP. However, in combination with the NADase active SPN cells underwent a glycolytic cell death through the depletion of NAD$^+$ and ATP. As a consequence of active SPN, these cells were unable to accumulate PAR despite PARP activation. These effects were associated with the release of HMGB-1 from the nucleus and low levels of IL-8 and TNF alpha production. In contrast, the NADase inactive SPN accumulated PAR and underwent a JNK mediated cell death. These events likely contribute to the increased IL-8 and TNF alpha production seen in these infections however HMGB-1 is not released as in the NADase inactive infections.

Taken together, these data indicate that the glycohydrolase activity of SPN is not the only contribution the toxin has to the pathogenesis of *S. pyogenes* and that both versions of SPN play an important role during infection.
Chapter I

Introduction
Classification

The Streptococcus genus is a gram-positive lactic acid group of bacteria classified in the phylum Firmicutes. The genus has been divided into Lancefield groups which are based on the their serologic typing of the cell wall group A carbohydrate (41). Streptococci are non-motile, chain forming, spherical shaped facultative anaerobes. They are further categorized by their ability to lyse red blood cells and fall into one of three classes - alpha, beta or gamma. Alpha hemolytic Streptococcus species appear greenish on blood agar plates. Beta-hemolytic species show complete clearance due to the presence of Streptolysin S and O, while gamma hemolytic species lack the ability to lyse red blood cells. *Streptococcus pyogenes*, a Lancefield Group A species, is categorized as beta-hemolytic (13).

Group A Streptococcus or *S. pyogenes* is also differentiated within its species into classes based on the major virulence factor M protein adhesion, which is found on the surface of the bacterium. The N-terminus of this protein is hyper variable and over 200 *emm* gene types have been found (6) (78). Since the advent of serological typing of M protein, another method has been employed, termed *emm* pattern. This pattern is based on a highly correlated nucleotide sequence that involves the 5’ end region of the *emm* gene that encodes the M protein as well as up three other genes which are encoded in the cell wall spanning domain (33, 34). Upon analysis of *S. pyogenes* strains, three main *emm* pattern groups have emerged and these are known as A-C, D, and E (8). It is also important to note the *emm* types are restricted to a single pattern meaning that no strain can fall into two different pattern groups. These groups have a strong correlation for tissue tropism. The *emm* patterns A-C have a strong propensity to cause throat infections while *emm* pattern D strains tend to cause impetigo (9). These strains are considered to be specialists due to their propensity to cause infections at a single site, the pharynx or the
skin, while emm pattern E strains are considered to be generalists since they cause infections at both the pharynx and the skin. A recent study of over a 1000 isolates found emm patterns D and E to be the most prevalent with only 20% of the strains emm pattern A-C. (50). Figure 1 depicts the difference between these emm patterns on the genotypic level.

**Figure 1.** Map of the emm patterns found in *S. pyogenes* strains. Represented are the most common gene combinations that consist of the *emm* and FCT region within different *emm* pattern grouping. Genes shown in blue are found in emm patterns A-C, D (84) and E (purple). Adapted from Ref (9).

**Diseases and Prevalence and Burden**

*Streptococcus pyogenes* is a significant human pathogen due to its versatility. It can cause more types of disease than any other bacterium and infects many different sites within the human host (Figure 2). This large spectrum of infections ranges from superficial infections, like
impetigo and pharyngitis, to systemic infections like toxic-shock-like syndrome, as well as the severe and invasive necrotizing fasciitis. Suppurative infections of the soft tissue including skin, tonsils and soft membranes can lead to more invasive infections, though these are less common. The most common infections are pharyngitis and pyoderma and occur most often in children (77) (25). Other infections include streptococcal toxic shock syndrome (STSS), cellulitis, bacteremia, pneumonia, and puerperal sepsis. Typically, these infections are treated with β-lactam antibiotics such as penicillin and cephalosporins.

![Figure 2: Pathogenesis of Streptococcus pyogenes infections.](image)

*Figure 2: Pathogenesis of Streptococcus pyogenes infections.* *S. pyogenes* can infect numerous sites within the host as depicted in the diagram. Adapted from (61)
In addition to these infections, post-infection sequelae can manifest and are due to autoimmune reactions by cross-reactive antibodies. These sequelae include rheumatic fever and post-streptococcal glomerulonephritis (23). Rheumatic fever can manifest 1 to 5 weeks after the initial pharyngeal infection and occurs due to an autoimmune reaction by T cells to streptococcal components and host tissue. This can lead to endocarditis, arthritis, subcutaneous nodules and erythema marginatum (79). Post-streptococcal glomerulonephritis occurs 1 to 4 weeks after the initial streptococcal infection and is characterized by edema, hypertension, and hematuria. It is also based on cross-reactive antibodies to both streptococcal and host antigens. \textit{S. pyogenes} is capable of both stimulating a strong immune response and in converse causing non-inflammatory infections. In many cases, these bacteria can be carried asymptotically. These features make \textit{S. pyogenes} an intriguing and formidable human pathogen.

The global burden of \textit{S. pyogenes} is significant and has a major role in mortality and morbidity. It is thought to cause over 700 million cases of superficial infections and over 18 million cases of severe infections each year ultimately causing over 500,000 deaths (15). Within the U.S., approximately 10,000 invasive cases of \textit{S. pyogenes} are reported with a 10% mortality rate (18). Necrotizing fasciitis accounts for 6-7% of these cases. Although invasive cases are relatively rare, once the infection has progressed mortality rates can reach up to 25%. In recent years there has been a dramatic increase in the number of severe invasive \textit{S. pyogenes} infections (57). There is also a significant economic burden in the U.S. costing between 224 million and 539 million US dollars for pharyngitis in children alone (63).

Though post-infection sequelae are not as common in developed countries, these are prevalent in developing countries. It is estimated that over 2 million children are affected with rheumatic heart disease with a total of 15 to 19 million people alive with rheumatic heart disease
(15). Around 80% of all rheumatic heart disease cases come from less developed countries. The same is true for glomerulonephritis. Around 400,000 cases occur per year with around 95% of these cases occurring in less developed countries (15). A more recently discovered sequela of *S. pyogenes* is the Pediatric Autoimmune Neuropsychiatric Disorders associated with streptococci or PANDAS. Although controversial, a link has been discovered between the streptococcal infection and obsessive-compulsive behavior that can be resolved with treatment with penicillin (45, 88).

In addition to causing active infections *S. pyogenes* can also be carried asymptptomatically and has been reported to be found in the throats of 15% of school-aged children. Other studies have shown that *S. pyogenes* can be found in the tonsils of asymptomatic carriers (60) (81). In addition to the nasopharynx, the vagina has been a site for asymptomatic carriage and the source of *S. pyogenes* outbreaks (7). Though vaginal carriage is typically low at 0.03%, the presence of *Streptococcus* in the vagina is increased after or during an episode of pharyngitis (4, 51). Occasionally, these carriers can give rise to outbreaks (76). Recently, the global transcriptional factor catabolite control protein A, CcpA was found to play an important role in asymptomatic murine vaginal colonization. CcpA has a major role in the regulation of virulence factor expression in response to the environment, thus implicating a function for virulence factors in carriage (87). Asymptomatic carriage allows for easier dissemination of the pathogen and can contribute to an eventual infection (49). It is thought that the failure to eradicate *S. pyogenes* after antibiotic treatment occurs in 5-30% of pharyngitis and this failure may contribute to asymptomatic carriage or recurrent infections (48, 64, 73). *S. pyogenes* is strictly a human pathogen, thus carriage becomes an important means for dissemination.
Thus far, *S. pyogenes* has not developed resistance to the β-lactam class of antibiotics although there are cases of macrolide resistance (26). Some models suggest *S. pyogenes* ability to maintain reservoirs despite antibiotic treatment is based on their ability to invade host epithelial cells. Electron microscopy and immunohistochemistry of pharyngeal epithelial cells from patients with tonsillitis demonstrated the presence of intracellular *S. pyogenes* (60). Other studies indicate that a specific transcriptional profile is necessary for survival within macrophages (31). While there are several studies suggesting *S. pyogenes* can be found intracellularly, it does not seem to be its primary or preferred environment since the majority of the bacteria are found extracellularly.

**Adhesion**

One of the most important interactions between *S. pyogenes* and the human cell is adherence. This initial interaction is the determining factor for successful colonization as the bacteria has to overcome several barriers such as the mucosal layer. For *S. pyogenes*, there are several factors that contribute to adhesion including M protein, F protein, lipoteichoic acid (LTA), hyaluronic acid and several others (10, 14, 59). Attachment to different cells is likely dependent on different sets of adhesions. Of these is M protein and can be grouped into different emm patterns (Figure 3). Specifically, M protein has been found to be important in keratinocyte adherence and binds to CD46 on the host cell membrane (58). This protein has a conserved carboxyl terminus consisting of C repeats and an amino terminus that is highly variable (Figure 3). Deleting C1 and C2 repeats of the M protein reduces adherence to keratinocytes (62). Additionally, the heterologous expression of M protein in *Lactococcus lactis* conferred adherence on A549 cells expressing CD46 (68). M protein has a multifactorial role due to its
ability to interact with multiple proteins on the host cell for adherence as well as to confer phagocytic resistance.

Figure 3. The structure of various M proteins grouped by emm pattern. The proline-glycine rich C terminus is covalently linked to the peptidoglycan. A, B, C, D represents blocks of amino acid repeats found in each M type. The C block is generally conserved while A and B blocks are variable between serotypes and linked to the N-terminal hypervariable region. Above are representative M proteins from each emm pattern group. Adapted from (74).

Another group of proteins made by *S. pyogenes* termed fibronectin binding proteins (FnBPs) bind to fibronectin, a large glycoprotein found in the extracellular matrix of human cells, to adhere to cells. Fibronectin interacts with membrane receptor proteins called integrins, which are important in cell-to-cell adhesion, growth and migration. *S. pyogenes* makes a diverse number of FnBPs and although not all isolates make this protein, it may contribute to tissue
tropism (19). Currently, 11 FnBPs have been discovered, most of which are bound to the bacterial wall via an LPXTG motif (90). Protein F, the first FnBP identified, has been shown to bind free fibronectin which subsequently inhibits the protein’s ability to bind to cellular fibronectin (1). Deletion of this Protein F also completely abrogates the ability of S. pyogenes to bind to epithelial cells even in the presence of M protein (30). This protein may act like a receptor for S. pyogenes in a two-step mechanism that depends on LTA as the initiating molecule. After this initial interaction between LTA and fibronectin, a second adhesin like protein F or M strengthens the bond to the host (19) (72). In addition to adherence, the interaction between FnBPs and fibronectin can stimulate signal transduction pathways in the host cell as well as help S. pyogenes evade the innate immune system (90).

Without adherence, S. pyogenes would not be able to cause an infection as it would likely slough off with the epithelium or mucosa. Adherence of the bacterium also allows for the onslaught of virulence factors S. pyogenes produces that affect so many processes within the host cell.

**Virulence Factors**

S. pyogenes is a versatile pathogen due to the impressive number of virulence factors it produces during the course of an infection (Figure 4) (22). These factors are often dependent on environment and the growth phase of the bacteria. One of these is the hyaluronic acid capsule, which adheres to cells expressing CD44 (5). In addition to contributing to adherence along with M protein, it is also required for resisting phagocytosis (89). The capsule consists of a high molecular mass polymer that is nearly identical to polysaccharides in the human host, thus facilitating host evasion (21) (22). Isolates of S. pyogenes can differ in the amount of capsule
made and knockout of the *has* operon responsible for the production of the capsules can reduce virulence the strain in certain types of infections (3).

Another mechanism for *S. pyogenes* to subvert the immune system is the production of several superantigens known as streptococcal pyrogenic exotoxin. These proteins enhance toxic shock by targeting T-cell receptors and MHC II molecules to cause these cells to proliferate and mount an excessive inflammatory response. The resulting cytokines can lead to multi-organ failure. Some evidence suggests these superantigens also play a role in contributing to autoimmune responses resulting in post-infection sequelae (86). Conversely, *S. pyogenes* also produces several proteins to subvert the immune system, such as C5a peptidase. This serine protease is anchored to the bacterial cell wall and can cleave the human complement protein C5a to prevent recruitment of immune cells. This is another barrier to the complement system and phagocytosis in conjunction with M protein (37).

One of the most abundantly made virulence factors is a broad-spectrum cysteine protease called streptococcal pyrogenic exotoxin or SpeB. During stationary phase, this protein is secreted through the sec machinery as a 40 kDa zymogen that is proteolytically cleaved to its active form at 28 kDa. Nutrient availability and environmental cues also contribute to activating the expression of SpeB (44). Although the *speB* gene is highly conserved and found in the majority of strains (91), it is not always expressed from these strains due to various factors. SpeB has multiple roles including cleaving both host and bacterial proteins. In terms of the host, SpeB cleaves or degrades several host immune factors including immunoglobulins, complement protein C3b, chemokines, fibrinogen, and integrins (56) (39). SpeB not only helps evade the host immune system by degrading these proteins but it also contributes to inflammation by cleaving pro-interleukin IL-1β into its active form (38). On the bacterial side, SpeB hydrolyzes adhesins
including M protein and protein F contributing to the release of the bacteria from the host cell membrane. In addition, SpeB cleaves other proteins secreted by the bacteria including virulence factors streptokinase, DNase SdaI, and Streptolysin O. This may contribute to both immune evasion as well as altering the function of these proteins during an infection (56). SpeB is a model for transcriptional regulation of a virulence factor as it is controlled by several different mechanisms that factor into how the protein contributes to virulence.

Streptolysin O and Streptolysin S (SLS) are two hemolysins that are capable of lysing cells, however they play distinct roles in pathogenesis. SLS is a small peptide typically bound to the bacterial cell presumably through an interaction with LTA (83). This non-immunogenic peptide is modified extensively post-translation before secretion (55). The toxicity of this oxygen stable protein is based on contact between the bacterial cell and host. SLS also affects the host’s ability to recruit neutrophils, possibly by affecting chemoattractants. In the zebrafish model, an SLS mutant was less virulent than the wild-type strain and exhibited an increase in neutrophil recruitment (43). Although capable of hemolysis, SLO is considerably different. This oxygen labile protein is a large cholesterol dependent cytolysin that is secreted from the sec machinery and required for the delivery of another virulence factor, SPN.
Figure 4. Virulence Factors produced *Streptococcus pyogenes*. Over 40 proteins are produced by the bacterium and contribute to the versatility of this pathogen. Several proteins are necessary for adhesion and colonization while others are necessary for host damage and immune evasion. Adapted from (54).

**Cholesterol-Dependent Cytolysins**

This large family of proteins is produced by many Gram-positive species including *Listeria*, *Arcanobacterium*, *Gardnerella*, and *Bacillus*. Since the discovery of pneumolysin, the CDC belonging to *Streptococcus pneumoniae*, over 25 more CDCs have been reported. These secreted proteins have 40-70% amino acid sequence similarity (35). These CDCs form large beta-barrel pore complexes that consist of 35-50 soluble monomers assembled on the host cell membrane to form pores between 25 to 30 nm in diameter. Cholesterol functions as a receptor for many of these proteins however other cellular structures function as the primary receptor as in the case of Intermedilysin, lectinolysin, and vaginolysin which bind CD59. Though in these cases, binding...
may still require the presence of cholesterol. Structurally, CDCs can be divided into four discontinuous domains based on the solved structure of *Clostridium perfringens* perfringolysin O (Figure 5) (63)(70). The mechanism of CDC pore formation is based on the extensive studies performed on this structure. Secreted monomers bind the host cell membrane and form oligomers before inserting into the membrane. The initial interaction is between the CDC and cholesterol is based on a conserved amino acid motif called the undecapeptide (ECTGLAWEWWR) found in domain 4 as well as the loops that anchor the monomer to the membrane (67). Monomers interact through this tip perpendicular to the membrane. The interaction with the membrane then leads to several conformational changes with several monomers to form the pre-pore complex that forms a ring without the insertion of the beta-barrel pore. Domain 3, positioned above the bilayer, unravels two alpha helices creating two transmembrane beta- hairpins (β1- β4). Specifically, the β1 strand of one monomer interacts with the β4 of the adjacent monomer. The prepore complex then inserts the amphipathic beta-hairpins into the membrane to finally form the pore (Figure 5).
**Figure 5. Structure of PFO and CDC pore formation.** The structure of PFO is divided into 4 domains (35). Once Domain 4 has anchored into the membrane domain 3 then undergoes a conformational change to insert beta-hairpins into the membrane. Domains 1 and 2 collapse to allow domain 3 to insert β-hairpins into the membrane to form the pore. Adapted from (32).

Although these CDCs share a large degree of similarity, their differences contribute variable interactions with the membrane and ultimately function. For example, ILY, a CDC that binds to CD59 only lyses human cells due to its unique domain 4 sequence (66). SLO is a unique CDC as it also possesses an 66 residue N-terminal extension (40)(52). This unique extension also contains a cleavage site for SpeB which leads liberates 47 amino acids from SLO (65). Each form of SLO is made abundantly and retains hemolytic activity. Although SLO binds cholesterol, its receptor is still unknown. SLO forms pores large enough to allow for passive diffusion of foreign proteins (85). Interestingly at sub-lethal concentrations of SLO, pores can be healed by the cell through the release of the damaged membranes in a calcium dependent manner (36, 85). Although this group of proteins is well characterized for their ability to form pores,
recent studies show there are alternative functions independent of pore formation. In the case of SLO, it also functions to translocate the NAD\(^+\)-glycohydrolase SPN into the host cell cytosol. This process has been shown to be pore independent indicating a multifunctional role for SLO (47).

**Cytolysin Mediated Translocation**

This injection-like mechanism is the first of its kind to be described in a Gram-positive bacterium. This process is much like the Type III secretion system (T3SS) found in many Gram-negative bacteria. The T3SS Gram-negative species largely contributes to virulence and effectors from this pathway often modify different processes within the host (29, 50, 78). This system is specialized in that it forms a needle-like complex that functions to secrete proteins through two bacterial membranes as well as the host cytoplasmic membrane (Figure 6)(18). Gram-positive bacteria lack this outer membrane and typically secrete proteins into the extracellular milieu. *S. pyogenes* specifically only possesses the Sec system of secretion thus all secreted proteins utilize this machinery. In *S. pyogenes*, once released from the secretion machinery, SLO interacts with SPN at the host cell membrane to translocate SPN into the host cell.
Figure 6. Type III secretion found in some pathogenic Gram-negative bacteria. Several proteins form this needle-like complex that traverses two bacterial membranes and the host membrane to allow for the translocation of effector proteins. Adapted from (20).

In the S. pyogenes genome, spn and slo genes are encoded in the same operon along with a third gene, ifs, which is the endogenous inhibitor of SPN (Figure 7). All strains examined to date possess the gene for spn (2, 69). Little is known about the regulation of this operon. Presently, it is thought a promoter is present upstream of spn which is expected to control all three genes. However, other studies have suggested slo has an autonomous promoter found within ifs sequence (71). The same study reported a negative regulator of SLO, sloR, found 19kb upstream of the slo gene. This regulator is thought to act solely on SLO and has no effect on the transcription of spn. Findings within our lab suggest all three are co-transcribed under the same promoter as a polar insertional activation of SPN abolished the expression of SLO (46).

The recent emergence of a highly virulent strain of S. pyogenes has given some insight into the regulation of the operon encoding SPN and SLO. This M1T1 strain is most often associated with invasive infections and carries a mutation in the two component regulatory
system CovRS. This mutation in the regulatory covR gene leads to increased production of several virulence factors including capsule, DNase Sda1, IL-8 protease SpyCEP, SLO and SPN (16, 80). Also associated with this is the loss of SpeB production, which can cleave SPN and SLO (17). *S. pyogenes* has numerous methods for transcriptional regulation and this is just one mechanism employed during the course of an infection.

**Figure 7. SPN, IFS and SLO operon.** The three genes involved in CMT are encoded in the same operon occupying approximately 3.7 Kb of the chromosome. Only SPN and SLO are secreted through the Sec machinery. Upstream of spn is nusG, a transcription termination factor not involved in CMT.

SPN and SLO possess a secretion signal at the N-terminus allowing for the secretion of these two proteins from the bacterial cytosol through the sec machinery and into the extracellular space. If a host cell is present, the interaction between these two proteins allows for the translocation of SPN through the cell membrane and into the cytosol (Figure 8). In the absence of SLO, translocation cannot occur. The deletion of SPN reduces cytotoxicity despite the presence of SLO pore formation (46). Together, SPN and SLO contribute to cytotoxicity in a synergistic manner.

In addition, CMT is dependent upon adherence of the bacteria to the host cell membrane as a deletion of M protein abolishes translocation in epithelial cells (46). It is well known SLO
can form pores large enough (30nm) for passive diffusion of proteins (85), however infection with isogenic slo and spn deletion mutants does not restore CMT indicating that this is an active process. Once secreted, approximately 80% of the total amount of SPN is translocated into the cytosol. This process is also dependent specifically on SLO, substituting with PFO, a highly similar CDC, does not confer CMT despite pore formation (52). The N-terminal of SLO is unique and removal of this sequence abolishes CMT. However, attaching this sequence to PFO also does not reconstitute CMT. Recently, our lab has shown that this complicated mechanism is not pore dependent. Mutating SLO to the monomer locked form (G398V/G399V) or the prepore locked form (Y255A) abolished the ability of SLO to form pores however despite this loss, SPN was still translocated into the cytosol (47). This prepore oligomer sits on the surface of the membrane and is based on the formation of hydrogen bonds between monomers (24). In the case of the prepore locked form, the amount of SPN found in the cell is near wild-type levels and approximately 50% of wild-type in the monomer locked SLO infection. Thus pore formation and translocation of SPN can be uncoupled. Taken together, CMT requires a specific interaction between SLO and SPN that occurs in close proximity at the host cell membrane. It is likely SPN also interacts with a different receptor on the host cell. This process is coordinated through the interactions between SPN and SLO as well as SLO and the host membrane.
Figure 8. Cytolysin Mediated Translocation. SPN, SLO and IFS are made within the bacteria during exponential growth. Within the bacterial cytosol, IFS can bind to SPN to prevent NAD$^+$ cleavage within the host cell. SPN and SLO are translocated from the bacterium through the sec machinery into the extracellular milieu. The bacteria can adhere to the host cell M protein and CD46. Secreted SLO monomers then oligomerize on the host membrane and form pores. The interaction between SLO and SPN on the host cell membrane allows for the translocation of SPN into the host cell cytosol and is not pore dependent.
SPN

SPN is the only known effector of CMT. SPN was first discovered for its NAD$^+$-glycohydrolase activity, able to cleave β-NAD$^+$ at the nicotinamide-ribosyl bond into two products: nicotinamide and ADP-ribose (Figure 9). This robust enzymatic activity is thought to contribute to virulence of *S. pyogenes* (2, 17, 36, 82). Prior to extensive studies done in the Caparon lab, it was previously reported that SPN also contained cyclase and ADP-ribosyl transferase abilities (2). However, since developing the capacity to purify SPN in an *E. coli* expression system, we have found that SPN ultimately lacks both of those activities as well as the inability to catalyze methanolysis or transglycosidation (27). SPN is a strict β-NAD$^+$-glycohydrolase and is unlike other classes of NAD$^+$ cleaving enzymes that are multi-functional and capable of further catalyzing products from the initial reaction. SPN is capable of cleaving NAD$^+$ at incredibly fast rates, greater than 8000 molecules per second, which is uncharacteristic of these multi-functional NAD$^+$ cleaving enzymes that typically cleave approximately 10 molecules of NAD$^+$ per second (27). SPN also has low product inhibition while nicotinamide can compete for the active site in these other classes.

![Figure 9. NAD+ cleavage](image)
Though we know SPN is translocated through its interaction with SLO during CMT, it is still unknown if CMT requires a signal sequence. It is also unknown if other effectors are injected in the host cytosol along with SPN. Thus far, studies within the Caparon lab have shown that the process of CMT is specific and discriminates between substrates. A study involving a different secreted toxin called mitogenic factor (MF) showed that MF is not translocated into the host cytosol unless it is fused with SPN in its entirety (28). Furthermore, any deletions within SPN also abolish CMT though deletions of up to 190 amino acids in the N-terminal portion of SPN do not abolish the NADase activity, thus uncoupling translocation from the NADase domain. Deletions in the C-terminus of SPN abolished NADase activity as well as CMT. To further elucidate SPN’s specificity for CMT, chimeras of MF and increasing amounts of N-terminus of SPN were constructed and it was found that none of these proteins could be translocated. Placing MF in the middle of SPN also abolished CMT, although it had no effect on the NADase activity. The only case in which mitogenic factor can enter the host cell is when it is positioned at the end of the entire SPN sequence. In this case, the chimera was both CMT competent and as well as NADase active (28). The fact that any deletion in SPN has a deleterious effect on CMT indicates that it is specific effector for this discriminating process. It also indicates an intact SPN is likely necessary to successfully interact with both the secretion system within the bacteria in addition to SLO on the eukaryotic membrane that ultimately leads to the translocation of SPN into the cytosol.

Based on the above data, SPN has two domains. The first 190 amino acids of SPN resemble a “jelly roll” domain, which likely binds to a target other than SLO on the host membrane that allows for translocation. The data mentioned above show that this N-terminus is necessary but not sufficient for CMT and that NADase domain can properly fold into an active
enzyme in the absence of the N-terminus. Analysis of the primary sequence of SPN shows that these 190 amino acids do not share any homology to other eukaryotic NAD\(^+\)-glycohydrolases. Taken together, SPN is a multi-domain toxin that interacts with the host cell in stages via SLO.

As robust NAD\(^-\)-glycohydrolase, SPN is also toxic to the bacteria. \(\beta\)-NAD\(^+\) is a co-factor for many metabolic factors in both the eukaryotic and prokaryotic systems. This made SPN impossible to clone in \textit{E. coli} until the discovery of the immunity factor of SPN termed IFS (53). This gene is located in between \textit{spn} and \textit{slo} in the same operon and is essential for the maintenance of NADase active SPN. IFS resides in the cytoplasm and acts on SPN in a one to one ratio by competitively blocking the active pocket from NAD\(^+\) thus neutralizing the toxic effects of SPN within the bacteria (Figure 10). It is also important to note there are two subtypes of both SPN and IFS (69). One subtype is the well-known NADase active enzyme that is dependent on the presence of IFS however another subtype exists that completely lacks this NADase activity and is associated with a truncated form of IFS. In this case, IFS is no longer necessary for the bacteria to maintain this NADase inactive form.

**Figure 10. The crystal structure of SPN and IFS.** SPN residues 191 to 451 were crystalized with IFS showing IFS binds to SPN blocking the active site for NAD\(^+\). SPN is presented in
yellow and IFS in blue. The first 38 amino acids are the signal sequence. Residues 39 to 190 could not be crystallized (green). Adapted from (75).

The NADase domain from residues 191 to 491 was crystallized along with IFS to determine how IFS binds to SPN and prevents NAD$^+$ cleavage (Figure 10). IFS undergoes major conformational change upon binding to SPN. Unbound IFS has a free C-terminal tail that when bound to SPN becomes a compact structure located on stop of the active site of SPN. Deleting various portions of the tail has been shown to cause toxicity to *E. coli* during the induction of SPN in an expression system (Chandrasekaran, unpublished). Structural analysis of SPN and its interaction with IFS has given us insight into how these two proteins interact as well as structural similarities of SPN with other NAD cleaving enzymes. Despite lacking the ability to transfer ADP-ribose to an acceptor molecule, SPN does share some major similarities. The overall structure of SPN overlays with two other bacterial ADP-ribosyl transferase families: the cholera toxin and diphtheria toxin. There are 3 motifs characteristic of this superfamily: the Arg/His, STS, and ADP-ribosyl turn turn motif (ARTT) that consists of two glutamic acids essential for the cleavage of NAD$^+$ in this family. An alignment of SPN with others in the ADP-RT family shows that it has a His of the A/H motif (His273) and the bi-glutamic acid motif (E389 and E391) (Figure 11). However, the STS motif is lacking in SPN though it does have amino acids similar in structure in place of these specific residues. Interestingly, mutation of the bi-glutamic acid motif in SPN does reduce the enzyme’s ability to cleave NAD at fast rates. Though it does completely abolish the NADase activity, an E391Q mutation reduces the rate of cleavage from approximately 8400 molecules per second down to 2 molecules per second. Another major difference between SPN and the ADP-RT family is the “scorpion” motif (42). This motif
consists of X-Y$_{10}$-X that contributes to substrate interaction and cleavage in ADP-RTs. In the cholera toxin family, the STS motif is found at one of this scorpion and acts as the attacker in the process of NAD$^+$ cleavage. Though SPN does not have a typical scorpion motif, it does have an elongated sequence similar to a scorpion that is flanked by the FVT sequence that replaces this STS motif. This structural analysis has given us insight into two families of NAD$^+$ cleaving enzymes and reasons as to why SPN is not able to transfer ADP-ribose groups to acceptor molecules.

![Figure 11](image.png)

**Figure 11.** The ARTT-like motif of SPN overlayed with ADP-ribosylating toxins in the dipherthia toxin family (DTx) and the cholera toxin family (CTx/C3). SPN residues corresponding to each motif is colored accordingly. The R/H motif is shown in orange, ARTT motif shown in green and the STS motif shown in purple. Adapted from (75).

Every strain of *S. pyogenes* examined to date maintains the full-length gene for SPN. A recent genotypic study on various *S. pyogenes* strains conducted in the Caparon lab has shown that both the NADase active and inactive versions of SPN are equally prevalent (69) (2). This collection consisted of 113 strains from various types of infections, locations and spanning over 67 years. SPN was also found to be evolving under positive selection at several residues thought
to be involved in the NADase activity. Four Bayesian clusters were identified based on 10 of the 23 polymorphic amino acid residues, two active and two inactive (Table 1).

Table 1. Polymorphic residues span the length of SPN. Four Bayesian clusters of SPN were discovered upon analysis of *S. pyogenes* strains. NADase activity was based on the presence of an aspartic acid at residue 330 rather than a glycine. These isolates were also associated with a truncated IFS indicating the loss of NADase activity. Adapted from (69).

<table>
<thead>
<tr>
<th>Bayesian cluster</th>
<th>NADase activity</th>
<th>Translocation domain</th>
<th>SPN amino acid</th>
<th>NADase domain</th>
<th>IFS</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>+</td>
<td>103 136 143 195</td>
<td>R G H I</td>
<td>L I R G I</td>
<td>Full length</td>
</tr>
<tr>
<td>2</td>
<td>+</td>
<td>103 136 143 195</td>
<td>R G Q M</td>
<td>L V L R G I</td>
<td>Full length</td>
</tr>
<tr>
<td>3</td>
<td>-</td>
<td>103 136 143 195</td>
<td>H R Q I</td>
<td>L V V K D Y</td>
<td>Truncated</td>
</tr>
<tr>
<td>4</td>
<td>-</td>
<td>103 136 143 195</td>
<td>R G Q M</td>
<td>L V L N D I</td>
<td>Truncated</td>
</tr>
</tbody>
</table>

* Bayesian clusters were identified through Bayesian analysis of population structure (BAPS).
* NADase activity is predicted based on the presence of a glycine at residue 330 in active strains (+) or an aspartic acid at residue 330 in inactive strains (−).
* Residues are numbered based on the SPN consensus sequence shown in Fig. 4.
* IFS domains are illustrated in Fig. 4.
* Indicates the ββ allele associated with the Bayesian *spa* cluster. Examples of full-length form and several truncated forms of IFS are illustrated in Fig. 2. Either the truncated 1 or the truncated 2 form of IFS may be paired with either Bayesian *spa* cluster 3 or Bayesian *spa* cluster 4. The two ββ alleles that encode a truncated 3 protein are found in strains with Bayesian *spa* cluster 4.

This finding correlates with the hypothesis that SPN is multifunctional and is preserved for reasons beyond the NADase activity. Despite the presence of full length SPN, IFS was found to be degrading as it is truncated when associated with SPN containing the G330D polymorphism that reduces NADase activity. The NADase activity of SPN could not be associated with severity of disease however one major finding did conclude that SPN could be correlated to tissue tropism. This study showed that the majority of NADase active strains were generalists (*emm* pattern E) able to cause infections at both the pharynx and the skin while the NADase inactive were associated with specialists (*emm* pattern A-C and D). Taken together, these data suggest a NADase independent role for SPN that has yet to be studied. IFS is under purifying selection while SPN is maintained in its entirety and is found equally among clinical
isolates. A NADase independent role of SPN must be contributing to the pathogenesis of SPN. This role is unknown and is the focus of the studies undertaken for this thesis. Prior to this work, few studies have been focused on how the NADase activity of SPN may contribute to virulence (11, 12) but none have examined the possibility of a NADase independent function to SPN.

AIM and SCOPE of THESIS

The goal of this thesis was to gain a greater understanding of SPN and how the toxin contributes to the pathogenesis of *S. pyogenes*. The work described here focuses on two aspects of the natural variants of SPN: the enzymatic activity and the effects of SPN on the host cell. Thus far, extensive studies on the residues important for the NADase activity and the differences between the two variants have not been conducted. Despite the presence of the NADase inactive SPN, research has not been conducted concerning possible roles for this version during an infection. The lack of NADase activity was also thought to be a lack of toxicity however research described in this thesis shows that is not the case and in fact, the NADase inactive SPN has a role in the pathogenesis of *S. pyogenes*. Both the active and inactive form of SPN generates different signaling events within the cell that eventually leads to two different types of necrosis. Thus, SPN is a multifunctional toxin and the work presented in this thesis highlights the versatility of *S. pyogenes* pathogenic abilities.
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Chapter II

Analysis of Polymorphic Residues Reveals Distinct Enzymatic and Cytotoxic Activities of the *Streptococcus pyogenes* NAD\(^+\) Glycohydrolase

Sukantha Chandrasekaran, Joydeep Ghosh, Gary Port, Eun-ik Koh, and Michael Caparon

The Streptococcus pyogenes NAD$^+$ glycohydrolase (SPN) is secreted from the bacterial cell and translocated into the host cell cytosol where it contributes to cell death. Recent studies suggest that SPN is evolving and has diverged into NAD$^+$ glycohydrolase inactive variants that correlate with tissue tropism. However, the role of SPN in both cytotoxicity and niche selection are unknown. To gain insight into the forces driving the adaptation of SPN, a detailed comparison of representative glycohydrolase activity-proficient and -deficient variants was conducted. Out of a total 454 amino acids the activity-deficient variants differed at only 9 highly conserved positions. Exchanging residues between variants revealed that no one single residue could account for the inability of the deficient variants to cleave the glycosidic bond of β-NAD$^+$ into nicotinamide and ADP-ribose. Rather, reciprocal changes at 3 specific residues were required to both abolish activity of the proficient version and restore full activity to the deficient variant. Changing any combination of one or two residues resulted in intermediate activity. However, a change to any one residue resulted in a significant decrease in enzyme efficiency. A similar pattern involving multiple residues was observed for comparison to a second highly conserved activity-deficient variant class. Remarkably, despite differences in glycohydrolase activity, all versions of SPN were equally cytotoxic to cultured epithelial cells. These data indicate that the glycohydrolase activity of SPN may not be the only contribution the toxin has to the pathogenesis of S. pyogenes and both versions of SPN play an important role during infection.
INTRODUCTION

Within a single bacterial species, allelic variation of a specific gene can arise through the process of niche specialization. For bacterial pathogens, this can occur when a generalist population diverges into distinct subpopulations with strong tropism for different hosts or for different tissues within the same host. Diversity emerges as continued selection pressure results in variants of virulence genes that function to increase fitness for infection of a particular niche. Understanding the functional consequences of these changes can provide important insights into how a specific virulence factor contributes to exploitation of a niche and its role in pathogenesis.

Variation in the *Streptococcus pyogenes* (group A streptococcus) NAD$^+$ glycohydrolase toxin (SPN, also known as NGA) has been associated with niche specialization. This Gram-positive pathogen is one of the most versatile pathogens of humans capable of causing both superficial and invasive diseases including pharyngitis, impetigo, and necrotizing fasciitis, as well as post-infection sequelae such as rheumatic fever and acute glomerulonephritis. Part of this versatility can be attributed to its ability to secrete a multitude of proteins that affect host cell function in numerous ways (8). One of these is SPN, which was originally identified by its ability to cleave the nicotinamide-ribosyl bond of β-NAD$^+$ to produce nicotinamide and adenosine diphosphoribose (ADPr). All *S. pyogenes* strains examined to date possess the gene encoding SPN (26). However, more recent studies have shown that these *spn* alleles exhibit diversity. Furthermore, SPN is evolving under positive selection and is diverging into multiple subtypes, including subtypes that lack its signature NAD$^+$ glycohydrolase (NADase) activity. Neither the function of the NADase-inactive subtypes nor the molecular basis of their loss of activity are well-understood.
However, it is understood that while there is not a clear consensus as to whether SPN subtypes have any association with isolates that can cause invasive disease, there is a strong association between SPN subtype and tissue tropism. Most cases of *S. pyogenes* infection are superficial and occur at one of two tissue sites: the throat (pharyngitis) or the skin (impetigo). Substantial epidemiological evidence indicates that there are distinct subpopulations of strains that are specialized for infection of just one of these two tissues (specialists). There is also a distinct subpopulation (generalists) that readily infects either tissue. Analysis of a collection of 113 strains that was assembled to maximize diversity revealed that intact alleles of *spn* were found in all strains and that NADase-active and –inactive haplotypes were equally prevalent (26). Of interest, tissue and throat specialist strains correlated with NADase-inactive SPN, while generalist strains correlated with NADase-active SPN. The reason underlying these associations is unknown. Furthermore, since there is evidence that SPN’s NADase activity can contribute to pathogenesis, the prevalence of NADase-inactive SPN is unclear.

The observation that NADase-inactive SPN remains under positive selection suggests that it does contribute to pathogenesis. Support for this idea comes through analysis of an endogenous competitive inhibitor of SPN’s NADase activity known as Immunity Factor for SPN (IFS). The gene encoding IFS is located immediately adjacent to *spn* and the ability of *S. pyogenes* to produce NADase-active SPN is absolutely dependent on IFS, which acts to inhibit self-toxicity that may arise from any SPN molecules that inadvertently fold prior to their export from the streptococcal cell (22). As expected for an essential activity, *ifs* has very little sequence divergence in these strains. However, NADase-inactive SPN haplotypes are associated with nonfunctional truncated alleles of *ifs* that are undergoing a pattern of random nucleotide change characteristic of a pseudogene. Thus, while *ifs* loses selective constraint and becomes non-
functional, the same degradation does not occur for NADase-inactive \textit{spn}, which remains under positive selection.

During infection, SPN undergoes complicated interactions with host cells that provide some clues to its role. While \textit{S. pyogenes} is an extracellular pathogen, SPN is delivered into the host cell cytoplasmic compartment by a process termed Cytolysin-Mediated Translocation (CMT). In this process, \textit{S. pyogenes} first attaches to a host cell and exports SPN via the general secretory pathway. It is then translocated across the host cell membrane by an unknown mechanism that requires a second streptococcal protein, the cholesterol-dependent cytolysin streptolysin O (20). Analyses of CMT have revealed that SPN has multiple domains, including an N-terminal domain required for translocation and a C-terminal enzymatic domain (10). Both active and inactive SPN haplotypes undergo CMT and \textit{S. pyogenes} mutants defective for expression of either SPN or SLO have a reduced cytotoxic effect for host epithelial cells. How these activities may contribute to niche specialization is not known.

Multiple enzymatic activities have been attributed to NADase-active SPN that could contribute to cytotoxicity, including ADP-ribosyl transferase, ADP-ribosyl cyclase and NADase activities (15, 30). The structure of the enzymatic domain of SPN has recently been solved and shows that it is related to the ADP-ribosyl transferase family of bacterial toxins (29). However, a re-evaluation of SPN’s enzymatic activity using highly purified recombinant protein has shown that SPN functions strictly as a NADase (9). Interestingly, comparison of NADase-active and –inactive subtypes reveals that the inactive haplotypes segregate into only 2 Bayesian clusters and these differ only minutely from the active haplotypes, varying at only 9-10 amino acid residues out of 454 total. Of these, only 5-6 polymorphic residues are located in the enzymatic domain, and all were identified as sites that are evolving under positive selection (26).
Although minor, these polymorphisms contribute to a significant difference in enzymatic activity and may affect pathogenesis and niche specialization. Thus, in order to gain greater insight into the function of SPN, its two phenotypic subtypes and the forces that are driving their evolution, we conducted a detailed analysis of the effect of polymorphism on enzymatic activity and the contribution of enzymatic activity to cytotoxicity. These studies revealed that differences in enzymatic activity were the result of polymorphism at not one, but rather multiple residues. Furthermore, this analysis revealed an unexpected NADase-independent cytotoxic activity that was retained by the enzymatically-inactive haplotypes. Together, these data reveal that SPN has multiple activities and provide insight into its molecular evolution.
RESULTS

SPN Diversity.

As discussed above, all S. pyogenes genomes analyzed to date possess the gene encoding SPN. However, it has been recognized that this population has distinct functional heterogeneity, including haplotypes that lack SPN’s signature NADase activity. Our prior analysis of 113 genomes from a strain collection assembled to reflect diversity indicated that these SPN haplotypes grouped into 4 Bayesian clusters 2 of which lacked detectable NADase activity (26). For the present study, we chose two strains (JRS4 and HSC5) whose SPN haplotypes were representative of the most common NADase active and inactive Bayesian Clusters (Clusters 2 and 3, respectively). A direct comparison between NADase-active SPN\(_{J4}\) (from strain JRS4), and NADase activity-deficient SPN\(_{H5}\), (from strain HSC5) revealed the presence of 9 polymorphic residues, each of which has previously been shown to be under positive selection in the activity-deficient haplotypes (26). Of these, three were located in the translocation domain with the remaining residues distributed throughout the enzymatic domain (Fig. 1). As is typical, the gene encoding the NADase-active enzyme (SPN\(_{J4}\)) is associated with an intact gene for IFS, the endogenous inhibitor of SPN, while that of the activity-deficient protein (SPN\(_{H5}\)) is associated with a truncated IFS gene (Figure 1) (26).
Figure 1. Distribution of polymorphic residues between two representative SPN alleles. The top of the Figure shows the domain structure of SPN from a strain (JRS4) with an active NAD$^+$-glycohydrolase (NADase) activity (SPN$_{J4}$). The signal sequence (SS) is not part of the mature protein. Indicated above the Figure are the locations of the various polymorphic residues when compared to SPN from a strain (HSC5) representative of the major class deficient for NAD$^+$-glycohydrolase activity (SPN$_{H5}$). The asterisks below show the location of two glutamate residues implicated in catalysis. The bottom of the Figure lists the identities of the polymorphic residues at each position. Also indicated is whether the gene encoding the SPN allele is associated with an intact (full-length) or degraded (truncated) gene encoding IFS, the endogenous inhibitor of SPN.

Multiple polymorphic residues at the C-terminal end of SPN contribute to enzymatic activity. To determine which polymorphic residues may affect NADase activity, mutations were made to switch residues in NADase-active SPN$_{J4}$ to their counterparts from activity-deficient SPN$_{H5}$. To provide a uniform background for comparison, the mutant genes were expressed from a plasmid vector that was introduced into a derivative of strain JRS4 (SPN1) engineered to
contain an internal deletion of its native *spn* chromosomal locus (20). All proteins were found to be expressed at equivalent levels following analysis of cell-free culture supernatants by Western blotting (data not shown). These supernatants were then subjected to a standard end-point titer assay to determine the relative levels of NADase activity produced by each protein. Individual swaps of the residues located in the translocation domain had no significant affect on the proteins’ ability to cleave a β-NAD$^+$ substrate (R103H, G136R, M195I; Fig. 2) as compared to the native SPN$_{J4}$ (Unmod, Fig. 2). Similarly, swaps of the 3 N-terminal residues of the enzymatic domain (L199I, Q253H, L280V; Fig. 2) also did not affect activity. In contrast, swaps of the 7$^{	ext{th}}$ and 9$^{	ext{th}}$ polymorphic residues (R289K, I374V) resulted in significant reductions in NADase activities ($P > 0.05$, Fig. 2). Consistent with prior reports (26, 32), a swap of the 8$^{	ext{th}}$ polymorphic residue (G330D) resulted in an undetectable level of NADase activity in these supernatants (Fig. 2). These data indicate that the activity of SPN$_{J4}$ is influenced to varying degrees by 3 different C-terminal polymorphic residues.

**Figure 2. Three residues are required to restore NADase activity to SPNH5.** Polymorphic residues in SPN$_{J4}$ were changed to the corresponding residue in SPNH5, and those in SPNH5
changed to their counterparts in SPN\textsubscript{J4}, as indicated by the grey and black bars, respectively for comparison to the unmodified proteins (Unmod). The genes encoding the mutations were expressed from a plasmid (Table S2) introduced into a derivative of JRS4 (SPN1) with a deletion of its resident gene. NADase activities were determined from cell-free culture supernatants as described in the Materials and Methods and are presented relative to SPN\textsubscript{J4}. A “BL” indicates that activity was below the limit of detection (<0.5%). A double asterisk indicates significantly less NADase activity when compared to unmodified SPN\textsubscript{J4} (\(P < 0.01\)). Data shown are the mean and standard error of the mean derived from at least three independent experiments.

**Conversion to a NADase-active enzyme requires three residues**

Reciprocal exchanges of the 3 C-terminal residues affecting activity of SPN\textsubscript{J4} were then made in NADase inactive SPN\textsubscript{H5}. Starting with the most N-terminal amino acids, a swap at residue 289 of SPN\textsubscript{H5} (K289R) did not increase the NADase activity of the resulting protein above background levels (Fig. 2). Also, despite its dramatic effect on reduction of SPN\textsubscript{J4} activity, the reciprocal exchange at residue 330 of SPN\textsubscript{H5} (D330G) resulted in only a very modest increase in NADase activity, to levels less than 3.0% of SPH\textsubscript{J4} (Fig. 2). An exchange of both of these C-terminal residues resulted in a protein with a significant increase in activity over the single-swap proteins (\(P < 0.01\)), however, the NADase activity of this protein was only at levels equivalent to approximately 30% of SPN\textsubscript{J4} (K289R + D330G, Fig. 2). Activity equivalent to that of SPN\textsubscript{J4} was obtained by the reciprocal swap of all 3 C-terminal residues (K289R + D330G + V374I, Fig. 2). Thus, all 3 of these residues make a critical contribution to the functional difference between these two naturally occurring variants of SPN.
**SPN\textsubscript{J4} single and double residue swap proteins have detectable activity.**

In our prior analysis of 131 clinical isolates, all alleles from NADase-active Bayesian clusters 1 and 2 contained the identical C-terminal residues at the positions analyzed above for SPN\textsubscript{J4} (26). Similarly, all activity-deficient cluster 3 proteins had the identical polymorphisms at these positions found in SPN\textsubscript{H5} (26). Thus, no naturally occurring intermediates containing just one or any combination of two of these polymorphisms were observed. To gain greater insight into this distribution, the properties of the engineered intermediate proteins were analyzed further. Recombinant versions of the intermediates were purified and their reaction products following incubation with \(\beta\)-NAD\(^+\) were analyzed by a more sensitive HPLC-based assay. After a one-hour reaction under these conditions, essentially all \(\beta\)-NAD\(^+\) was converted to nicotinamide and ADP-ribose by SPN\textsubscript{J4}, while all \(\beta\)-NAD\(^+\) remained uncleaved following reaction with SPN\textsubscript{H5} (Fig. 3A, 3B). In contrast to the assay described above, the R289K protein did not appear different from unmodified SPN\textsubscript{J4} and the G330D protein readily consumed substrate, although to a much lower extent than SPN\textsubscript{J4} (Fig. 3A, 3B). The ability of a double swap protein with these substitutions (R289K + G330D) to consume substrate was further reduced than by either single swap, although it was still more active than SPN\textsubscript{H5} (Fig. 3A, 3B). Similarly, the introduction of the I374V along with G330D resulted in a more inactive enzyme than the G330D protein alone (Fig. 3A, 3B). Only when all 3 polymorphisms were combined in the same protein did activity become indistinguishable from SPN\textsubscript{H5} (Fig. 3A, 3B). When combined with the results described above, these data suggest that the single and double swap proteins retain at least partial activity.
Figure 3. SPNJ4 single and double swap proteins have NADase activity. The various polymorphic residues from SPN_{H5} were introduced into SPN_{J4} as indicated in the Figure. The NADase activity of purified proteins was then analyzed using an HPLC-based assay. A. Representative HPLC chromatograms showing the products of enzymatic cleavage of β-NAD⁺ following a one hour reaction at 37°C. Also evaluated was a reaction mixture lacking protein (NAD control). Identities of the various products, including β-NAD⁺ (NAD), Nicotinamide and ADPribose (ADPr) are indicated at the top of the Figure. B. Relative activity of each of the proteins shown in Panel A quantitated as the percentage of the β-NAD⁺ substrate remaining uncleaved following a one hour reaction. A “BL” indicates that values were below the limit of detection (<0.05%) and an asterisk indicates that significantly more substrate was consumed.
when compared with SPN_{H5} (P < 0.05). Data presented are the mean and standard error of the mean derived from at least three independent experiments.

The NADase-deficient Bayesian cluster 4 protein has partial activity.

As discussed above, Bayesian cluster 4 proteins represent a second distinct cluster of NADase-deficient SPN in clinical isolates with a truncated immunity factor. These cluster 4 proteins differ from the activity proficient enzymes at 2 important residues. Firstly, they share the G330D polymorphism with the cluster 3 SPN_{H5} (Fig. 4A). Secondly, instead of the lysine for arginine substitution found in SPN_{H5}, the cluster 4 proteins substitute an asparagine (Fig. 4A). Finally, unlike the cluster 3 proteins, the cluster 4 proteins do not differ from the activity-proficient (SPN_{J4}) proteins at residue 374 (Fig. 4A). Single and double swaps of these polymorphic residues were introduced into SPN_{J4}, followed by purification and analysis utilizing HPLC-based assay as described above. Similar to the cluster 3 R289K polymorphism, the cluster 4 R289N single swap protein retained an appreciable ability to consume substrate (Fig. 4B). However, when combined with G330D, the resulting double swap protein failed to consume substrate and was indistinguishable from SPN_{H5} (Fig. 4B). Nevertheless, upon an extended period of incubation (up to 20 hrs), the cluster 4 double swap protein did demonstrate some activity, while both SPN_{H5} and its corresponding triple swap protein (R289K + G330D + I374V) failed to consume any $\beta$-NAD$^+$ (Fig. 4C). Taken together, these data indicate that polymorphism at two positions contribute to the NADase-deficient property of the cluster 4 protein, although it is more active than the cluster 3 protein.
A. 

<table>
<thead>
<tr>
<th>Cluster/NADase</th>
<th>103</th>
<th>136</th>
<th>195</th>
<th>199</th>
<th>253</th>
<th>280</th>
<th>289</th>
<th>330</th>
<th>374</th>
</tr>
</thead>
<tbody>
<tr>
<td>2/Active</td>
<td>R</td>
<td>G</td>
<td>M</td>
<td>L</td>
<td>Q</td>
<td>L</td>
<td>R</td>
<td>G</td>
<td>I</td>
</tr>
<tr>
<td>4/Deficient</td>
<td>R</td>
<td>G</td>
<td>M</td>
<td>L</td>
<td>Q</td>
<td>L</td>
<td>N</td>
<td>D*</td>
<td>I</td>
</tr>
</tbody>
</table>

B. 

Retention Time (min.)

C. 

% NAD remaining

- NAD control
- SPN14
- R289N
- R289K + G330D
- SPN14 + E374V
Figure 4. Cluster 4 swaps have low, but detectable NADase activity. A. Residues polymorphic between the Bayesian Cluster 2 (SPN\textsubscript{J4}) and the Cluster 4 proteins are shown. An asterisk indicates that the Cluster 4 residue corresponds to the residue in the Cluster 3 NADase activity deficient (SPN\textsubscript{H5}) protein (compare to Fig. 1A). B. Polymorphic residues from NADase activity deficient Cluster 4 were introduced into SPN\textsubscript{J4} as indicated and analyzed as described in Fig. 3. Shown are representative HPLC chromatograms indicating the products of enzymatic cleavage of β-NAD\textsuperscript{+} following a one hour reaction. The NAD\textsuperscript{+} control is a reaction that does not include enzyme. C. Relative activity of selected proteins following the extended incubation periods shown in the Figure. Data presented are the mean and standard error of the mean derived from at least three independent experiments.

Each C-terminal polymorphism reduces the rate of enzymatic activity.

In order to gain greater insight into the effect of each polymorphic residue on enzymatic activity, the kinetic properties of the various swap proteins were analyzed. Analysis of the SPN\textsubscript{J4} derivative modified at residue 289 revealed a modest (< 2-fold) decrease in $k_{cat}$ (R289K, R289N; Table 1). However, despite being able to cleave substrate at a fast rate, alterations at this position did have a marked effect on catalytic efficiency for the R289K protein, resulting in over a 10-fold increase in $K_m$ and an approximately 20-fold decrease in efficiency ($k_{cat}/K_m$, Table 2). The modification at residue 330 was more inhibitory, resulting in over a 5,000-fold decrease in $k_{cat}$ (G330D, Table 1) and in combination with other cluster 3 polymorphisms resulted in up to a 8-fold further decrease in $k_{cat}$ (Table 1). In the enzyme modified with both of the polymorphic residues of the cluster 4 protein, $k_{cat}$ was reduced nearly 60,000-fold, while for the enzyme with all 3 of the cluster 3 residues, $k_{cat}$ was below the limit of the assay (reduced over 2 x 10\textsuperscript{8}-fold vs
For all proteins containing the G330D polymorphism, it was not possible to determine $K_m$ at the concentrations of $\beta$-NAD$^+$ obtainable in the assay. Taken together, these data implicate a role for these 3 polymorphic residues in influencing substrate affinity.

Table 1. Enzymatic properties of $\text{SPN}_{14}$ variants

<table>
<thead>
<tr>
<th>Variant$^a$</th>
<th>Bayesian Cluster$^b$</th>
<th>$k_{cat}$ (min$^{-1})^c$</th>
<th>Fold Difference$^d$</th>
</tr>
</thead>
<tbody>
<tr>
<td>SPN$_{14}$</td>
<td></td>
<td>$2.21 \pm 0.25 \times 10^5$</td>
<td>-</td>
</tr>
<tr>
<td>R289K</td>
<td>3</td>
<td>$1.40 \pm 0.12 \times 10^5$</td>
<td>1.58</td>
</tr>
<tr>
<td>R289N</td>
<td>4</td>
<td>$1.26 \pm 0.08 \times 10^5$</td>
<td>1.75</td>
</tr>
<tr>
<td>G330D</td>
<td>3, 4</td>
<td>$39.6 \pm 1.5$</td>
<td>5580</td>
</tr>
<tr>
<td>G330D + I374V</td>
<td></td>
<td>$11.5 \pm 0.7$</td>
<td>$1.9 \times 10^4$</td>
</tr>
<tr>
<td>R289K + G330D</td>
<td></td>
<td>$6.3 \pm 0.2$</td>
<td>$3.5 \times 10^4$</td>
</tr>
<tr>
<td>R289N + G330D</td>
<td></td>
<td>$3.8 \pm 0.4$</td>
<td>$5.8 \times 10^4$</td>
</tr>
<tr>
<td>R289K + G330D + I374V</td>
<td></td>
<td>$&lt;1.0 \times 10^{-3}$</td>
<td>$&gt;2.6 \times 10^8$</td>
</tr>
</tbody>
</table>

$^a$Variants are derived from $\text{SPN}_{14}$. Recombinant proteins are purified from $E. coli$ as described in the Experimental Procedures.

$^b$Based on Riddle et al [2]. The number indicates the cluster(s) from which the polymorphism is derived.

$^c$Determined using the HPLC-based assay modified as described in the text. $k_{cat}$ of variants were derived from data in Supplemental Figure 1. Data represents mean ± SEM from at least three independent experiments.

$^d$Relative to $\text{SPN}_{14}$

Table 2. Catalytic Efficiency of $\text{SPN}_{14}$ and R289K

<table>
<thead>
<tr>
<th>Variant$^a$</th>
<th>$K_m$ ($\mu$M)$^b$</th>
<th>$k_{cat}/K_m$ $^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td>SPN$_{14}$</td>
<td>175 ± 30</td>
<td>1263</td>
</tr>
<tr>
<td>R289K</td>
<td>2140 ± 290</td>
<td>65</td>
</tr>
</tbody>
</table>

$^a$Variants and enzyme assays are described in Table 1.

$^b$Determined using the HPLC-based assay modified as described in the text. Data represents mean ± SEM from at least three independent experiments.

$^c$k$_{cat}$ from Table 1
**IFS is not essential in strains encoding minimally active SPN.**

In order to protect its own pool of intracellular $\beta$-NAD$^+$, *S. pyogenes* encodes IFS (Immunity Factor for SPN), an inhibitor of SPN’s NADase activity (22). However, in strains with a NADase-negative allele, the gene encoding IFS has accumulated mutations and has become a pseudogene (22, 26). Thus, to gain insight into the co-evolution of IFS and SPN, it was of interest to determine the requirement for IFS in strains of intermediate NADase activity. A test for essentiality was conducted using two different mutational strategies to determine if the IFS gene could be replaced or insertionally-inactivated in strains expressing various SPN alleles. Consistent with prior results (22), it was not possible to replace or inactivate *ifs* in a strain expressing SPN$_{J4}$ (JRS4, Table 3). However, when *spn* was deleted from this strain, mutations in *ifs* were readily obtained (SPN1, Table 3). Similarly, *ifs* was not essential for expression of the activity-deficient triple swap protein or any of the intermediate strains expressing a low activity SPN (Table 3). The exception was the intermediate strain of highest NADase activity (R289K) where no *ifs* insertion or replacement mutants could be recovered (Table 3). These results show that IFS is not absolutely required for expression of NADase-active SPN, but is required when a certain threshold of activity is exceeded.
NADase-deficient SPN is cytotoxic to eukaryotic cells.

The observation that both clusters of NADase activity-deficient SPN are maintained under positive selection, while the gene encoding IFS degrades into a pseudogene (26), suggests that the cluster 3 and cluster 4 proteins make an important contribution to pathogenesis independent of NADase activity. One documented function for NADase-proficient SPN is that it is cytotoxic for mammalian cells when translocated into their cytosolic compartment via CMT (3, 23). To gain insight into the role of NADase activity in this context, the native spn allele of JRS4 (Fig. 5A) was modified so that various SPN proteins containing an influenza hemagglutinin (HA)-epitope tag (Fig. 5B) would be expressed from an identical host background for comparison to a derivative with an in-frame deletion (Fig. 5A, SPN1, (20)). These included a triple mutant of SPN_{J4} (R289K + G330D + I374V) and SPN_{H5} (Fig. 5B), which were as efficiently translocated into A549 cell cytosols following infection as the SPN_{J4} derivative (Fig.

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Table 3. Essentiality of IFS in strains encoding various SPN alleles

<table>
<thead>
<tr>
<th>Strain</th>
<th>SPN allele</th>
<th>NADase Activity</th>
<th>IFS Essentiality</th>
</tr>
</thead>
<tbody>
<tr>
<td>Joy1</td>
<td>SPN_{J4}</td>
<td>+++</td>
<td>Yes</td>
</tr>
<tr>
<td>Suki3</td>
<td>SPN_{R289K}</td>
<td>++</td>
<td>Yes</td>
</tr>
<tr>
<td>Suki4</td>
<td>SPN_{G330D}</td>
<td>+</td>
<td>No</td>
</tr>
<tr>
<td>Suki5</td>
<td>SPN_{R289K/G330D}</td>
<td>+</td>
<td>No</td>
</tr>
<tr>
<td>Suki2</td>
<td>SPN_{TM}</td>
<td>–</td>
<td>No</td>
</tr>
<tr>
<td>Joy110</td>
<td>SPN_{H5}</td>
<td>–</td>
<td>No</td>
</tr>
<tr>
<td>SPN1</td>
<td>ΔSPN</td>
<td>–</td>
<td>No</td>
</tr>
<tr>
<td>HSC5</td>
<td>SPN_{H5}</td>
<td>–</td>
<td>No</td>
</tr>
</tbody>
</table>

---

*a* Recipient of IFS-inactivating plasmids. Strains were generated as described in Table S3.

*b* Defined as described in Table 1 as fold change in k_{cat} relative to SPN_{J4} as follows: (+++), equal to SPN_{J4}; (++), 1-2 fold lower; (+), 10^3 fold lower; (+), 10^8 fold lower.

*c* Defined by the ability to recover a viable mutant with a deletion or insertion mutation in ifs as described in the “Experimental Procedures.” Yes, IFS is essential; No, IFS is dispensable.
5C, Inset). Surprisingly, while the deletion strain was minimally cytotoxic as expected (Fig. 5C, SPN1), the strains expressing both NADase activity-deficient proteins were as cytotoxic as the strain expressing the SPN$_{J4}$ protein, with nearly all A549 cells showing evidence of membrane compromise following 7 hours of infection (Fig. 5C). These data suggested that NADase activity could be uncoupled from cytotoxic activity revealing it is not the sole function of SPN that contributes to cytotoxicity.

**Figure 5.** NADase-deficient SPN remains cytotoxic to A549 cells. A. The chromosomal *spn* loci of JRS4 and an in-frame deletion mutant of JRS4 (SPN1, (4)) are shown. Adjacent to the gene encoding SPN$_{J4}$ (spn), ifs encodes Immunity Factor for SPN and slo encodes Streptolysin
O. **B.** Strain Joy1 is a derivative of JRS4 expressing SPN\textsubscript{J4} with an HA epitope tag (black box) from the native \textit{spn} locus. Derived from Joy1 are strains Suki2 and Joy110, which express the chimeric SPN proteins listed in parentheses at the right of the Figure. Regions and residues derived from SPN\textsubscript{H5} are shown by shaded boxes and in italics, respectively. **C.** The strains expressing the proteins listed in parentheses were used to infect A549 cells and cytotoxicity evaluated at the indicated times by fluorescent microscopy following Live/Dead\textsuperscript{TM} staining. SPN1 does not express SPN protein (ΔSPN). Refer to Table S3 for detailed construction of these strains. Data presented represents the mean and SEM derived from at least 3 independent experiments. BL, below the limit of detection (< 0.3%). The Inset shows a Western blot analysis of A549 cytosolic fractions prepared after 5 hrs of infection developed with an HA-epitope antiserum. Lanes: 1, MW standards; 2, Uninfected; 3, Joy1(SPN\textsubscript{J4}); 4, SPN1(ΔSPN); 5, Suki2 (SPN\textsubscript{TM}); 6, Joy110 (SPN\textsubscript{H5}). The molecular weight of selected standards are indicated at the left of the inset.

To confirm this, the bi-glutamic acid motif previously shown to be essential for catalysis (9) was exchanged for glycines in all 3 of these proteins and cytotoxicity assessed. Again, all derivatives demonstrated an ability to damage A549 cells as compared to the deletion strain (Fig. 6). The strains expressing the proteins derived from either the triple mutant or SPN\textsubscript{H5} were not as cytotoxic as the SPN\textsubscript{J4} derivate, but this likely reflects a lower level of translocation into the host cell cytosols (Fig. 6, Inset).
Figure 6. Catalytic bi-glutamic acid residues are not required for cytotoxicity. The bi-glutamic acid residues involved in catalysis (see Fig. 1) were changed to glycines in SPN proteins SPN\textsubscript{J4}, SPN\textsubscript{TM} (R289K + G330D +I374V) and SPN\textsubscript{H5} at the chromosomal SPN locus in strain JRS4 to generate strains SPN\textsubscript{J4-GG}, SPN\textsubscript{TM-GG} and SPN\textsubscript{H5-GG}, respectively. Refer to Table S3 for detailed construction of these strains. The strains expressing the proteins listed in parentheses shown in the Figure were used to infected A549 cells and cytotoxicity monitored at the indicated times as described for Fig. 5. SPN1 does not express SPN protein (ΔSPN). The data presented shows the mean and SEM derived from at least 3 independent experiments. The inset shows a Western blot analysis of the cytosolic fraction of A549 cells following a 5 hr infection using an HA epitope antiserum. Lanes: 1, MW standards; 2, Uninfected; 3, Joy1 (SPN\textsubscript{J4}); 4, SPN1 (ΔSPN); 5, Suki6 (SPN\textsubscript{J4-GG}); 6, Suki7 (SPN\textsubscript{TM-GG}); 7, Suki8 (SPN\textsubscript{H5-GG}). The molecular weight of selected standards are indicated at the left of the inset.
DISCUSSION

Understanding the functional consequences of polymorphism can provide insight into how a specific virulence factor has been adapted in disparately evolving lineages, including those that are diverging with regards to niche selection. The present analysis of SPN has revealed that differences in enzymatic activity between haplotypes cannot be attributed to a single polymorphic residue. Rather, the transition from an enzymatically active to an inactive protein required changes at multiple positions among polymorphic residues universally conserved in both activity-deficient haplotype clusters 3 and 4. Furthermore, despite a precipitous loss of NADase activity, these variants retained their cytotoxic activities for cultured epithelial cells. These data provide insight into the evolution of SPN by demonstrating that this toxin has at least two distinct activities, a NADase activity whose loss has been selected for in certain lineages, and a NADase-independent cytotoxic activity. An important and conserved role for this latter activity may explain why SPN remains under positive selection even in those lineages that lack NADase activity.

Most analyses of selection with regard to niche specialization in S. pyogenes have focused on events that have been facilitated by this organism’s high rate of recombination. Prominent among these are horizontal gene transfer events, including interspecies transfer of pathogenicity islands (34) and toxin alleles (14), recombination between orthologous regulatory genes (18), and the gain or loss of adhesins at defined loci (2, 16). However, the fact that the SPN haplotypes lacking NADase activity are found only in S. pyogenes, that intermediate variants containing only a subset of the activity-associated polymorphic alleles have never been observed, and that these alleles are always associated with a degraded variant of IFS (26) suggest that recombination may not have played a major role in the evolution of these variants. Thus,
how these variant haplotypes may have descended from the activity-proficient versions is not clear. It can be speculated that because multiple residues are involved and because the two haplotypes share features in common, adaptation may have followed a step-wise course. Both NADase-inactive haplotypes share the D330G polymorphism, suggesting that a change at this position may be the progenitor of both alleles. However, as shown in this study, intermediate combinations of these activity-associated residues, including the G330D change, retain partial activities. Given the extremely high NADase activity of SPN, the partial activities observed in the engineered intermediate forms remain relatively high when compared to other classes of enzymes that share this activity, including NAD ribosyl transferases and NAD cyclases (9). This suggests that additional alterations are required for complete loss of NADase activity. Since both variant haplotypes share polymorphism at residue 289, this may have been the next residue to come under selection. In the case of the Bayesian cluster 4 variant, the R289N variation further reduced NADase activity to levels that are not toxic for the streptococcal cell, since this haplotype no longer requires a functional immunity protein for its expression.

The evolution of IFS is likely intimately linked with that of SPN. Consistent with the scheme described above, IFS is no longer essential when paired with a SPN that has the G330D polymorphism. The development of this variant would then release \textit{ifs} from selective pressure to begin its transition to a pseudogene. If correct, this model implies several things about SPN-IFS co-evolution. Firstly, the fact that \textit{ifs} has become a pseudogene in the absence of robust NADase activity indicates that inhibition of this activity is its principal function, rather than having any additional contributions to virulence, including SPN secretion or the CMT injection process. Secondly, that the fact that \textit{S. pyogenes} can tolerate a low level of NADase activity in the absence of IFS indicates that alterations to \textit{ifs} itself was not a major driver of SPN’s continuing
evolution to an activity-deficient phenotype. Instead, the lack of intermediates for the SPN NADase-deficient haplotypes in the *S. pyogenes* population structure suggests that the near to complete loss of NADase activity in these lineages was driven exclusively by selective pressure imparted by some virulence-related function associated with how SPN interacts with host cells.

While their origins may not be clear, insight into the mechanistic role of polymorphism on function can be gained from analysis of the structure of SPN. The three-dimensional structure of the enzymatic domain of SPN has recently been determined, revealing that it is structurally related to the broad family of NAD*+* ribosyl transferases (29). These proteins share a conserved core structure consisting of seven β strands arranged in two perpendicular β sheets that bracket the β-NAD*+* binding pocket. In addition, SPN and the NAD*+* ribosyl transferases share an active site ADP-ribosyl turn-turn (ARTT) motif that contains a catalytically essential glutamate residue (E391 for SPN), that lies at the base of a bowl-like substrate binding pocket (Fig. 7A). The three positions of interest are all situated in the wall of the substrate binding pocket, although two of these are distal to the active site and are located near the lip of the bowl. One of these is G330, which hydrogen bonds with Q216 from the opposing surface to form one of the outside walls of the bowl (Fig. 7B). Molecular modeling of the glutamate polymorphism reveals significant steric clashes with Q216, which likely lead to a significant distortion of this wall, possibly leading to a reorientation of the two halves of the enzyme relative to each other. Consistent with this, the G330D change had the highest impact on NADase activity as compared with changes at the other two sites, and likely radically altered substrate binding, as the Km was raised to a level that was difficult to determine with precision. On a different face of the wall, R289 interacts with D286 and K288 to form a surface that molecular modeling suggests may be involved in positioning the ribose moiety of the β-NAD*+* substrate in the active site cleft (Fig. 7C). While the
lysine polymorphism in the cluster 3 variant SPN also represents a highly conserved substitution, the reduced ability of lysine to form hydrogen bonds likely results in some disruption of the extensive network of interactions contributed by arginine at this position. Consistent with this, the lysine substitution had the least effect on NADase activity, but was still substantial, resulting in a near 20-fold decrease in enzymatic efficiency. The much greater decrease in NADase activity found in the cluster 4 variants can be explained by the considerable loss of interactions that would result from the substitution of glutamine. The third position of interest, I374, is located at the bottom of the bowl near catalytic E391. However, while the latter’s side chain extends into the lumen of the bowl, the side chain of I374 is buried in the hydrophobic core (Fig. 7D). Molecular modeling of the I374V substitution reveals extensive steric clashes with F199 and Y243 from two adjacent helices, which likely would distort the position of the side chain of E391 to an orientation less favorable for interaction with the glycosidic bond of the β-NAD\(^{+}\) substrate.
Figure 7. Polymorphic residues that influence catalysis are located in the substrate binding pocket of SPN. A. Orthogonal view of the enzymatic domain of SPN (residues 191-451) modeled in complex with β-NAD⁺. The model was developed from a structural alignment with cholera toxin bound to β-NAD⁺ (PDI accession 2a5f) followed by superimposition of β-NAD⁺ into the binding pocket of SPN. Residues of the ARTT motif, catalytic residues and relevant polymorphic residues are labeled and are highlighted in red, β-NAD⁺ is shown in blue. B. G330 forms a hydrogen bond with Q216 to form one wall of the binding pocket. C. Proximity of R289 to adjacent residues D286 and K288, which form a surface that may position β-NAD⁺ in the binding pocket. D. I374 is in proximity to catalytic residue E391, but lies buried in an adjacent hydrophobic pocket that includes F200 and Y243.

The fact that adaptation produced two distinct variant haplotypes suggests this event was subject to considerable selective pressure. Why this process involved selection at multiple residues is not clear, since a single mutation at residue E391 would lead to a drastic reduction in NADase activity. It is possible that significant structural constraint was imposed by a dual
requirement for loss of NADase activity and selection for the cytotoxic activity. However, this scenario would imply a larger conserved role for E391 beyond cytotoxicity because mutation at this position did not influence cytotoxicity for cultured epithelial cells. A pathway of multiple changes may have been necessary because any single mutation resulted in a less stable protein. Some evidence for this comes from the observation that the enzymatic activity of some of the engineered intermediate forms was more erratic using low salt conditions that had no effect on activity of the SPN$_{34}$ enzyme. However, all engineered intermediate forms were readily expressed by either $S.$ pyogenes or $E.$ coli and none exhibited any obvious differences with solubility or degradation relative to the unmodified proteins. Thus, a more detailed understanding of adaptation will require uncovering the mechanism of the NADase-independent cytotoxic activity.

Multifunctionality is not an uncommon property of many bacterial toxins that are recognized to be subject to ongoing adaptation (7). For example, the VacA vacuolating cytotoxin of Helicobacter pylori is an important determinant of colonization, persistence and pathogenesis of its human host and exhibits considerable diversity. Like SPN, VacA diversity is restricted to specific regions of the toxin and generates a wide range of toxin efficacy, from variants that produce extensive vacuolation, to those that only produce vacuolation in a limited range of cultured cells, to variants that fail to cause any detectable cytotoxicity in $in vitro$ assays (7, 12). Also like SPN, variation has a functional consequence, although for VacA this appears to correlate with the risk of developing certain complications of long-term colonization like peptic ulcer disease or gastric cancer (1, 33). However, VacA is multifunctional and in addition to cytotoxicity has been reported to alter membrane permeability, to target and damage mitochondria, and to activate numerous host cell signaling pathways (7). Similar to SPN, it is
thought that this multifunctional nature likely explains why VacA is found in essentially all *H. pylori* strains that infect humans, despite extensive variation in vacuolating ability (1).

A common property of multifunctional toxins exhibited by VacA is the ability to cause distinct cellular effects by acting at different cellular locations (7). Whether SPN acts at different cellular locations is not clear. Translocated SPN is found exclusively in the cytoplasmic compartment (4, 20), although the presence or absence of SPN in defined mutants has been reported to affect the invasiveness of *S. pyogenes* during infection of cultured epithelial cells (3), a property that could contribute to niche selection. SPN itself is a multi-domain protein and in addition to its NADase domain, has an N-terminal domain that is essential for its translocation (10). While the structure of this domain has not been solved, homology modeling indicates that it adopts a “jelly-roll” fold common to many carbohydrate-binding proteins that suggests it may promote interaction at the cell surface (10). The role of this domain in cytotoxicity remains to be determined. Prior reports suggested that SPN may have multiple enzymatic activities that could produce differential cellular effects, including ADP-ribosyl transferase and cyclase activities (15, 30). However, more refined analyses of highly purified recombinantly-produced SPN have failed to confirm these additional activities (9). It is possible that there are two modes of SPN cytotoxicity, a NADase-dependent activity in the activity-proficient haplotypes and a gain-of-function cytotoxic activity that results from the multiple polymorphisms in the NADase-deficient haplotypes. Support for a role for NADase activity in cytotoxicity comes from studies in *E. coli* and yeast that show that expression of SPN in the absence of IFS, the endogenous inhibitor of its NADase activity, is lethal (9, 23). In contrast, expression in yeast of an activity-deficient haplotype is not lethal (9). Yeast may lack a target required for the NADase independent activity or this may be due to the observation that full penetrance of the cytotoxic effect on epithelial
cells requires a synergistic interaction with the pore-forming activity of the streptococcal SLO protein (21).

The studies described here have expanded our understanding of SPN structure and function and the molecular basis for its evolution. However, several important questions remain, including the structural and molecular basis of its NADase-independent cytotoxic activity and how its various activities contribute to tissue tropism. Several studies have suggested that the number of *S. pyogenes* isolates that demonstrate NADase activity has been increasing since the late 1980’s and that the activity-proficient SPN haplotypes have entered into some lineages that are now more frequently isolated from invasive disease (30-32). Thus, a detailed understanding of SPN function and evolution will further our understanding of how *S. pyogenes* populations continue to evolve.
Supplemental Figure 1. Bayesian clusters and the corresponding polymorphic amino acids.

Diagrams are based on (26). Data was determined by Bayesian analysis of 113 clinical isolates.

The first 6 polymorphic residues vary between NADase types however the 3 C-terminal residues are conserved to each Bayesian cluster.
Supplemental Figure 2. Graph of slopes used to determine NAD⁺ cleavage rates of variants of SPN<sub>J4</sub>. Various protein concentrations were used with 2.5 mM β-NAD⁺ incubated at 37°C. Rates were determined as Moles of NAD⁺/Minutes/Moles of SPN. See Methods for details.
MATERIALS AND METHODS

Bacterial Strains and Media. The studies with *S. pyogenes* utilized the M serotype 6 strain JRS4 (28) and derivatives including SPN1 (Δspn, (20)) and JOY1, which expresses SPN with a carboxy-terminal HA epitope tag. Chromosomal DNA from strain HSC5 (11) was used as a template in PCR reactions for molecular cloning of SPN<sub>H5</sub>. Construction of all strains is described in Table S3. Other molecular cloning and protein expression studies utilized *E. coli* TOP10 (Invitrogen). Routine culture of *S. pyogenes* and *E. coli* was conducted using Todd Hewitt Yeast Extract and Luria-Bertani media, respectively, as described (9). Where appropriate, antibiotics were used at the following concentrations: chloramphenicol, 7.5 µg/ml for *E. coli* and 3 µg/ml for *S. pyogenes*; erythromycin, 500 µg/ml for *E. coli* and 1 µg/ml for *S. pyogenes* and carbenicillin, 50 µg/ml for *E. coli*.

Manipulation of DNA- Transformation of *E. coli* used the method of Kushner (17) and *S. pyogenes* was transformed by electroporation (6). Plasmid DNA was isolated by standard techniques and all enzymes, including restriction endonucleases, ligases (New England Biolabs), and polymerases (Pfx, Invitrogen) were used according to the manufacturers’ recommendations. All site-specific mutations described in the text were generated by PCR with the mutagenic oligonucleotide primers listed in Tables S1 and S2 and the *Dpn*I digestion method to degrade template DNA using a commercial kit (QuikChange XL kit, Stratagene). Fidelity of all DNA sequences generated by PCR was verified by DNA sequence analyses performed by commercial vendors (SeqWright, Galveston, TX; GeneWiz, South Plainfield, NJ).
**Expression of SPN derivatives in *S. pyogenes***. Plasmids for expression of SPN in *S. pyogenes* were based on pJOY3 (10) and include pJOY7, which expresses SPN_{J4} with a carboxy-terminal Influenza Hemagglutinin epitope tag (HA epitope tag). A fragment containing SPN_{H5} was amplified from HSC5 chromosomal DNA using primers oJOY5 and oJOY6 (Table S1) and introduced between the *Eco*RI and *Bst*EII sites of pJOY7. The resulting plasmid (pJOY39) expresses SPN_{H5} with a carboxy-terminal HA-tag (Table S1). The various site-specific mutations were then introduced into pJOY7 and pJOY39 by PCR using the primers listed in Table S1. For expression and analysis, each plasmid was used to transform the SPN deletion strain SPN1 and the resulting strains were analyzed by the assays described below. Plasmid constructions are summarized in Table S1.

**Expression and purification of recombinant SPNs.** PCR was used to introduce the various site-specific mutations of interest into a derivative of the *E. coli* expression plasmid pBAD (Invitrogen) that encodes a His\_6-tagged SPN_{J4} (pMAM3.18, (22)). Mutagenic primers and the relevant features of this panel of expression plasmids are summarized in Table S2. Expression and purification of the His\_6-tagged recombinant proteins was performed as previously described (9). As a final step, the purified proteins were dialyzed at 4°C against a buffer consisting of 50 mM potassium phosphate and 100 mM sodium chloride. Purity of the 48.6 kDa proteins was routinely assessed by SDS-PAGE and staining with Coomasie Brilliant Blue and protein concentrations determined using a BCA assay (Pierce) with a BSA standard.

**Single-copy *S. pyogenes* SPN expression strains-.** SPN_{J4} and SPN_{H5} were amplified by PCR from templates pJOY7 and pJOY39, respectively, using primers oJOY180 (5' CGGTGTTTAC
TCGAGAAACAAAAAGTAACATTAGC-3') and oJOY6 (Table S1). The resulting fragments were inserted between the \textit{XhoI} and \textit{PstI} sites of pJRS233 (25) to produce pJOY109 and pJOY110. A mutant of SPN\textsubscript{J4} containing the cluster 3 polymorphisms (“triple mutant”) and a carboxy-terminal HA tag was produced by sequential site directed mutagenesis of pJOY7 using primer pairs oJOY85/oJOY86 (R289K), oJOY89/oJOY90 (G330D) and oJOY93/oJOY94 (I374V) as detailed above (primers are described in Table S2). The modified SPN gene of the resulting plasmid (pASuki2) was amplified using oJOY180 and oJOY6 and inserted between the \textit{XhoI} and \textit{PstI} sites of pJRS233 to produce pJSuki2. Site directed mutagenesis of pJoy109 was performed to generate the single (R289K, G330D) and double polymorphic mutants (R289K+G330D) in SPN\textsubscript{J4} (primers and strains described in Table S3). The five plasmids (pJOY109, pJOY110, pJSuki2, pJSuki3, pJSuki4, pJSuki5) were then used to replace the native SPN allele in JRS4 by a standard method (13) to generate strains designated SPN-J4, SPN-H5 and SPN-TM, SPN-R289K, SPN-G330D, and SPN - R289K/G330D. Chromosomal sequences were verified by DNA sequence analysis of PCR products generated using appropriate primers.

\textbf{Tests for \textit{ifs} essentiality.} The requirement for \textit{ifs} to support viability of strains expressing SPN of differing NADase activities was assessed by two complementary mutational strategies. In the first, an attempt was made to replace \textit{ifs} with the \textit{aad9} spectinomycin-resistance gene. In the second, an attempt was made to insertionally-inactivate \textit{ifs} using an integrational plasmid. Both strategies were based on established methods using plasmids with conditional temperature-sensitive replication (25) (19). The construction of the two mutagenic plasmids and steps involved in mutagenesis are described in detail in the Supplemental Experimental Procedures. It was concluded that \textit{ifs} was essential in a given strain if both strategies failed to generate an \textit{ifs}-
deficient mutant. In successful *ifs* mutants, DNA sequence analyses of *spn* and its promoter each mutant retained the expected SPN allele.

**β-NAD\(^+\) Glycohydrolase Activity of SPN from *S. pyogenes*-.** A fluorimetric assay was used to analyze the β-NAD\(^+\) glycohydrolase activity in culture supernatants from *S. pyogenes* strains expressing the various SPN constructs (9). Concentrations of SPN in culture supernatants were normalized by immunoblotting to detect an HA epitope tag as previously described (9). Specific activities of each SPN mutant is reported relative to wild type JRS4 as described (10). Data presented are derived from three independent experiments, each performed in triplicate.

**β-NAD\(^+\) glycohydrolase activities of recombinant enzymes.** Rates of β-NAD\(^+\) cleavage were determined by analytical HPLC as described (Supplemental figure 1) (9). In initial trials, it was found that inconsistencies in results obtained from several of the low activity enzymes could be eliminated by the inclusion of NaCl in the reaction mixture (100 mM). Since this had no effect on the *K_m* of SPN\(_{J4}\), all analyses were conducted using this modification. Briefly, the molar concentrations of SPN indicated in the text were incubated with 2.5 mM β-NAD\(^+\) in 100 µl of reaction buffer (50 mM potassium phosphate, 100 mM sodium chloride, pH 7.4, 0.5 mg/ml BSA) at 37°C. Reactions were quenched by the addition of 100 µl of 20% ice-cold perchloric acid and incubated on ice for 30 min to precipitate protein and BSA. Precipitates were removed by centrifugation, supernatants diluted into 980 µl of deionized water and the mixture subjected to reversed-phase HPLC (SunFire, C18 column, Waters, 5 µm, 4.6 x 250 mm) developed isocratically with 1% (v/v) acetonitrile in buffer A (10 mM diammonium phosphate buffer, pH 6.4) with a flow rate of 1.5 ml/min over 22 min. Quantitation of reaction products and
determination of initial reaction rates and other kinetic parameters were performed as described in detail elsewhere (9). Data presented are derived from three independent experiments, each performed in triplicate.

**End point β-NAD⁺ glycohydrolase activity assay.** A single end point assay to determine the amount of uncleaved β-NAD⁺ remaining following reaction with various SPN derivatives was conducted as follows: Enzyme (20 picomoles) was incubated in reaction buffer (50 mM potassium phosphate, 100 mM sodium chloride, pH 7.4, 0.5 mg/ml BSA) with 1 mM β-NAD⁺ for 1, 5, 7 and 20 hrs at 37°C. Reactions were quenched by chromatography using a centrifugal column (Millipore, 3 kDa exclusion). The flow-through fraction was diluted into deionized water and analyzed by reverse-phase HPLC as described above. Data presented are derived from three independent experiments, each performed in triplicate.

**Infection of epithelial cells.** Analysis of cytolysin-mediated translocation (CMT) and cytotoxicity were assessed following infection of A549 epithelial cells, conducted as described in detail elsewhere (20). Briefly, various *S. pyogenes* strains were cultured overnight, back diluted, and grown to mid-logarithmic phase and used to infect confluent monolayers of A549 cells. Following incubation for 285 min., cells were lysed by the addition of Saponin to a final concentration of 0.1%, the lysate fractionated by ultracentrifugation and the concentration of SPN in the cytosolic fraction determined by immunoblotting to detect the HA epitope tag. Efficiencies of CMT were quantitated relative to a strain expressing SPN₅₄ as described (10). Cytotoxicity of various SPN-expressing *S. pyogenes* strains was assessed by the ability of infected cells to exclude the membrane-impermeable fluorescent probe ethidium homodimer-1.
(Live/Dead®, cat. #L3224, Invitrogen) as described previously (20). Data presented represents
the mean and standard error of the mean derived from at least 3 independent experiments.

**Computational methods and statistical analyses.** Molecular modeling employed the
PyMOL Molecular Graphics System (Version 1.5.0.1, Schrödinger, LLC) and the structure of
the enzymatic domain of SPN (PDB accession 3PNT) determined by Smith et al. (29). Where
indicated, differences between observed experimental mean values were tested for significance
using Tukey-Kramer Multiple Comparisons Test. The null hypothesis was rejected for $P < 0.05$.

**SUPPLEMENTAL EXPERIMENTAL PROCEDURES**

**Tests for ifs essentiality.** A plasmid was constructed for replacement of *ifs* (SPy_0166) with the
gene encoding the *aad9* spectinomycin-resistance determinant as follows: A DNA fragment that
included sequences extending 229bp upstream and 231bp downstream of the 486bp *ifs* ORF was
amplified and inserted into the temperature-sensitive shuttle vector pJRS233 (25) by overlap
extension PCR (5) using primer pair GP972/GP973 (Table S4). The resulting plasmid
(pGCP858) has the *ifs* fragment located between the M13F and M13R primer binding sites of the
pJRS233 vector. Next, *aad9* lacking a promoter sequence was amplified from pSPC18 (19)
using primer pair GP921/GP922 (Table S4) by PCR and then overlap extension PCR was used to
flank promoterless *aad9* with chromosomal sequences from upstream and downstream of *ifs*
using primer pairs GP972/GP925 and GP926/GP973. The resulting fragment was used to replace
the *ifs* open reading frame in pGCP858 by overlap extension PCR. The resulting plasmid
(pGCP867, Fig. S3A) was then used to attempt to replace *ifs* in the chromosome of various
strains using a standard method (27) with selection for erythromycin-resistance (1 μg/ml) at the
permissive temperature (30°C) followed by selection for resistance to both erythromycin and
spectinomycin (100 μg/ml) at the non-permissive temperature (37°C). This latter culture was
plated to obtain individual spectinomycin-resistant colonies and 3 independent colonies were
picked and cultured at the permissive temperature in media containing spectinomycin, but
lacking erythromycin. Every 24 hrs, each of these cultures was plated for single spectinomycin-
resistant colonies and also diluted 1:1000 in fresh medium with spectinomycin and the
incubation continued. Since aad9 lacks its own promoter in pGCP863, recombination with the
chromosomal copy of ifs will place aad9 under the control of the SPN-SLO operon promoter
(Fig. S3A). At least 150 colonies from each time point were replica plated to identify those
resistant to spectinomycin, but sensitive to erythromycin, which were then screened for allelic
replacement by PCR using primer pair GP956/GP957 and DNA sequence analysis with primers
GP200 and GP250. Successful allelic replacement typically required 2-5 passages (Table S5).
However, a conclusion that allelic replacement was not possible was only made following at
least 16 passages with screening of a minimum of 900 individual colonies (Table S5).

While sensitive, this method involves competition between the mutant, the intermediate
merodiploid and wild type, and a significant difference in fitness can result in a failure to recover
the mutant leading to a false assignment of essentiality. Therefore, these data were confirmed by
a second strategy involving a single-step insertional inactivation of ifs, conducted as follows: a
246 bp fragment of the ifs open reading frame (nucleotides 83-246) lacking its 5’ and 3’ ends
was amplified from a JRS4 chromosomal DNA template and inserted into the temperatur-
sensitive shuttle vector pGCP213 (24) by overlap extension PCR using the primer pair
GP879/GP880 (Table S4). In the resulting plasmid (pGCP773) the ifs fragment is located
between M13F and M13R primer binding sites (Fig. S3B). To test for essentiality, pGCP773 was used to transform the various *S. pyogenes* strains to resistance to erythromycin and plated for single colonies at the permissive temperature (30°C). For each strain, 6 colonies were picked and cultured individually overnight at the non-permissive temperature (37°C) in the presence of erythromycin. Cultures were then diluted 1:1000 in fresh medium containing erythromycin and cultured overnight at the non-permissive temperature. Cultures that continued to grow at 37°C for a minimum of 3 successive passages were screened by PCR to confirm the loss of a full length *ifs* open reading frame, for disruption of *ifs* and for integration of the plasmid into the *ifs* locus (Table S5). DNA sequence analyses of *spn* and its promoter confirmed that each *ifs* mutant retained the expected SPN activity phenotype.
Supplemental Figure 3. *ifs* mutagenesis plasmids. Two plasmids were generated to test for essentiality of IFS in strains of intermediate NADase activity. (A) The plasmid pGCP867 containing a promoterless *aad9* spectinomycin-resistance gene flanked by 229bp and 231bp DNA regions homologous to those immediately upstream and downstream of *ifs*, respectively, was constructed for a two-step replacement of *ifs* (SPy_0166). A promoterless copy of *aad9* was utilized to enrich for spectinomycin resistant transformants in which the first crossover event occurs upstream of *ifs* (labeled 1) placing *aad9* under the transcriptional control of the SPN-SLO operon promoter. Upon plasmid excision but maintained spectinomycin resistance selection, a second crossover event occurs downstream of *ifs* (labeled 2) resulting in replacement of *ifs* for *aad9*. (B) The plasmid pGCP773 containing a 246bp internal fragment of the *ifs* open reading frame was constructed for a single-step insertional inactivation of *ifs*. 
### SUPPLEMENTAL TABLES

<table>
<thead>
<tr>
<th>Table S1.</th>
<th>Construction of plasmids for protein expression in S. pyogenes</th>
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<sup>a</sup> Nucleotides sequences are 5' to 3'.

<sup>b</sup> All plasmids express full length IFS and SPN with indicated mutation.

<sup>c</sup> All secreted proteins have a C-terminal HA tag.
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^a Nucleotide sequences are 5’ to 3’
^b All plasmids express full length IFS without a signal sequence
^c All proteins have a N-terminal 6xHis tag
### Table S3. Mutagenesis of the spn chromosomal locus

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<sup>a</sup> All strains are derivatives of JRS4.

<sup>b</sup> Sequence listed in Table S2; oJOY156, oJOY157, oJOY160, oJOY161 are from Reference (9).

<sup>c</sup> All plasmids are derivatives of pJRS233 (14) using indicated primers to generate specified SPN mutations.

<sup>d</sup> All SPN alleles contain a C-terminal HA tag. Suki2, Suki3, Suki4, and Suki5 contain mutations to swap residues in SPN<sub>H3</sub> to SPN<sub>H5</sub>. SPN<sub>TM</sub> is SPN<sub>H4</sub> with the following point mutations: R289K, G330D, I374V. SPN<sub>H4.GG</sub>, SPN<sub>TM.GG</sub>, SPN<sub>H5.GG</sub> indicates the addition of E389G and E391G mutations to the designated SPN allele.
Table S4. Mutagenesis of the ifs chromosomal locus

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*Primers were used as described in "Supplemental Experimental Procedures" for construction of ifs mutagenesis plasmids pGCP867 and pGCP773 or for DNA sequence analysis of the SPN-SLO operon.

*Nucleotide sequences are 5' to 3'

Table S5. IFS mutations generated in S. pyogenes

<table>
<thead>
<tr>
<th>Mutation (Plasmid)</th>
<th>SPN allele (activity)</th>
<th>Host (Parent)</th>
<th>Strain</th>
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<tr>
<td>ΔIFS::aad9 (pGCP867)</td>
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<td>Suki4 (JRS4)</td>
<td>GCP897</td>
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<tr>
<td></td>
<td>SPNR289KGG330D (+)</td>
<td>Suki5 (JRS4)</td>
<td>GCP898</td>
</tr>
<tr>
<td></td>
<td>SPN_{TM} (-)</td>
<td>Suki2 (JRS4)</td>
<td>GCP896</td>
</tr>
<tr>
<td></td>
<td>SPN_{H5} (-)</td>
<td>Joy110 (JRS4)</td>
<td>GCP895</td>
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*ΔIFS::aad9 and ΩIFS::ermC are deletion and insertion mutations, respectively, generated using pGCP867 (for ΔIFS::aad9) and pGCP773 (for ΩIFS::ermC).

*NADase activity defined as described in Table 3 as fold change in k_{cat} relative to SPN_{J4}.

*Host strain is the recipient of the mutagenic plasmid and is derived from the parent indicated in parentheses where appropriate. The construction of all Host strains is described in "Experimental Procedures" and listed in Table S3 except SPN1 and HSC5 which have been described previously (11, 20).
ACKNOWLEDGMENTS

We thank Craig Smith for sharing his structural insights and his assistance with molecular modeling and Tamara Doering for the use of her HPLC system. We also thank Zachary Cusumano for his technical guidance on protein purification. This study was supported by Public Health Service Grant AI064721 from the National Institutes of Health.
REFERENCES


CHAPTER III

SPN activity modulates PARylation in epithelial cells influencing the release of the pro-inflammatory molecule HMGB-1
SUMMARY

The interaction between virulence factors produced by a pathogen and the affected host cell can determine the success of the pathogen. *S. pyogenes* employs several virulence factors during an infection making it one of the most versatile bacterial pathogens. One strategy involves modulating host response through cytolysin-mediated translocation (CMT). CMT involves two toxins, Streptolysin O and the NADase SPN, that contribute to pathogenesis in multiple ways.

Recent data showed that both the NADase active and inactive SPN are cytotoxic to epithelial cells. In this study, we find the NAD$^+$ glycohydrolase SPN has a multifunctional role in terms of both the active version capable of cleaving NAD$^+$ and the inactive version capable of eliciting a different cellular response. We found *S. pyogenes* to be capable of activating the eukaryotic enzyme Poly-ADP-ribose Polymerase-1 in an SLO dependent manner however the activity of this regulatory enzyme was influenced by the NADase activity of SPN. Remarkably, the NADase active SPN was not only capable of cleaving NAD$^+$ in the cells but also has the potential to cleave PAR polymers, the product of PARP-1 activation. This may contribute to the release of the pro-inflammatory protein HMGB-1 from the nucleus, which does not occur in the NADase inactive SPN. In contrast, the SPN deletion and the NADase inactive SPN allows for the accumulation of PAR through the sustained activation of PARP-1. Normally PARP activity is transient and fleeting, however in these infections PAR accumulation was cyclical and sustained. Massive accumulation of PAR is often associated with cellular signaling and AIF translocation leading to cell death. In these infections, we found that AIF is not translocated and another mode of cell death is likely occurring in these infections.

Together, this data indicates one virulence factor, SPN, in conjunction with SLO can influence the cellular response to an infection through its enzymatic activity.
INTRODUCTION

For an organism to become a successful pathogen it must overcome host barriers, which often is achieved through the production of virulence factors. For bacteria, these factors are often secreted proteins that affect various processes in the host creating a battlefield between host and pathogen. Evolution and divergence of these proteins also play an important role in the ability of the microorganism to continue causing disease. For example, *Legionella pneumophila* encodes over 200 effectors many of which are redundant but are thought to allow the bacteria to thrive in various hosts like amoebas and alveolar macrophages (20). This redundancy also allows for *L. pneumophila* to evade the host in multiple ways. Understanding the diversity and the cellular consequences of these proteins can provide insight into how microbes can subvert the host and cause destruction.

*S. pyogenes* is a versatile and successful pathogen on many levels which can largely be attributed to the wide variety of virulence factors the bacterium can produce during the course of an infection. This Gram-positive organism can cause a range of diseases including superficial infections such as pharyngitis and cellulitis as well as more invasive infections like necrotizing fasciitis. These initial infections can also lead to post-infection sequelae such as rheumatic fever and acute glomerulonephritis. This disease range also includes both inflammatory and non-inflammatory responses making this bacterium especially unique. *S. pyogenes* is known to secrete dozens of proteins affecting multiple processes within the host cell, modulating them to allow for further pathogenesis. One such protein is the *S. pyogenes* NAD⁺ glycohydrolase (NADase) known as SPN (or NGA). During an infection, *S. pyogenes* can adhere to the host cell through adhesive protein-protein interactions such as M protein to the eukaryotic receptor CD46. Through a general secretory pathway, SPN and a cholesterol dependent cytolysin termed...
Streptolysin O (SLO) are secreted into the extracellular milieu and the interaction between these two proteins on the host cell membrane allow for the translocation of SPN into the host cell cytosol (16). This mechanism has not been completely elucidated however we know that it is not dependent on the pore formation of SLO as non-pore forming mutants of SLO still allow for the translocation of SPN (17).

Originally, this enzyme was identified for its ability to cleave NAD$^+$ into nicotinamide and adenosine diphosphoribose (ADPr). Since then, multiple subtypes of SPN have been discovered including those that lack the characteristic NADase activity and have been shown to be evolving under positive selection. Analysis of a collection of diverse isolates revealed that all strains examined encoded the full-length gene for SPN and that both active and inactive haplotypes were equally prevalent (23). Variations in the enzymatic domain of SPN are also associated with tissue tropism indicating a functional role for these variants. Those strains with a NADase inactive allele were strongly associated with the ability to cause strictly pharyngeal or skin infections termed specialists while those strains with the NADase active allele were associated with the ability to cause disease in both sites termed generalists. Also of note is the presence of the endogenous inhibitor of SPN (IFS) necessary for *S. pyogenes* to maintain the NADase active allele. This gene is located within the same operon along with SLO, the necessary component for translocation of SPN into the host. It is thought that IFS resides within the bacterial cytosol where it can bind to SPN in a one to one ratio preventing NAD$^+$ from interacting with the enzymatic pocket of SPN. IFS is truncated in those strains with NADase inactive SPN however SLO and SPN despite loss of NADase activity remain completely intact further indicating there is a role for NADase inactive SPN (23).
The differences between these two haplotypes of SPN can be categorized into four Bayesian clusters and further dissection shows the differences between the NADase active and inactive forms are simply 10 polymorphic amino acid residues. Upon close examination, the polymorphic residues responsible for the enzymatic activity were found to be three specific residues in the C-terminal end of the protein. Previously, we found NADase active SPN contributed to cytotoxicity when compared to both the SPN deletion and SLO deletion in HaCat keratinocytes (16). We examined the NADase inactive SPN’s contribution to cytotoxicity in epithelial cells and found this version to be equally cytotoxic as the NADase active form thus uncoupling the NADase activity from cell death. Little is understood about the function of this inactive version though we believe there is role in virulence since S. pyogenes has maintained both variants in its full length despite the active loss of IFS. It is thought the active form could kill cells by cleaving NAD\(^+\) stores within the cell yet the presence of the inactive form indicates another function for SPN. The role of SPN in cell death is dependent on SLO as knockouts of SLO are less cytotoxic than the single deletion of SPN alone. Studies examining SLO pore formation and the translocation of SPN has shown that translocation is not dependent on pore formation as both prepore and monomer locked SLO mutants can perform CMT (17). In the case of prepore locked SLO, SPN is translocated to wild type levels in the cytosol while monomer locked translocates about half that amount. In both cases, cytotoxicity assays showed that cells are still viable and membranes were intact suggesting that pore formation in conjunction with SPN is necessary for cell death.

We know that SLO and SPN are both necessary for toxicity to cells but we do not fully understand how each of these factors contribute to cell signaling and subsequently cell death. It is thought the NADase active SPN cleaves \(\beta\)-NAD\(^+\) stores in the cell creating both metabolic
dysfunction and signaling molecules from the cleavage reaction. However this does not explain how the NADase inactive version may be contributing to toxicity. The road to cell death is complicated and is the result of several different reactions the cell undergoes that eventually lead to its demise. Often these are stress responses made by the cell necessary to rapidly adapt to environmental cues.

One such reaction is the activation of the nuclear protein Poly-ADP-ribose polymerase (PARP). This protein resides in the nucleus where it performs a broad spectrum of functions including DNA damage control, transcriptional activation, chromatin modulation, cell division, stress response and inflammation (8). PARP-1 is an enzyme that cleaves NAD$^+$ to create ADP-ribose chains on target proteins in a process called poly ADP-ribosylation or PARylation. These poly-ADP-ribose (PAR) polymers are produced and removed rapidly in response to various stimuli. A counteracting enzyme called Poly-ADP-ribose glycohydrolase or PARG works to removed these scaffolds. The over-activation of PARP has multiple consequences that can each lead to cell death through different mechanisms. One pathway includes the depletion of NAD$^+$ and ATP which leads to cell death (1). A second mechanism involves an overabundance of PAR polymers created by PARP and released by PARG that become signaling molecules contributing to a different form of cell death called parthanatos. This mechanism involves the release of PAR from the nucleus into the cytosol where it can interact with the mitochondria leading to the release of the protein Apoptosis Inducing Factor (AIF) from the membrane and can then travel to the nucleus to cause DNA damage and eventually cell death (28, 29). A third pathway PARP contributes to involves programmed necrosis through cell signaling and the production of ROS (3, 26).
Aside from the negative effects of over-activation of PARP, it also plays role in the inflammatory response. It is also involved in the transcriptional regulation if iNOS and NF-κB (11, 30). One inflammatory protein that is directly modulated by PARP is the High Mobility Group Box-1 protein (HMGB-1). Normally, this protein resides in the nucleus where it helps to maintain chromatin stability however this protein can be released during necrosis and contribute to inflammation through its interaction with TLR 2, TLR 4 and the RAGE receptor (21, 25). However, for HMGB-1 to be released from the chromatin it depends on PARP-1 for both chromatin modification and PARylation (6).

Little is known about the stress response to S. pyogenes in the context of both NADase variants of SPN and SLO. Though both versions of SPN are equally cytotoxic, their enzymatic differences likely contribute to pathogenesis through different pathways. Virtually nothing is known about how the NADase inactive version of SPN contributes to pathogenesis as it was always thought to be inert. Here, we report that PARP is activated in an SLO dependent manner and PARylation is modulated the NADase activity of SPN. The presence of the NADase active SPN likely has a dual role in the lack of PARylation seen in these infections: one is cleavage of NAD$^+$ and second is the cleavage of PAR. This modulation by the NADase of SPN produces a different inflammatory response as the NADase active version of SPN also induces the release of HMGB-1 while SPN deletion and inactive SPN variant do not. These results are the first to elucidate differences between the NADase active and inactive SPN and may have implications in the unidentified role of NADase inactive SPN.
RESULTS

PARP is induced during an infection in an SLO dependent manner

To try and determine how the cells response to SPN variants and SLO we looked at the activation of PARP after an infection with strains of *S. pyogenes* with NADase active SPN (SPN-J4), SPN deletion (SPNΔ), NADase inactive SPN (SPN-H5) and SLO deletion (SLOΔ). PARP responds to many different stimuli leading to its activation, which involves the addition of poly-ADP-ribose polymers to itself and other proteins. A Western blot analysis of HeLa lysates showed that the 116 KDa enzyme PARP is activated during an infection with *S. pyogenes* in an SLO dependent manner (Figure 1). SPN-J4, ΔSPN, SPN-H5 all express SLO. While a band for PARP is present in the SPN-J4 infection, it seems to be masked by auto-ribosylation in the SPN deletion and SPN-H5 infection as indicated by the PAR polymers (Figure 1B). During apoptosis, cleavage of PARP by caspase 3 can occur as control mechanism to prevent the overactivation of PARP. This process cleaves the 116 kDa protein into an 89 kDa and 27 kDa protein rendering it inactive. Cleavage of PARP can be seen in staurosporine treated cells, a known inducer of PARP and caspase 3, however this cleavage is not seen in any of the infections (Figure 1A) (2). However, despite the activation of PARP in the NADase active infection, the presence of poly ADP ribose (PAR or PARylation) is only present in the SPN knockout infection and the NADase inactive SPN infection (Figure 1B). PARylation appears as high molecular weights between 116 kDa and 200 kDa. Neither a band for PARP nor a band for PARylation could be seen in the SLO deletion infection indicating that SLO is necessary for PARP activation and PARylation.
Figure 1. PARP is activated in an SLO dependent manner. A. HeLa cells were infected for 5 hours with the indicated strains. Lysates were assessed by Western blots for PARP and actin for a loading control. Control indicates uninfected control cells. St represents cells treated with 1 uM staurosporine. B. The activity of PARP was assessed in the same HeLa lysates by Western blot analysis for poly-ADP-ribose polymers (PAR). Blots are representative of at least 3 independent experiments.

To understand the importance of SLO and the activation of PARP, purified SLO was used to determine if SLO alone is enough to induce PARylation. Purified SLO was obtained from an E. coli expression system and hemolytic activity was verified. Various concentrations of purified SLO were used to treat HeLa cells. This included low amounts from one nanogram to 100 nanograms (ng) (Figure 2A) for 5 hours. High concentrations of SLO from 100 ng to 2000 ng were also used to treat HeLa cells (Figure 2B). Concentrations higher than these cause
immediate sloughing of cells. None of these concentrations were capable of activating PARP thus indicating SLO is necessary but not sufficient for PARylation.

Figure 2. Purified SLO is not sufficient to induce PARylation. A. Westerns blot of HeLa lysates after treatment with different concentrations of purified SLO at low concentrations. PAR was absent at the indicated molecular weights. A non-specific band at 70 kDa appears in lysates and is used to verify equal loading. B. Western blot analysis of HeLa cells treated with buffer, or 100ng to 2000 ng of SLO. C indicates untreated control cells and B indicates cells treated with buffer alone. Amounts of SLO are designated above the blot in nanograms. Blots are representative of at least 3 independent experiments.
To address whether pore formation specifically is necessary for PARylation, non-pore forming SLO locked mutants were used to infect HeLa cells and PARylation was assessed by Western blots. Neither the monomer locked nor the prepore locked SLO were able to PARylate indicating that SLO pore formation in the context of an infection is necessary for PARylation (Figure 4A). Furthermore, these strains maintained a NADase active SPN, which could be depleting NAD\(^+\). These locked SLO mutants are still capable of translocating SPN into the host cell cytosol. The prepore locked SLO, which can still form oligomers, can translocate SPN at wild-type levels while the monomer locked can translocate to 50% of wild-type levels (17). To address whether the lack of PARylation in the cells is due to NADase active SPN, an NADase inactive form of SPN was also derived by mutating the three amino acid residues responsible for the NADase activity as previously discovered (Chapter 2). These mutations include R289K, G330D and I374V at the C-terminus of SPN. This set of mutations is referred to as the triple mutant or TM. The monomer locked (Mono-TM) and prepore locked (Prepore-TM) strains lacking NADase activity were then used to infect HeLa cells for 5 hours and lysates were subjected to Western blots analysis (Figure 3B). Again, PARylation was absent in these infections confirming that pore formation is necessary for PARylation to occur during an infection.
Figure 3. Monomer and Prepore locked SLO cannot induce PARylation. A. A PAR Western blot analysis after a 5 hour infection using monomer and prepore locked SLO strains with NADase active SPN. Non-specific band at 70 kDa appears to verify presence of lysate. B. PAR western blot with control untreated cells (C), SPN deletion (ΔSPN), SPN-H5, Monomer locked SLO with NADase inactive SPN (Mono-TM) and Prepore locked SLO with NADase inactive SPN (Prepore-TM). Images are representative of at least 3 independent experiments.

One common mode of PARP-1 activation is DNA damage. To address whether or not extensive DNA damage is present in these infection we isolated DNA from HeLa cells after a 5 infection. At this time point, high levels of PAR is present and the majority of cells are dying. Agarose gel analysis indicated that neither apoptotic nor extensive necrotic DNA was present at this time point(Figure 4A). To verify this we also analyzed HeLa lysates for phosphorylated H2AX (gamma H2AX). This histone is a checkpoint marker which is phosphorylated when double stranded DNA breaks occur (31). The phosphorylated form of H2AX could be detected
in an SLO dependent manner however it was hard to detect in most replicate experiments. It is also possible this phosphorylation is occurring because cells are dying and are not necessarily the cause of PARP-1 activation or cell death.

![Figure 4](image)

**Figure 4. Extensive DNA damage is not present during an infection.** A. A 2% agarose gel with 1 ug of DNA from HeLa cell after a 5 hour infection with the indicated strains. B. Western blot analysis of HeLa lysates after a 5 hour infection with the indicated strains for phosphorylated H2AX. Representative images are from at least 3 independent experiments.
**PARP activation is cyclical and sustained**

The normal cycle of PAR formation and degradation is quick often within minutes of each other (26, 33). The primary PAR glycohydrolase, PARG, quickly cleaves PAR with its endoglycohydrolysis and exoglycohydrolysis activity (8). This activity can be seen almost as quickly as synthesis of PAR. Since PARP-1 has so many roles in cell regulation PAR production is thought to be tightly controlled and transient. To determine when PAR formation appeared we used western blot analysis after an infection with varying O.D.s of SPN-J4, ΔSPN, and SPN-H5 to determine if a minimum amount of bacteria is necessary to induce this response. (Figure 5A). The standard assays utilizes an O.D. of 0.5 and causes massive cell death by 5 hours however PARylation can be seen with an O.D. as little as 0.1 which is not sufficient to cause cell death at this time point. At 5hrs, all concentrations of the SPN-J4 strain lacked PARylation. However, infections with the SPN deletion and the NADase inactive SPN-H5, PAR levels varied with O.D. Part of this variation could be due PARylation occurring in a cyclical manner as seen in the time course. A time course at an O.D. of 0.5 was also utilized to determine if PAR formation is transient or sustained (Figure 5B). During these infections we can see PARylation as early as 30 minutes. It also appears that PARylation is cyclical since levels appear to decrease and increase again over time. This process is also sustained in the SPN deletion and the NADase inactive SPN-H5 strains. Remarkably, in the NADase active infection PARylation does occur early but eventually disappears. This is likely due to the NADase activity of SPN and is addressed later in this chapter.
Figure 5. PARylation is cyclical and is sustained for hours. A. Western blot analysis of PAR after an infection with different O.D.s of indicated strains. The bracket denotes PAR on the blot. Actin Western blots are shown below each PAR Western blot. The O.D.s are indicated at the bottom of the panel B. A Western blot analysis of PAR during a time course infecting HeLa cells with the same strains. Actin Western blots were used as a loading control. Representative blots of 2 independent experiments.
SPN modulates PARylation through its NADase activity

As seen in the PAR western blot in Figure 1B, the NADase active SPN lacks PARylation despite activating PARP. There were two hypotheses we tried to address. One of which includes the possibility that all available NAD\(^+\) has been depleted thus prohibiting PARP from using NAD\(^+\) to form PAR. This study is detailed later in the chapter. The second possibility is that SPN is actively cleaving these polymers. Studies involving a detailed analysis of SPN's enzymatic activity showed that SPN has an affinity to ADP ribose and does not have product inhibition with nicotinamide (13). To determine if the NADase activity directly affects PAR levels, SPN mutants with varying levels of NADase activity were used to infect HeLa cells. Single and double polymorphic mutations that reduce the NADase activity were introduced into SPN through allelic replacement (Figure 6A). These mutants included single mutations R289K (SPN-R289K) and G330D (SPN-G330D) as well as the combination of the two (SPN-RK/GD). The reduction in the ability to cleave NAD\(^+\) molecules per minute ranged from 1.58 fold lower up to 30,000 fold lower (Figure 4A). In addition to these 3, the triple mutant lacking any NADase activity, SPN-TM, was also utilized. Cytolysin-mediated translocation was verified after an infection with all variant SPN strains (Figure 6B). We then tested whether the levels of PAR correlated with SPN's ability to cleave NAD\(^+\) (Figure 6C). As seen in the Western, the amount of PAR present in the lysates was dependent on the activity of SPN. The highest amounts of PAR were present in infections with the least active SPN variants. This indicates that SPN can modulate PAR levels through its NADase activity.
Figure 6. The NADase active SPN reduces PAR levels through its enzymatic activity.

A. Single and double mutations were made in the \textit{S. pyogenes} SPN-J4 chromosome to create mutants with reduced NADase activity. Fold differences in the rate of catalytic activity are
shown in reference to wild-type NADase active SPN. These values were determined in chapter 2
All strains are HA at the C-terminus. B. Cytolysin-mediated translocation of SPN was assessed
by HA antibody after an infection with strains indicated above the blot. C. PAR levels were
assessed after infecting HeLa cells with the indicated strains. The mean intensities of 3
experiments are shown below with standard deviation.

The reduced levels of PAR could also be due to the ability of SPN to cleave PAR polymers. To test this hypothesis, we performed an assay by infecting HeLa cells with the two strains that induced high levels of PARylation: the SPN deletion and the NADase inactive SPN-H5. Cells were then lysed and purified SPN was then added to these lysates and incubated at room temperature for 90 minutes. Western blots indicated only the NADase active form of SPN was able to reduce the amount of PAR in lysates post infection as compared to the NADase inactive SPN, buffer alone and those lysates saved without incubation (Figure 7). Treatment of lysates with NADase active SPN reduced PAR up to 50% in both lysates compared to starting PAR levels indicating that SPN has a PARG-like activity. PAR levels with the cell play an important role in cell signaling and metabolic homeostasis. Within the cells, PAR levels are regulated by Poly-ADP-ribose glycohydrolase (PARG), which breaks down PAR using both an endoglycosidase and exoglycosidase mechanism. It is possible SPN is also acting as PARG-like enzyme thus reducing the PAR levels. This has implications in terms of both cell signaling and cell survival since PAR is a charged and bulky molecule. The lack of PARylation in the NADase active SPN infection could also be due to the depletion of NAD$^+$ by SPN.
Figure 7. **Purified NADase active SPN can reduce PAR in lysates post-infection.** Lysates from SPN deletion and SPN-H5 infections were treated with buffer alone, 5 ug of purified SPN_{J4} or SPN_{H5} for 90 minutes at room temperature. The treatment of both types of lysates are indicated in the box below the column. Time 0 indicates an equivalent portion of lysate saved immediately following infection. Black columns represent SPN deletion lysates and gray columns represent NADase inactive SPN-H5 lysates. Actin was used as a loading control. Western blot is a representative of at least 3 independent experiments. Below the Western is the blot intensity and S.D. from those experiments.

**NAD^+ and ATP levels are affected by S. pyogenes and SPN**

Since active SPN and PARP can cleave NAD^+, intracellular NAD^+ levels were assessed in HeLa cells at three, five, and seven hours post infection. Infections with *S. pyogenes* showed
that NAD\(^+\) levels increase nearly two-fold at the five hour time point unless the NADase active SPN is present with the SPN-J4 infection. These levels remained at or near control uninfected levels at all time points (Figure 8A). A treatment with staurosporine, which activates PARP-1 showed a gradual decrease in NAD\(^+\) levels over time. Monomer and Prepore locked SLO with NADase active SPN (SPN\(_{J4}\)) also had a decrease in NAD\(^+\) levels while the NADase inactive SPN (SPN\(_{TM}\)) with the SLO mutants showed an increase in NAD\(^+\) levels (Figure 8B). This spike in NAD\(^+\) levels is likely due to a stress response as this increase is not dependent on SLO or SPN. To verify this, RT-PCR was performed to verify if the transcription of any NAD\(^+\) biosynthesis genes were upregulated during an infection. These included 3 isoforms of NMNAT. NMNAT-1 is present in the nucleus, NMNAT-2 in the mitochondria and NMNAT-3 in cytosol as well as NAMPT another prominent cytosolic enzyme (12). Analysis of these mRNAs indicated that NAMPT transcription is up regulated two-fold in all infections including the NADase active strain while NMNAT isoforms were all at control uninfected levels (Figure 8C). This was in contrast to treatment with staurosporine which had a dramatic increase in NMNAT-1, a nuclear enzyme, which has been shown previously to respond to an over activation of PARP-1.
**Figure 8. NAD⁺ levels increase during an infection with *S. pyogenes* in an SLO independent manner.** A. Intracellular NAD⁺ levels were assessed at 3, 5, and 7 hours in HeLa cells after an infection with various strains or treatment with 1 uM of Staurosporine, a PARP-1 activator. **B.** NAD⁺ levels in monomer locked and prepore locked SLO mutants with either NADase active SPN (SPN_{J4}) or NADase inactive SPN (SPN_{TM}) were assessed at 5 hours. **C.** Total RNA was isolated from HeLa cells after an infection and used for real time RT-PCR. Transcript levels for the NAD⁺ biosynthesis genes NMNAT-1, NMNAT-2, NMNAT-3, and NAMPt were assessed and compared to uninfected control. The dotted line represents no fold change from the control uninfected cells. Data shown is the mean and standard deviation of at least 3 independent experiments performed in triplicate.

Since these cells were up-regulating NAD⁺ and ATP is necessary for the generation of NAD⁺, ATP levels were evaluated after a 5 hour infection where the greatest amounts of NAD⁺ were observed. ATP levels were reduced in all infections but were significantly reduced in the NADase active SPN infection (Figure 9). This suggests the cells are attempting to increase NAD⁺ levels and are failing due to the presence of the enzymatically active SPN. In this attempt, these cells are also depleting ATP making the active SPN much like an over-activated PARP where NAD⁺ and ATP are depleted causing metabolic cell death.
**Figure 9. ATP levels are decreased in a NADase active infection.** HeLa cells were lysed and ATP levels were assessed after a 5 hour infection with indicated *S. pyogenes* strains. Asterisk indicates the ATP level is significantly lower than control levels. Student T-test (P <0.05). At least 3 independent experiments were performed and normalized to control.

Not only can the over-activation of PARP deplete NAD$^+$ stores and ATP but it also create an abundance of PAR which can interact with the mitochondria, release AIF from the mitochondrial membrane that can translocate to the nucleus, cause DNA damage and ultimately lead to cell death known as parthanatos (28). This type of cell death was assessed since SPN-J4 and SPN-H5 infection leads to cytotoxicity. In the case of SPN-H5 there is an accumulation of PAR, which could lead to AIF translocation from the mitochondria to the nucleus. To evaluate whether parthanatos cell death was occurring, immune-fluorescence of AIF was carried out to visualize the localization of AIF. AIF appeared to localize with mitochondria and did not localize to the nucleus in any *S. pyogenes* infections indicating that AIF does not contribute to cell death in any of these infections (Figure 10).
Figure 10. AIF is not translocated into the nucleus during an infection with *S. pyogenes*.

Immunofluorescence of AIF was performed after a 5 hour infection with indicated strains. Nucleus is stained with DAPI (blue) and AIF in green. Images are representative of at least 3 experiments.

**HMGB-1 is released from the nucleus in NADase active infections.**

To try and assess what other effects PARylation has on the cell we evaluated cells for the release of High Mobility Group Box-1 (HMGB-1) after an infection. HMGB-1 is both a marker for necrosis and an inflammatory molecule. In healthy cells and during apoptosis, this protein resides in the nucleus tightly bound to chromatin. During necrosis, this protein can translocate out of the nucleus into the cytosol and ultimately be released into the extracellular milieu to interact with TLR2, 4, and RAGE receptors further contributing to inflammation through NF-kB activation (25). This molecule is also one of the few endogenous signals marking self-injury.
For HMGB-1 to be released from the chromatin, it first must be PARylated (6). PARylation occurs early on in all SLO infections but is sustained in the SPN deletion and the SPN-H5. Since PARylation is modulated in our infections with the SPN variants we looked for the release of HMGB-1 from the nucleus into the cytosol. Infections with NADase active SPN had elevated levels of HMGB-1 in the cytosol as detected by immunofluorescence (Figure 11). In contrast this re-localization was not seen to the same levels in the SPN knockout, the NADase inactive SPN, nor the SLO knockout. The eventual release of this protein into the extracellular media was detectable by western blot in the NADase active infection (Data not shown) though not consistently. This was surprising as there is less PARylation in the NADase active infections however there is a cycle of PAR accumulation that occurs early infection (Figure 5) and could be involved in this release of HMGB-1. Taken together, this data implicates the NADase activity of SPN modulates the inflammatory response through its effects on PARylation.
Figure 11. HMGB-1 is released from the nucleus during NADase inactive infections. A. Immunofluorescence of HMGB-1 in HeLa cells after a 4 hour infection. Nucleus in blue is stained with DAPI. HMGB-1 is in red. The nucleus of cells that have a loss of HMGB-1 appears blue while co-localization appears magenta. B. Quantification of cells with HMGB-1 localized to cytosol. Cytoplasmic HMGB-1 is represented with the dark gray bar. Nuclear HMGB-1 is represented with the white bars. Student t-test indicates difference is extremely significant (P=0.0002). Three independent experiments were performed.
DISCUSSION

The studies undertaken in this chapter give us insight into the impact of the SPN activity and SLO on the cell during an infection. The function of NADase active SPN in infection had always thought to be simply the depletion of NAD$^+$ and ATP leading to cell death. However this enzymatic activity has a greater impact on the cell than previously thought. PARP-1 is an important regulator in stress, transcriptional regulation and inflammation and SPN’s ability to influence PARP’s activity has several implications. The findings in this study not only show that NADase active SPN can cleave cellular stores of NAD$^+$ but it also has the potential to cleave the product of PARP-1, PAR polymers. How SPN is capable of cleaving PAR is unclear but this is a novel finding for a NAD$^+$-glycohydrolase. An in depth study of the NADase activity of SPN showed that it has little to no product inhibition. However, at high concentrations ADP ribose is capable of having an effect on SPN’s ability to cleave NAD$^+$ (7). Perhaps this is an indication that SPN can interact with ADP ribose and is capable of cleaving polymers of ADP-ribose. Assays done prior to this study showed that SPN localizes to the cytosol (Supplemental Figure 1) therefore if SPN is cleaving PAR it is doing so in the cytosol. This may be another clue that PAR exits the nucleus to enter the cytosol during these infections. This has implications on cell signaling as well since PAR acting as a signaling molecule (27). In this case it seems to lead to a difference in downstream effects between these two infections like the release of HMGB-1.

In the NADase active infection, the release of HMGB-1 from these cells indicates that enough PARylation has occurred to have some effect on the outcome of the cell and the inflammatory response. These cells are likely signaling to the neighboring cells to respond to this danger signal. The consequence of the differential PAR levels is likely greater than what we have explored in this chapter. PARylated HMGB-1 has implications in the context of different cell
types since it has been found that the release of PARylated HMGB-1 inhibits efferocytosis, the process in which macrophages phagocytose apoptotic cells (5). Perhaps, in vivo the release of HMGB-1 affects the recruitment and function of immune-modulating cells. It has been shown to aid in the recruitment and maturation of immune cells (4) (18).

In contrast to the NADase active SPN, the inactive SPN-H5 accumulates PAR readily as does the SPN deletion. PARP-1 responds to many types of stress including genotoxic, oxidative, thermal, metabolic and microbial (15). This response is SLO dependent and could be activated by the damage this cytolysin can cause to the membrane. Some evidence of this can be seen in the lack of PARylation seen with prepore and monomer locked SLO, which cannot induce PARylation but can interact with the host membrane. Though SLO is necessary to induce PARP activation it is not sufficient implying that more than factor is needed for this activation. Perhaps the pore cannot be healed as easily during an infection or the localization of the pore also plays a factor in this response. It is also possible, the pores are allowing for passive diffusion of other bacterial products or simply allowing for an influx of ions like calcium, which may also contribute to a stress response. Previously, we found that SLO pore formation in conjunction with SPN is necessary for cell death. The monomer locked and prepore locked SLO can translocate SPN however there is virtually no cell death in these infections (17). Pore formation could lead to an assortment of responses that are needed in conjunction with SPN’s activity to kill cells.

Another interesting finding is that these cells have a considerable spike in intracellular NAD\(^+\) levels that can be attributed to NAMPt, a cytosolic enzyme. Though NAD\(^+\) increase was not SLO dependent it does seem to signify a stress response made by the cells to \textit{S. pyogenes}. NAMPt was the only NAD\(^+\) biosynthesis gene up-regulated in response to \textit{S. pyogenes} despite
PARP-1 activation in some infections. NAMPt is a cytosolic enzyme and responds to the levels of NAD\(^+\) in the cytosol, which could also indicate that SPN is acting in the cytosol. Previously it was found that NAMPt is more than just a biosynthetic enzyme. It was originally identified as a cytokine and has pro-inflammatory properties when released from the cell including the recruitment of neutrophils (24). Though this was not examined in these studies it would be interesting to know if the release of this enzyme from dying cells has any implications on the inflammatory response of the surrounding population of cells. In retrospect, the increase in NAD\(^+\) biosynthesis is not extraordinary, as these cells need this co-factor for many processes involved in metabolism and stress response. One reason some NAD\(^+\) remains in the NADase active infection could be due to the compartmentalization of NAD\(^+\) within the cell. The pools of NAD\(^+\) within the cell reside in the mitochondria, cytosol and nucleus. The highest concentration is in the mitochondria with no exchange between this pool and the cytosolic pool (14). However, there is crosstalk between the cytosolic and nuclear NAD\(^+\) that affect each other during NAD\(^+\) depletion. PARP-1 and NADase active SPN can affect both of these pools but it is unlikely the mitochondrial pool is available to either of these enzymes.

These studies indicate SLO is necessary to activate PARP-1 however we have not uncovered the exact mechanism for activation. Many studies involving PARP-1 activation utilize DNA damaging agents, however major DNA damage does not seem to be occurring in these infections. Aside from DNA damage, multiple factors have been uncovered which can activate PARP-1 including histone association, mono-ribosylation by other enzymes, acetylation and phosphorylation by Mitogen Activated Protein Kinases (MAPK) (8). The role of MAPK activation will be addressed in the next chapter. The fact that PARP-1 can be stimulated by many different factors which can lead to several different outcome highlights the importance of PARP-
1 to cell integrity and stability. Though PARP-1 is not essential for life of the cell it still contributes to a great number of processes within the cell with not only stress but also general transcriptional activation programs. The roles for PARP-1 in the cell continue to be revealed with more extensive studies.

Other bacteria have been shown to activate PARP-1 however this list has only recently started to grow. One bacteria known to cause activation is *Helicobacter pylori* with the multifaceted VacA toxin (22) and a heat and protease sensitive factor found in the *H. pylori* culture filtrate (HPCF) (19). *H. pylori* was the first bacteria discovered to activate PARP-1 directly and is a newly discovered strategy for host pathogen interactions. Although this is a new pathway to be discovered in terms of host pathogen interactions it is not new that PARP-1 is important in inflammatory responses as it is also involved in the activation of inflammatory responses including iNOS production, NF-κB activation and cytokine production (9-11, 30, 32).

The differential release of HMGB-1 from NADase active SPN infection gives us two clues to the differences between the variants of SPN. One is the indication necrosis is occurring in the NADase active infection but also that NADase activity of SPN may be dictating the inflammatory outcome of an infection. The next chapter will address these possibilities including the role of the MAPK JNK, its role in cell signaling and the inflammatory response. The discoveries in this chapter are the first elucidating the differences between the NADase activities of SPN and how they might contribute to pathogenesis. Further studies are necessary to determine how each contributes to cell signaling and cell death.
Acknowledgments

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**Supplemental Figure 1.** A549 cells were infected with either SPN-J4 or ΔSPN. The T indicates total cell lysates after an infection with SPN-J4. Cytosol and nuclear fractions are indicated. Anti-GAPDH was used to verify purity of cytosolic fractions and anti-HDAC for nuclear fractions. Control indicates uninfected cells. Anti-HA was used to detect SPN.
MATERIALS AND METHODS

**Bacterial Strains and Media.** *S. pyogenes* utilized were of the M serotype 6 strain JRS4. This includes all derivatives utilized in this work. SPN-J4, SPN-H5, SPN-R289K, SPN-G330D, SPN-R289K/G330D and SPN-TM express SPN with a carboxy-terminal HA epitope tag. The construction of these strains are described in Chapter 2. Routine culture of *S. pyogenes* was conducted using Todd Hewitt Yeast Extract supplemented with 0.2% yeast extract.

**Infection of HeLa cells.** *S. pyogenes* strains were inoculated in ThyB and grown overnight at 37°C. The following day, strains were sub-cultured 1:10 and allowed to double twice. Cultures were then subjected to centrifugation, washed with phosphate saline buffer (PBS), and re-suspended in tissue culture media. HeLa cells were then infected with an OD$_{600}$ of 0.5 for 5 hours unless otherwise specified.

**Isolation of DNA from HeLa cells.** DNA was isolated from HeLa cells after an infection using Qiagen’s DNeasy Blood and Tissue kit. Briefly, cells were washed twice with D-PBS, scraped and centrifuged at 500 x g to collect cells. Cells were then treated per manufacturer’s protocol. One ug of DNA was dry-loaded into a 2% agarose gel for electrophoresis.

**Treatment of cells for Western Blot analysis.** After infection, cells were scraped and centrifuged at 700xG for 10 min. Supernatants were discarded and cells were lysed with M-PER (Thermo scientific) by incubating in appropriate volumes based on number of cells at room temperature for ten minutes and then centrifuged for 10 min at max speed typically 20K x G. SDS-loading buffer (4x) was added, samples boiled and saved at 20°C until further analysis.
Antibodies. Antibodies for PARP, H2AX-p, AIF and HMGB-1 were purchased from Cell Signaling Technologies. PAR antibody was purchased from BD biosciences and Actin mouse monoclonal and anti-HA was purchased from Sigma.

Purification of SLO: WT SLO was expressed in E. coli by using the pTrc vector. The bacteria were grown in Luria Bertani medium containing 50 ug/ml Kanamycin and induced with 1mM IPTG. The cells were pelleted, resuspended in buffer (50 mM sodium phosphate buffer) incubated on ice with lysozyme (10 mg/mL) in the presence a protease inhibitor, sonicated and centrifuged for supernatants. Supernatants were used in purification by metal ion affinity chromatography using commercial matrix (Cat. No. 635503, Clontech). Protein concentrations were determined using a BCA assay (Pierce). Hemolytic activity was verified through a red blood cell lysis assay as described before (17).

Purification of SPN. Expression and purification of the His6-tagged recombinant proteins was performed using a periplasm preparation as previously described (13). As a final step, the purified proteins were dialyzed at 4°C against a buffer consisting of 50 mM potassium phosphate and 100 mM sodium chloride. Purity of the 50 kDa proteins was routinely assessed by SDS-PAGE and staining with Coomassie Brilliant Blue and protein concentrations determined using a BCA assay (Pierce) with a BSA standard.

Intracellular NAD⁺ Quantification. After the infection, HeLa cells were washed twice with D-PBS and harvested according to manufacturer’s instructions (Biovision, NAD⁺/NADH Quantification kit). Briefly, cells were resuspended in NADH/NAD buffer and extracted by
freezing on dry ice and thawing at 37°C twice. Cells were then vortexed and centrifuged at maximum speed for 5 minutes. To remove any NAD$^+$ consuming enzymes, samples were filtered through a 3 kd molecular weight cut off column. For total NAD$^+$ detection, 50 microliters of the samples were placed into the 96 well in duplicates. To detect NADH, 200 microliters were aliquoted to tubes and heated at 60°C for 30 minutes to decompose NAD$^+$. Samples were cooled and 50 microliters were aliquoted into the 96 well in duplicates. The NAD cycling enzyme mix (100 microliters) was added to each sample and NADH standards. Plates were incubated at room temperature for 5 minutes and 10 microliters of NADH developed was added. The cycling reaction proceeded for one hour at room temperature and plates were read 450nm on a Tecan M200 Pro Infinite plate reader. Calculations were based on the standard curve and normalized to control uninfected cells.

**ATP analysis.** To determine ATP levels in HeLa cells, cells were seeded in a 96 well black clear flat bottom plate prior to the infection. Post infection cells were lysed by the addition of 50 microliters of the mammalian cell lysis solution provided by the manufacturer (Perkin Elmer, ATPlite) directly to 100 microliters of media. The plate was placed on an orbital shaker at 700 rpm for 5 minutes. Then 50 microliters of substrate solution containing luciferase and luciferin was added and plate was placed on an orbital shaker for 5 minutes. Plate was then dark adapted for ten minutes and luminescence was measured on the Tecan M200 Pro Infinite plate reader. ATP levels were calculated using a standard curve and then normalized to control uninfected levels.
**Immunofluorescence.** Two days prior to infection cells were seeded into 8 well chamber slides. Confluent cells were then infected for specified times and subsequently washed with PBS before fixing with 4% paraformaldehyde for 15 minutes at room temperature. Cells were washed with PBS twice and permeabilized with 0.5% saponin/PBS. After three PBS washes, cells were blocked with 1% BSA diluted into PBS-Tween 0.1% (PBS-T/BSA). Primary antibodies were diluted into PBS-T/BSA 1:100 and incubated overnight at 4°C. Fluorescent secondary anti-rabbit antibodies, Alexa-fluor 594 or 488 (Invitrogen) 1:300, were incubated for two hours at room temperature in the dark. Following this incubation, cells were washed in PBS and the chamber removed. Ten microliters of Prolong Gold with DAPI was placed on each chamber square and a glass slide overlayed and set overnight. Cells were imaged using a Leica DMRE2 fluorescent microscope and images were obtained using Openlab software (Provision).

**RNA isolation and Real-Time PCR.** Total cellular RNA was isolated from HeLa cells after the indicated infection or treatment using Qiagen RNeasy. cDNA was created by subjecting RNA to reverse transcription using iScript (BioRad) as per manufacturer’s instructions. Real-time PCR analysis of cDNA samples was carried out using iTaq SYBR green supermix (Bio-Rad) using primers listed in Table S1. Relative transcript levels were analyzed using the \( \Delta\Delta C_t \) method with \( \beta\text{-actin} \) as a control standard. Data presented are the mean and standard error of the mean resulting from triplicate analysis of samples prepared from at least three independent experiments.
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CHAPTER IV

SPN influences cellular signaling and death in epithelial cells
SUMMARY

Virulence factors are often multifunctional and contribute to pathogenesis through different mechanisms. SPN and SLO are two factors that each has more than one role in the pathogenesis of *S. pyogenes*. SLO not only forms pores but also allows for the translocation of the NAD$^+$-glycohydrolase SPN. This pore contributes to cell death along with SPN but is not necessary for the translocation of SPN. SPN exists as two subtypes, one that exhibits this NADase activity and one that lacks this function. Despite this loss of enzymatic activity, *S. pyogenes* has continued to maintain SPN in its entirety and is associated with tissue tropism. Recent data indicates that each SPN can modulate different pathways with in epithelial cells after an infection. Both versions of SPN can cause cell death to equal degrees however little is known about the NADase inactive SPN or the mechanism of cell death. The active SPN can cleave NAD$^+$, eventually causing the depletion of both NAD$^+$ and ATP, likely creating a metabolic cell death. This does not explain how the NADase inactive SPN can contribute to cell death. The studies described in this chapter suggest that SPN is capable of eliciting necrosis through two distinct mechanisms: metabolic and programmed. *S. pyogenes* activates the MAPK JNK in an SLO dependent manner. This activation is sustained in all infections with pore forming SLO. Silencing JNK increased cell survival only in the NADase inactive SPN. Silencing JNK also increased mitochondrial membrane potential in the infections involving SPN. Silencing PARP did not increase cell survival in any infection however it did increase mitochondrial potential. These differences also led to distinct inflammatory profiles for the NADase active and inactive SPN. The inactive SPN created more IL-8 and TNF alpha while the NADase active had little to no cytokine production. Taken together, this data indicates a role for each SPN during an infection and likely contributes to how the bacteria interact with the host cell.
INTRODUCTION

Pathogenic bacteria must evolve to adapt to the host they live in often developing sophisticated means to create an advantage against the host. Multi-functional toxins allow bacteria to maximize these tools needed to colonize and infect their host. *S. pyogenes* is a strict human pathogen thus making their pathogenic mechanisms specific to human cell biology. *S. pyogenes* produces two toxins, SPN and SLO that not only work together in causing cytotoxicity to human cells but also maintain multifunctional roles in their interactions with the host.

The relationship between SPN and SLO continues to be unraveled. Recent studies have revealed that pore formation is not necessary for the translocation of SPN however is it necessary for cell death. SPN is also multifunctional as 2 subtypes of this enzyme exist however little is known about how these variants interact with the host cell. The previous chapter highlighted the differences in the cascade following PARP activation after an infection with either the NADase active SPN or the NADase inactive SPN. An important partner in these findings is SLO. We know that both are necessary to cause cell death as deletions of either reduce cytotoxicity and we now know that SLO pore formation is also necessary to induce responses that are subsequently modulated by SPN.

Questions remains regarding how the NADase inactive SPN contributes to cell death. Several studies have been done focused on SLO and various cell types. One study showed that SLO plays an integral role in causing apoptosis in macrophages however these studies were done in the absence of either versions of SPN (33). Another study in macrophages showed *S. pyogenes* can induce pyroptosis in an SLO dependent manner (16). A study in keratinocytes reported apoptosis occurred in an SLO dependent manner however this study also ignored the role of SPN.
Macrophages and epithelial cells are programmed to deal with pathogens in distinct manners making the comparison between these two types of cells difficult. In light of this evidence in conjunction with recent data indicating the NADase inactive SPN is equally toxin, it has become important to study the role of both variants of SPN and SLO in epithelial cell death. In the context of an infection, these cells are often the first barrier the bacteria need to overcome, colonize and disseminate. To date, there have been no studies on the possible role of NADase inactive variant and how either SPN in conjunction with SLO contributes to cell signaling and ultimately cell death.

NADase active SPN has a high catalytic rate and is capable of cleaving NAD$^+$ at fast rates. Studies done by others and work described in the prior chapter showed that NADase active SPN reduces NAD$^+$ levels in the cell as well as ATP levels (23). It is likely these cells are undergoing metabolic catastrophe. However this does not provide an explanation for the possible mechanism of cell death in the NADase inactive infection. This SPN has no detectable NADase activity yet it is capable of killing cells to the same capacity as the NADase active SPN. Much remains to be understood about the function of the NADase inactive form of SPN, why it is maintained in clinical isolates and why it is associated with tissue tropism. We now know that these cells also have sustained PARP activation and high levels of PAR however we don’t know how this SPN contributes to cell signaling and eventually cell death.

Although cell death can be broadly categorized into a few groups recent in depth studies have shown these processes to be complicated within these classes. It was once thought that cells either die by apoptosis or necrosis but now we have a greater understanding and have since discovered other pathways including pyroptosis, programmed necrosis and even the combination of the two – pyronecrosis (40). Each pathway has a defining set of characteristics (11). For
example, apoptosis is a programmed non-inflammatory mode of death owing to the activation of various caspases depending on the stimulus. This cascade leads to quiet break down of cells into smaller apoptotic bodies that are eventually taken up by resident macrophages. The release of caspase-1 is a defining characteristic of pyroptosis that leads to an inflammatory outcome involving the production of IL-1β and IL-8 and is largely found to be a mechanism of cell death in immune competent cells such as macrophages (12). Finally, necrosis, an inflammatory outcome, and can be further characterized into two distinctive groups, programmed and un-programmed. Several different stimuli and signaling cascades can lead to this outcome. Recently, the term programmed cell death or necroptosis has been recently been elucidated since a specific set of proteins has been found to be involved in necrotic cell death via receptor signaling including RIP1 and TRAF (9). These two proteins are involved in several different pathways involving several different partners and events that ultimately lead to necrosis.

Dysfunction within the cell can lead to cell death however the path can involve several different factors. One of these includes the regulatory eukaryotic protein PARP-1, which can cleave NAD⁺ and form poly ADP ribose polymers (PAR) on itself and substrates. Normally, this protein oversees and recruits DNA repair proteins to sites of DNA damage (22). It also plays a role in transcriptional regulation, chromatin reorganization and DNA replication thus making the protein multifunctional. Over-activation of this protein can lead to death through the depletion of NAD⁺ and subsequently ATP in effort to restore NAD⁺ levels. The antagonizing enzyme Poly ADP-ribose glycohydrolase (PARG) degrades and releases these PAR polymers to bring the state back to normal. When abundant, these PAR polymers can be released from the nucleus and subsequently lead to several transduction pathway including inflammation and cell death. Previously, we found that this protein is activated in an SLO dependent manner and the
formation of PAR polymers is modulated by SPN’s NADase activity. Though we found that PARylation does not cause parthonatos, a form of cell death involving PAR induced AIF translocation, it does not rule out a role for PARP in cell signaling that can add to cell death. In fact, PARP-1 has been shown to be involved in RIP1 mediated necroptosis often involving the activation of the MAPK JNK (10, 41).

There are several activators of PARP, one of which includes the multifunctional MAPK JNK (15, 45). JNK has previously been shown to play a decisive role in cell fate. Depending on the presence of other signaling cascades as well as the stimulation of the TNF receptor, JNK activation can have multiple outcomes including cell survival, apoptosis or necrosis (24). This cascade also involves cross talk with the transcriptional activator NF-κB that can prevent JNK mediated cell death by conducting a different transcriptional activation program that leads to cell survival. Several of these gene products involve inhibiting the production of reactive oxygen species (43) that can cause necrosis. Though JNK can activate PARP-1, PARP-1 activation can also precede of JNK activation (41). The factors that decide the timing of these activation events are unclear.

So far we have found PARP-1 activation is SLO dependent but have not determined the exact mechanism for this activation. Cytolysin dependent cytolsins (CDCs) including SLO have been shown to activate MAPKs, specifically p38. As mentioned above, MAPKs are involved in several signaling pathways including PARP-1 activation and cell death. Little is known about how the interplay of these pathways during an infection with *S. pyogenes*. Though SLO can activate MAPKs, nothing is known about the role of both SPN and SLO in regard to MAPK activation during an infection (18, 20, 27). With this information, we decided to study what roles
MAPKs and PARP-1 had with each other and if these two proteins participate in cell signaling and cell death during an infection with SLO and the NADase SPN variants.

Our study shows that in epithelial cells SLO was necessary to activate JNK. Unlike other studies, the activation of p38 or ERK was not dependent on SLO but rather a general response to infection. The synergistic relationship between SPN and SLO leads to necrosis but the NADase activity of SPN dictates the inflammatory profile of these dying epithelial cells. NADase inactive SPN undergoes a JNK mediated cell death as silencing JNK leads to increased cell survival. Though silencing PARP did not increase cell survival, it did increase mitochondrial potential in the NADase inactive infection indicating PARP has a factor in the demise of these cells. These findings ultimately tell us that although both types of SPN cause necrosis, they each create divergent pathways that lead to two different types of necrosis: metabolic and programmed. This data has given us clues to why NADase inactive SPN is still under positive selection.
RESULTS

SPN and SLO act synergistically to cause necrosis in epithelial cells

Previously, we showed SLO and SPN are both necessary to cause accelerated cell death as knocking out either protein reduced cytotoxicity in epithelial cells. Strains with wild-type SLO but lacking SPN are more cytotoxic than a SLO knockout strain however the kinetics of this are slower than then infecting with strains including both SLO and SPN. This outcome is not dependent on the NADase activity of SPN since removing this function of SPN does not inhibit cell death indicating that SPN has a dual role in the pathogenesis of S. pyogenes. To determine how epithelial cells are affected by SLO and its relationship with variants of SPN we performed several assays to determine what pathway of cell death these cells undergo. The enzymatic activity of SPN is likely influencing how these cells die, however the robust NADase activity cannot be the only contributing factor of SPN. Assays were done to establish what mode of cell death is occurring during these infections including apoptosis, pyroptosis, and necrosis and to differentiate between these two naturally occurring variants of SPN. Prior to our study, pore formation in macrophages during an S. pyogenes infection was found to be an inducer of pyroptosis, a caspase-1 dependent process of cell death (12). Studies to determine whether pyroptosis occurred during an infection were concluded as negative since this mechanism could not be induced in HeLa cells suggesting this is not a characteristic mode of cell death in epithelial cells. Other studies have suggested that SLO alone is necessary to cause apoptosis in keratinocytes (8). Though we know that SLO is vital to cell death, our assays indicate that apoptosis is not occurring in our infections. Hallmarks of apoptosis such as nuclear fragmentation, impermeable membranes and caspase activation were assessed after a 5 hour infection with the NADase active SPN-J4, the NADase inactive SPN-H5, SPN deletion (ΔSPN)
and SLO deletion (ΔSLO). The presence of SPN increased cell permeability greatly compared to the SPN knockout infection (Figure 1A). Over 90% of cells are compromised in comparison to either the SPN deletion with about 10% permeability and SLO deletion that is almost completely impermeable. This was also compared to staurosporine treated cells, a known inducer of apoptosis. The membranes of these cells remained intact however their morphology showed a significant change. To get a more detailed look at the cells during an infection, we assessed the status of both the mitochondria and the nucleus after a 5 hour infection. Both SPN variants also affected mitochondrial potential (Figure 1B). Using TMRE as an indicator for membrane potential, the presence of either SPN variant abolished the mitochondrial potential equally while the SPN deletion and the SLO deletion affected the mitochondria. DAPI staining of the nucleus showed that nuclear fragmentation does not occur during these infections in contrast to staurosporine treated cells, which undergo apoptosis causing DNA condensation (Figure 1C). Both variants of SPN had a greater effect on cells than with the SPN knockout that has SLO alone. In order to verify apoptosis did not occur in these infections, the presence of activated caspase in HeLa lysates was assessed. Using a synthetic substrate of caspase 3, a converging player in both the intrinsic and extrinsic apoptotic pathway, we found none of the strains could stimulate activation of these caspases (Figure 1D). To verify this, caspase 8 activation was examined as it can be activated by TNF alpha-receptor stimulation. Again, this was compared to staurosporine treatment of cells, which can activate caspase 3 and 8. Taken all together, we can conclude that these cells are undergoing a mode of necrosis dependent on both SLO and SPN, eventually leading to compromised cell membranes and loss of mitochondrial potential. As recent literature indicates, this is not a simple pathway to death nor is it confined to one modality.
Figure 1. SPN induces necrosis

A. HeLa cells were stained the membrane permeable dye Calcien AM (green) and the impermeable dye ethidium homodimer-2 (red) after a 5 hour infection with the indicated strains. Live cells are stained green and dead cells red. B. The mitochondrial dye TMRE (red) was used to assess mitochondrial membrane polarization. Images are overlaid with brightfield images of cells. Mitochondria with membrane potential retain this dye and appear red. C. The nuclei of cells were stained with DAPI and images were taken at 63x. Strains used are indicated above the image. D. Caspase 3 and Caspase 8 activation was assessed by the addition of caspase 3 and 8 synthetic substrates to lysates of infected HeLa cells and read at 405nm. Data presented is the mean and S.D. of 3 independent experiments.
**S. pyogenes induces JNK activation in an SLO dependent manner**

Previously, we found that PARP is activated in an SLO dependent manner. The most well studied activator of PARP is DNA damage however we found that was likely not the case. MAPK activation is also known to activate PARP and MAPK activation has been shown to occur in a CDC dependent manner. Previously, pore forming toxins from a variety of gram-positive bacteria including SLO were shown to activate p38 (18, 20, 27, 31). To examine what role SPN and SLO may play with MAPK activation during in an infection we examined HeLa cells for the phosphorylated forms of p38, ERK and JNK after infection. All three pathways were activated during an infection however only JNK was activated in a strictly SLO dependent manner (Figure 2). This was similar to our findings with PARP. Pore formation was necessary for this activation, as the prepore locked SLO could not activate JNK to the same levels as wild-type SLO. The prepore locked SLO induced some JNK activation but at low levels and is likely due to membrane perturbation. Monomer locked SLO had near background levels of activated JNK compared to those infections with wild type SLO (Figure 2B)
Figure 2. *S. pyogenes* activates several MAPKs however JNK activation is SLO pore dependent. **A.** Western blot analysis of HeLa lysates after a 5 hour infection with the indicated strains. Antibodies for the activated MAPKs were used as probes. Actin was used as a loading control. The C indicates uninfected control. St indicates treatment with 1 uM of staurosporine. **B.** Strains expressing monomer locked and prepore locked SLO in the JRS4 background were also assessed and compared to both the NADase active (SPN-J4) and inactive (SPN-H5) variant. Both express SLO capable of forming pores. Actin was used as loading control. Blots are representative of at least 3 independent experiments.

The activation of JNK in healthy cells is often transient and it is thought the sustained activation of JNK leads to deadly cellular outcomes (34, 45). This activation has an important function in cell differentiation and stress response. The duration of activation of these kinases help determine the cell fate. Differentiation tends to have transient JNK activation while sustained JNK activation can lead to cell death (19). To assess the time course of JNK activation in HeLa cells after an infection, we used Western blot analysis after several time points as well
as several optical densities of bacteria (OD_{600}) to determine if there is a minimum amount of bacteria needed to activate this pathway. Lower amounts of bacteria could stimulate JNK activation (Figure 3A). We also found that it took 3 hours to activate JNK at an OD of 0.5 (Figure 3B). During these infections, activation of JNK was not transient and was sustained for long periods of time, a known marker for cell death.

Figure 3. JNK activation is O.D. and time dependent. A. Western blot analysis of HeLa cells for phosphorylated JNK after a 5 hour infection using several indicated ODs. Strains used are indicated to the left of the blot. B. Phosphorylated JNK was also assessed after each indicated time point for SPN-J4 and SPN-H5. Actin was used as a loading control and blots are presented below each pJNK Western. Westerns are representative of at least 2 independent experiments.
**JNK and PARP contribute to mitochondrial depolarization**

As mentioned before the activation of JNK has multiple outcomes, which include NF-κB activation, apoptosis and necrosis. Interestingly, PARP can also contribute to these same outcomes through different pathways (3, 17, 39, 41). In an effort to reveal the role of JNK in SPN mediated cell death, we utilized a siRNA targeted to JNK1 and PARP-1. We also used a combination of a non-targeted siControl and siGLO, a fluorescent transfection efficiency indicator. These controls were used at the same concentration as targeted siRNAs (100nM). siGLO indicated that almost 100% of cells were transfected (Data not shown). As indicated in Figure 3, efficient knockdown was achieved in both JNK silenced cells (98% knockdown) and PARP-1 (undetectable PARylation activity) (Figure 4A). Control, uninfected cells were compared to cells infected with the SPN-H5 strain to verify both PAR and JNK1 was not present as a SPN-J4 infection does not accumulate PAR. Cytolysin-mediated translocation of SPN was unaffected by these siRNAs (Figure 4B). The silencing of JNK1 also provided evidence that JNK1 does not activate PARP since this blot also indicates knockdown of JNK1 does not prevent PARylation. We could not assess through Western blot analysis whether the silencing of PARP affected JNK1 activation since all isoforms of JNK (1, 2, and 3) are phosphorylated at the same residues making it impossible to create specific antibodies for pJNK1.
Figure 4. Silencing PARP and JNK is efficient and does not inhibit CMT. A. Western blot analysis of total JNK1 and PAR in PARP silenced and JNK1 silenced cells. Control indicates uninfected cells. siControl are cells treated with a non-targeted siRNA and siGLO a transfection indicator to measure efficiency. Efficiency of knockdown was measured using Actin band intensities. B. Western blot analysis of HeLa lysates using anti-HA to verify SPN is translocated into the host cell cytosol. Blots are representative of 2 independent experiments.
Both activated JNK and PAR polymers can be released from the nucleus, interact with the mitochondrial membrane and cause membrane depolarization (5, 37). To evaluate the possibility that these molecules contribute to depolarization we stained the mitochondria to assess membrane potential after a 5 hour infection in JNK and PARP silenced cells. As with an infection with strains expressing either SPN, the siControl and GLO treated cells lost mitochondrial potential with both the NADase active and inactive SPN strains. In contrast, mitochondrial membrane potential was protected in JNK silenced in the NADase inactive SPN-H5 infection and to a lesser degree in the NADase active SPN-J4 infection (Figure 5). Interestingly, silencing PARP also increased mitochondrial polarization in the NADase inactive infection where PAR levels can be high however it had no effect in the NADase active infection. This is not surprising, as there is virtually no PAR in these infections at this time point and these cells are undergoing metabolic dysfunction through the loss of NAD⁺ and ATP. This data indicates both PAR polymers and activated JNK have an effect on the mitochondria during an infection with either SPN variants.
Figure 5. Mitochondrial polarization increases with JNK and PARP silencing. A. TMRE was used to stain mitochondria (red). Healthy, polarized mitochondria are able to retain this fluorescent dye. First panel are cells treated with siControl and siGlo, second panel are cells treated with siPARP and third panel is treated with siJNK 72 hours prior to infection to ensure efficient silencing as seen in Figure 3. Indicated within the image is the strain used to infect HeLa cells. Control indicates uninfected cells. B. Quantification of healthy mitochondria capable of retaining TMRE. White bars represent siControl treated cells, gray bars represent siPARP treated cells, and black bars represent siJNK treated cells. Data represents 2 independent experiments.
JNK contributes to cytotoxicity in NADase inactive infections

Since these molecules have an effect on the mitochondrial membrane and are known contributors to cell death we examined cytotoxicity in the PARP and JNK silenced cells after an infection. The reduction of PARP did not increase cell survival in the SPN infections that typically cause massive cell death (Figure 6). Remarkably, silencing JNK did increase cell survival drastically in NADase inactive SPN infections with approximately 60% cell survival. Survival was increased to a smaller degree in the NADase active infection with about 25% cell survival. This data further solidifies that the NADase active SPN infection is undergoing metabolic catastrophe despite active JNK signaling and that silencing JNK can save some population of the cells. With the NADase inactive SPN infection, this data indicates that these cells are experiencing signaling mediated cell death with JNK1 as a major contributor to this outcome. Cells in the SPN deletion were unaffected by either PARP or JNK silencing (Data not shown).
Figure 6. Silencing JNK, but not PARP, increases cell survival. Cytotoxicity was assessed after a 5 hour infection using calcein-AM which stains live cells and ethidium homodimer-2 which stains dead cells. Cells were treated with siRNA 72 hours prior to infection. The ** indicates P < 0.01 and *** indicates P < 0.001 using a Student t-test. Data represents at least 3 independent experiments.

The NADase activity of SPN affects the downstream events of JNK and PAR

JNK and PARP activation occurs when SLO is present and both of these proteins are well knock players in various signaling cascades. Based on our earlier studies, we could conclude that apoptosis is likely not occurring leaving NF-κB activation and programmed necrosis as possible outcomes of this activation. Prior reports showed SLO alone can activate the transcription of inflammatory cytokines however studies involving SPN have not been done (28). We then asked the question how does the NADase activity of SPN effect cell signaling. NADase active SPN depletes the cell of NAD⁺, ATP, and lacks PAR which can lead to different outcome from those
infections that have either a SPN knockout or NADase inactive SPN that have an abundance of these three metabolites. We examined the presence of inflammatory markers to determine what cytokine profile each of these infections present. Previously, we found that High Mobility Group Box-1 (HMGB-1), a marker for necrosis and an inflammatory molecule, was released into the cytosol in NADase active SPN infections. In contrast this re-localization was not seen to the same levels in the SPN knockout, the NADase inactive SPN, nor the SLO knockout. During necrosis, this protein can be released into the extracellular milieu to interact with TLR2, 4, and RAGE receptors further contributing to inflammation through NF-κB activation (21, 25). This data implicates not only is necrosis occurring but also a different signaling cascade is likely occurring. Since HMGB-1 can interact with several receptors that eventually lead to the activation of NF-κB. Aside from HMGB-1, PARP1 also activates NF-κB adding to the list of proteins that could contribute to inflammation (44). In unstimulated cells, this subunit is found in the cytosol however once this pathway is activated, the NF-κB inhibitor degrades releasing the p65 subunit to allow for its translocation to the nucleus where it acts a transcriptional activator for several genes involved in immune response. We used immunofluorescence to detect the translocation of the NF-κB p65 subunit into the nucleus after an infection with each SPN variant, the SPN deletion and the SLO deletion. Activated NF-κB could be visualized in the majority of cells infected with either the SPN knockout or the NADase inactive SPN and about half the cells in a NADase active infection (Figure 7). Though there was a reduction in the number of cells with translocated p65 subunit in the NADase active infection, it was not statistically significant.
Figure 7. **NF-κB is activated in an SLO dependent manner.**

**A.** Immunofluorescence for the translocation of the NF-κB p65 subunit in HeLa cells after a 5 hour infection. Nuclei are stained with DAPI (blue) and NF-κB p65 (red).

**B.** Quantification of 3 independent experiments. Nuclear translocation of the p65 subunit is represented by dark gray and cytosolic NF-κB p65 is represented by light gray.
To verify the significance of this translocation, RT-PCR was performed to detect the up-regulation of cytokines that are regulated by NF-κB such as IL-6, IL-8 and TNF alpha. In correlation with activated NF-κB, transcripts of cytokines were also augmented in the SPN knockout infection and NADase inactive SPN. Compared to the uninfected control these transcripts were also elevated in the NADase active SPN infection however not to the same levels as the SPN knockout or NADase inactive SPN (Figure 8A). Specifically, IL-8 and TNF alpha were highly up regulated in those two infections compared to the NADase active SPN. To verify transcript levels correlated to levels of protein produced, ELISAs of supernatants after an infection were performed to detect the presence of cytokines (Figure 8B). In correlation with transcript data, IL-8 and TNF alpha cytokines were present in the supernatants of NADase inactive and SPN deletion infections at significantly higher levels than the uninfected control and NADase active infection. However, IL-6 protein levels did not differ between any of the infected or uninfected condition. SPN deletion and NADase inactive SPN-H5 create more cytokines than the NADase active SPN-J4 implying these cells have a greater capacity to signal neighboring cells. Taken together, this cytokine profile indicates the enzymatic activity of SPN can modulate the downstream signaling events that lead to different inflammatory cascade. SLO plays an important role in this stimulation as the SLO knockout had reduced amounts of NF-κB translocation and thus cytokines compared to the other infections.
Figure 8. Differential cytokine production in NADase active and inactive SPN infections.

A. Cells were infected with indicated strains. Total RNA was isolated and used for quantitative RT-PCR for the indicated genes. Transcript levels were normalized to B-actin compared to control uninfected cells. Data indicates fold change.

B. Cytokine levels were assessed in culture supernatants after a 5 hour infection with indicated strains. Concentrations are in pictograms per milliliter. All experiments are the S.D. of 5 independent experiments.
DISCUSSION

Ultimately, our findings suggest that SPN is necessary to modulate cell signaling that is initiated by SLO leading to necrosis. Though both variants of SPN lead to necrosis we found major differences between these two infections that affect the steps leading to cell death (Figure 9). This is also the first report that SPN can cause necrosis via 2 mechanisms. This data gives insight into why a NADase inactive version of SPN is maintained and perhaps why every strain maintains SPN and SLO. One virulence factor, SPN, dictates the inflammatory profile through the presence or absence of NADase activity thus signifying a role in the pathogenesis of S. pyogenes.
**Figure 9. A Model of Necrosis Mediated by SPN and SLO.** In an SLO dependent manner, PARP and JNK are activated leading to NF-κB activation. However, with the translocation of NADase active SPN, NAD$^+$ and ATP are depleted leading to metabolic necrosis. The lack of PAR accumulation could be due to both the activity of SPN as well as lack of NAD$^+$ substrate. These processes lead to HMGB-1 release. With the translocation of NADase inactive, PAR is allowed to accumulate and in conjunction with activated JNK lead to mitochondrial depolarization, cytokine release and eventually JNK signaling mediated cell death.

The NADase active SPN has an obvious function – to cleave available NAD$^+$ leading to several downstream effects. The first and foremost is the effect of depleting a co-factor that is important in several metabolic processes necessary. The cell then attempts to replenish NAD$^+$ stores however this attempt is futile eventually depleting the cell of both NAD$^+$ and ATP. Another downstream effect is that the cleavage of NAD$^+$ creates two small molecule products could be involved in intracellular signaling: ADP ribose and nicotinamide. Nicotinamide is an inhibitor of ADP-ribosylating proteins but is also the starting molecule needed to make NAD by NAMPt (2). Free ADP ribose can activate the TRPM2 channel allowing an influx of calcium that amplifies several signaling pathways including ERK and cell death (32, 42). The ADP-ribose formed from PARP-1 activity has been associated with TRPM2 activation thus this could also be occurring during our infections (13). The implication that SPN could also be cleaving PAR polymers also adds to the list of differences between signaling molecules present in NADase active infections versus the inactive infection. An in depth study involving any of these pathways would give us more insight into how the molecules modulated by SPN contribute to inflammation and death.
The function of the NADase inactive SPN remained a complete mystery until these studies were undertaken. Prior to this study, we found the NADase inactive SPN was not toxic to yeast as it was readily expressed by *S. cerevisiae* (Figure S1). In contrast, plasmid with NADase active SPN could not be transformed to create viable yeast. Although we have yet to determine the secondary target of SPN, we now know the inactive SPN contributes to cell death through JNK signaling and subsequently leads to greater cytokine production. SPN modulation of PARP induction is also of consequence. A study by the Dawson group showed that the introduction of PAR polymers is toxic to cells at certain concentrations (1). PAR polymers may also be associated with mitochondrial depolarization since silencing PARP increasing the mitochondrial potential during a NADase inactive infection. The interaction between these molecules and the mitochondria is not uncommon. Silencing PARP does not increase cell survival and it may even have the opposite effect than initially expected. As discussed in the previous chapter, PARP is a multifaceted enzyme and is involved in numerous processes including transcriptional control and genome integrity. Removing this protein from the cell’s arsenal has many effects aside from simply a loss of PAR polymers. PARP-1 is also involved in cell division and spindle segregation (30) (7). Immunofluorescent staining of these cells indicate that both cell shape and nucleus are dysmorphic suggesting that loss of PARP has many different effects on the cell (Data not shown).

Initially, the activation of MAPKs was pursued to identify if these proteins were activating PARP-1. We found that JNK1 was activated in an SLO dependent manner as was PARP-1 activation however Figure 3 shows us that silencing JNK1 has no effect on the activation of PARP-1 as PAR formation was abundant. PAR formation also occurs early and before JNK activation. In this case, it is possible PARP-1 and JNK1 are stimulated by two
independent pathways, eventually converging to contribute to cell death potentially through mitochondrial depolarization. One possible convergence point is the production of reactive oxygen species (43). Both the abundance of PAR and activated JNK1 has been shown in stimulate the mitochondria and produce ROS to the point of cell death (41, 45). The order of these events can dictate whether apoptosis or necrosis occurs (24, 26, 36). JNK activation can stimulate ROS production and has been associated with TNF alpha induced necrosis (36) while JNK activation due to ROS production has been linked to apoptosis (26, 29). While we know that JNK activation and necrosis occur in our infections, we have yet to elucidate the intermediates steps between these two factors. Programmed necrosis, termed necroptosis is a well-orchestrated form of necrosis and involves the serine/threonine kinase RIP1. This kinase is downstream of TNF receptor, TLRs, and PARP activation and is at the crossroads of several different signaling cascades that all have the potential to lead to necrosis (10). Necroptosis and necrosis share many morphological and biochemical features however the inhibition of RIP1 cannot prevent un-programmed necrosis like necrosis founded on metabolic dysfunction (35). It is striking that one virulence factor, SPN, has the ability to provoke both types of necrosis depending it on its NADase activity. How the NADase inactive SPN is capable of stimulating necrosis remains unknown but it is clear JNK plays a major role. A closer look at the role of RIP1 is needed to further validate the NADase inactive SPN promotes necroptosis.

Phosphorylated JNK has been shown to directly interact with the mitochondria. Results from a study using anisomycin stressed HeLa cells suggested that cellular stress altered mitochondria, which caused JNK to translocate to the mitochondria creating a large amplification of ROS (4). The mitochondrial binding partner for JNK is Sab and its been shown that interfering with this interaction can reduce ROS production (6). In our studies we found that
silencing JNK increases mitochondrial potential in both the NADase inactive and to a lesse degree in the active infections despite the lack of cell survival in the NAD active infection. This data suggests that activated JNK interacts with the mitochondria and this interaction plays a larger role in cell death in the NADase inactive infections. The production of ROS during these infections is the next step to understand cell signaling in these cells. *S. pyogenes* also produces an enzyme called lactate oxidase (*lctO*), which generates large amounts of hydrogen peroxide. It would be interesting to determine what role LctO plays in cell signaling and if this directly induces PARP-1, JNK1 and necrosis.

The outcome of JNK activation is dictated by the activation of NF-κB. JNK is capable of causing either apoptosis or necrosis however the transcriptional activator NF-κB can suppress these outcomes through the activation of several genes that work to counteract different aspects of TNF receptor activation including ROS production. In our infections, we see that SPN deletion and the NADase inactive SPN-H5 have similar effects on the cell: PAR formation, NF-κB activation and cytokine production. However the SPN deletion infections are not as cytotoxic to these cells in this time frame. Perhaps the differences between these infection at these early times points can be attributed to the pro-survival genes up-regulated by NF-κB. NF-κB activation can lead to cell survival through the production of several genes that combat JNK activation as well as the production pro-inflammatory cytokines to recruit immune to cells to control infection. It is possible during the SPN deletion infection some SLO pores are healed allowing for prolonged cell survival. In fact the resealing of SLO pores has been accompanied by NF-κB activation (38). The differences contributing to cell death in the NADase inactive SPN infection versus the SPN deletion is subject to further study.
These studies have given us further understanding into how these SPN variants affect the cell during an infection and why they are both maintained in clinical isolates. The differences in inflammatory profiles may indicate why two variants of this NADase activity exist. The NADase active SPN is associated with generalist strains while the NADase inactive SPN is associated with specialists and perhaps this inflammatory response influences the strains ability to cause infections at different sites. Conceivably, dissemination and versatility of the bacteria would be altered if a greater inflammatory response occurs bringing in immune cells to police the infection. While we have discovered differences in cellular response between these two types of SPN the list of experiments to fully elucidate the mechanism of cell death continues to grow. There is much to be discovered in terms of how these two function in the cell, how it contributes to the pathogenesis of S. pyogenes and how SPN factors into tissue tropism.
Acknowledgments

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Figure S1. NADase inactive SPN is not toxic to *S. cerevisiae*. A. Western blot analysis of whole cell extracts prepared from *S. cerevisiae* yeast expressing SPN that lacks NAD+-glycohydrolase activity or the plasmid vector alone (vector). SPN is expressed from the pYES2 vector under a galactose-inducible promoter. Expression was assessed at the times indicated (in hours) following growth in the presence of 2% glucose (Glc) or 2% galactose (Gal). B. Growth of the indicated strains in the presence of 2% galactose. Viable yeast transformants with plasmids expressing NADase active SPN were unobtainable. Adapted from (14).
MATERIALS AND METHODS

Bacterial Strains and Media. *S. pyogenes* utilized were of the M serotype 6 strain JRS4 and derivatives including SPN1 (*spnΔ*), SLO1 (*sloΔ*) SPN-J4, and SPN-H5 which expresses SPN with a carboxy-terminal HA epitope tag. The construction of these strains are detailed in chapter 2. Routine culture of *S. pyogenes* was conducted using Todd Hewitt Yeast Extract supplemented with 0.2% yeast extract.

Infection of HeLa cells. *S. pyogenes* strains were inoculated in ThyB and grown overnight at 37°C. The following day, strains were sub-cultured 1:10 and allowed to double twice. Cultures were then subjected to centrifugation, washed with phosphate saline buffer (PBS), and resuspended in tissue culture media. HeLa cells were then infected with an OD<sub>600</sub> of 0.5 for 5 hours unless otherwise specified.

Cell lysis for Western Blot analysis. After infection, cells were scraped and centrifuged at 700 x g for 10 minutes to pellet intact cells. Supernatants were discarded and cells were lysed with M-PER (Thermo scientific) by incubating in appropriate volumes at room temperature for ten minutes and then centrifuged for 10 minutes at max speed typically 20K x g. SDS-loading buffer (4x) was added, samples boiled and saved at 20°C until further analysis.

Antibodies. Antibodies for P-JNK, P-ERK, P- p38, JNK1, NF-κB p65 were purchased from Cell Signaling Technologies. PAR antibody was purchased from BD biosciences. Actin AC-40 was used as a loading control. Anti-hemagglutinin antibody was purchased from Sigma.
**Caspase Activation Assay.** Caspase activation was assessed using a colorimetric substrate assay (Alexis ALX-850-228). HeLa cells were infected for 5 hours and lysed according to manufacturer's instructions. Briefly, 2x reaction buffer with DTT was then added to lysed cells. Five microliters of the 4 mM pNA conjugated substrates (200 uM final) for Caspase 3 and 8 were added and incubated for 1 hour at 37°C. Samples were read in a 96 well plate read (Tecan M200 Infinite Pro) at 405 nm.

**Cell Permeability and cytotoxicity Assay.** After an infection, HeLa cell membrane integrity was assessed by the cell’s ability to exclude the membrane impermeable fluorescent dye ethidium homodimer-1 by using a commercial probe (Live/Dead, Invitrogen) as described previously. Live cells were stained with the green fluorescent calcein-AM probe.

**Fluorescent staining.** For nuclear staining, Prolong Gold with DAPI was used from Invitrogen. For mitochondrial membrane potential detection, tetramethylrhodamine ethyl ester (TMRE) was diluted into D-PBS for a final concentration of 150 nM. For a positive mitochondrial membrane potential, carbonyl cyanide 3-chlorophenylhydrazone (CCCP) was used at 50 uM for 1 hour prior to staining. After an infection, cells were washed with D-PBS once and TMRE was incubated with the cells for 20 minutes at 37C. After this time, the stain was removed and replaced with D-PBS. Cells were imaged using a Leica DMRE2 fluorescent microscope and images were obtained using Openlab software (Provision).

**Immunofluorescence.** Two days prior to infection cells were seeded into 8 well chamber slides. Confluent cells were then infected for specified times and subsequently washed with PBS before
fixing with 4% paraformaldehyde for 15 minutes at room temperature. Cells were washed with PBS twice and permeabilized with 0.5% saponin/PBS. After three PBS washes, cells were blocked with 1% BSA diluted into PBS-Tween 0.1% (PBS-T/BSA). Primary antibodies were diluted into 0.1% PBS-T/1%BSA 1:100 and incubated overnight at 4°C. Fluorescent secondary anti-rabbit antibodies, Alexa-fluor 594 or 488 (Invitrogen) 1:300, were incubated for two hours at room temperature in the dark. Following this incubation, cells were washed in PBS and the chamber removed. Ten microliters of Prolong Gold with DAPI was placed on each chamber square and a glass slide overlayed and set overnight. Cells were imaged using a Leica DMRE2 fluorescent microscope and images were obtained using Openlab software (Provision).

**siRNA silencing of JNK and PARP.** siRNAs knockdown was performed with 100 nM of siRNA duplexes targeting JNK1 and PARP-1 purchased from Cell Signaling. siControl and siGlo were purchased from Dharmacon and used at a total concentration of 100nm. Lipid and siRNA complexes were made per manufacturer’s instructions using Lipofectamine 2000 and OPTI-MEM I from Invitrogen. Using a reverse transfection method, these complexes were added to trypsinized cells in each well in complete media. Cells and complexes were incubated for at least 24 hours up to 48 hours before media was removed and fresh complete media added. Cells were infected 72 hours after treatment with siRNAs.

**RNA isolation and Real-Time PCR.** Total cellular RNA was isolated from HeLa cells after the indicated infection or treatment using Qiagen RNeasy. cDNA was created by subjecting RNA to reverse transcription using iScript (BioRad) as per manufacturer’s instructions. Real-time PCR analysis of cDNA samples was carried out using iTaq SYBR green supermix (Bio-Rad) using
primers listed in Table S1. C1000 Thermal cycler with CFX96 Real Time System was used for analysis. Relative transcript levels were analyzed using the ∆∆C_t method with β-actin as a control standard. Data presented are the mean and standard error of the mean resulting from triplicate analysis of samples prepared from at least three independent experiments.

**ELISA.** For Il-6 (44 HS600B) and Il-8 (44D8000C) Quantikine ELISA kits were purchased from R and D Systems. TNF alpha Ready-Set-Go ELISA (Cat. No. 88-7346-22) was purchased from eBiosciences. The manufacturer’s protocol was followed. After a 5 hour infection, cell supernatants were collected, subjected to centrifugation at 6000 x g rpm for 10 minutes, and stored at -80°C for analysis. The data presented represent the mean and the standard deviation of 5 experiments.

**Table S1. RT-PCR Primer Table**

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<td>β-Actin Reverse</td>
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accumulation of ROS that mediate prolonged MAPK activation and necrotic cell death.

The EMBO journal 22:3898-3909.


Chapter V

Conclusion and Future Directions
CONCLUSIONS

Pathogens maintain tools to either subvert the host or establish a commensal-like relationship. In the case of *S. pyogenes*, the secretion of several virulence factors throughout the infection allows the bacteria to be versatile. The ability to infect or colonize several sites within the host as well as cause both inflammatory and non-inflammatory infections make this bacteria especially unique. One process *S. pyogenes* utilizes against the host is cytolysin-mediated translocation (CMT). Studies within the Caparon lab continue to unravel how the process occurs and reveal more functions for the proteins involved. We know the process involves an intimate interaction between SLO and SPN at the host membrane allowing for the translocation of SPN into the host cell cytosol. Previously, we found the process of CMT is not pore dependent indicating that SLO has two distinct roles: to form pores and to translocate SPN (4). This finding also revealed that pore formation in conjunction with SPN is necessary for cell death since SPN in the cell alone did not kill cells.

In addition to SLO’s multiple functions, SPN also appears to have dual roles. Analysis of over a hundred strains indicated a NADase negative version of SPN that was equally prevalent associated with tissue tropism (7). In these strains, IFS was truncated while SPN was preserved despite the loss of this enzymatic activity suggesting that SPN was under positive selection. Prior to these studies, the NADase inactive SPN was thought to be inert and unable to contribute to pathogenesis. Analysis of the polymorphic residues between these two alleles of SPN showed that only 9 amino acids differ between these subtypes and was under positive selection. This work determined the three polymorphic residues at the C-terminus contributed to the NADase activity. Each change in the amino acid residue contributed to enzymatic activity to varying degree. One polymorphic residue that drastically reduces enzymatic activity, G330D, was once
used as a marker for NADase inactive SPN. Interestingly, two alleles of the NADase inactive SPN was found one of which only had two polymorphic residues from the active SPN-J4 including G330D residue. Closer examination of the NADase activity of this SPN showed that it actually had minimal activity, over 60,000 fold slower than the active SPN. This allele was the only intermediate version of SPN found suggesting that near complete loss of NADase activity has selective advantage that eventually leads to the loss of IFS. The loss of IFS is likely to be secondary to the loss of NADase activity. IFS was found to be essential to maintain only the fully active SPN-J4 and SPN-R289K, the next most active version of SPN. This indicates that less activity than SPN-R289K is tolerable. However, these combinations of SPN and loss of IFS are not seen in clinical isolates suggesting there is no advantage to maintaining these intermediate types of SPN for \textit{S. pyogenes}.

Most remarkable was that both the NADase active and inactive SPN expressed from the same strain were capable of killing epithelial cells to the same degree. This gave us a definitive clue that there is another function to SPN aside from the NADase activity. This led to more in depth studies regarding cellular responses and cell death. A closer look at how SPN’s activity affects the cell showed the NADase activity modulates several processes creating divergent cell signaling pathways ultimately leading to necrosis. Even more interesting is the method by which SPN causes necrosis, as there seems to be two distinct mechanisms depending on the NADase activity of SPN. While the NADase activity of SPN can cleave NAD$^+$ eventually causing the depletion of ATP killing the cell metabolically, the NADase inactive SPN undergoes a JNK mediated cell death. Even aside from cell death, these two versions of SPN create different stress responses including the modulation PARP activation product PAR that may eventually contribute to cell death. The active SPN depletes NAD$^+$ but also seems to cleave PAR polymers
a known signaling molecule (3) while these two molecules are abundant in the inactive SPN infection. PARylation seems to have some part in decreasing mitochondrial function since the silencing of PAR increases mitochondrial potential in the NADase inactive infection.

The results of these differences led to distinct inflammatory responses with these two SPN variants. While all infections with wild-type SLO led to the activation of JNK1 and PARP-1, SPN was capable of modulating these events causing the release of different pro-inflammatory molecules. The SPN deletion infection and the NADase inactive SPN had many of the same characteristics during an infection: PARylation, JNK activation, lack of HMGB-1 release, increase in NAD$^+$ levels and cytokine release. However, the SPN deletion infection does not kill cells within the same time frame. The exact mechanism of how NADase inactive SPN is capable of causing JNK mediated cell death is unknown. Determining this may give us insight into how cells infected with the SPN deletion strain survive for a longer period of time.
FUTURE DIRECTIONS

Several questions remain concerning how SPN causes toxicity on both the host side as well as the non-NADase function of SPN. While we know of some key components to cell death many experiments remain to determine what other factors play a role in this outcome. As mentioned before RIP1 is a key mediator of several pathways that involve MAPKs, NF-κB activation, apoptosis, and necrosis. These signaling cascades involve many different aspects of cellular biology including the production of acid sphingomyelinase, phospholipases, ROS generation, proteases, and mitochondrial dysfunction that can all contribute to programmed necrosis. There is not just one described pathway and it is likely more than one of these mechanisms is occurring during necroptosis. Data thus far points to necroptosis as the mechanism of cell death for the NADase inactive SPN but more experiments are needed to confirm this hypothesis.

Figure 1. A diagram depicting RIP1 as the central player of several pathways that lead to necrotic cell death. The stimulation of multiple receptors converges on RIP1, which is central
to numerous aspects of cellular function that contribute to death. Dying cells can release cytokines due to the activation of NF-κB and MAPKS that can occur downstream of RIP1. The mitochondria, ROS generation and Ca^{2+} influx can also take part in multiple pathways. Figure is adapted from (1).

Finding another component of the cell death signaling pathway would validate our current results. Preliminary validation of JNK silencing yielded varying results with Chapter 4. A pool of siRNAs targeted to JNK1 from another company (Dharmacon) silenced JNK1 efficiently however it did not result in a survival phenotype. To try and determine if the single siRNAs were as efficient as the pool, we also tested each one individually and pooled. Using the single siRNAs from this pool alone at the same concentration (100 nM) did not give an efficient knockdown of JNK1 and gave variable results with cytotoxicity. It is important to note this pool is based on an algorithm and not validated by Dharmacon. The siRNA used in Chapter 4 is a single sequence from a different company and was efficient at 100 nM. One follow up method would be to utilize another siRNA to validate the findings in Chapter 4. The most direct method would involve infection of JNK1^-^- cells and verify the death phenotype through the reintroduction of JNK expression on a plasmid. In any case, determining what other factors aside from JNK1 contribute to death would give us more insight into the role NADase inactive SPN has during an infection and help determine if programmed necrosis is occurring.

Experiments to determine if ROS generation contributes to cell death is the next logical step since this process involves both JNK activation and the mitochondria (6, 11). Determining the sequence of events between which occurs first would also be helpful to understand cellular signaling. ROS can activate JNK but the activation of JNK can stimulate the mitochondria to
generate ROS. Both events can be inhibited by NF-κB activation (5, 9). Oxidative stress could be fueling the sustained JNK activation in these infections that ultimately contributes to programmed necrosis. Along the same lines, understanding the role of calcium would also be informative since it is likely SLO pore formation allows for an influx of this ion into the host cell and likely contributes to cell signaling. In addition to SLO pore formation, the cleavage of PAR and NAD$^+$ leads to increase in ADP ribose, which can also cause an influx of calcium the TRPM channel (2). Prior attempts to study calcium have proven difficult as removing calcium from the media during an infection causes S. pyogenes to halt growth. Perhaps understanding the role of Ca$^{2+}$ by studying the influx through the SLO pores and the TRPM channel would give us more insight.

The most central factor in programmed necrosis is the kinase RIP1. The silencing of RIP1 could provide some clues however there is a risk that NF-κB activation would also be affected, removing the cells chance at survival. One method to try and determine what genes are important in necrosis mediated by either types of SPN would be to perform a siRNA screen using a cytotoxicity assay as a read out. This would be comprehensive and yield many more targets than simply silencing one predicted partner at a time. Another benefit of the screen will be the potential identification of targets that are important for cytolysin-mediated translocation of SPN.

Another remaining question from these studies involves identifying the factor that activates PARP-1. We originally pursued MAPKs and focused on JNK since both the activation of PARP and JNK were SLO dependent. However, we saw that the silencing of JNK1 did not prevent PARylation from occurring. ERK1/2 can also activate PARP and though it is not activated in an SLO dependent manner it cannot be ruled out since we do see activation with an
infection with *S. pyogenes*. Another possibility for the activation of PARP is the entry of bacterial products through the SLO pore. This pore is large and can easily allow for the entry of proteins and ions (10). As mentioned in Chapter 2, *S. pyogenes* makes large amounts of hydrogen peroxide, a known stimulator of PARP and cell death. Though the cause of PARP-1 activation remains elusive, much remains to be understood with signaling pathways in the cell.

Yet another comprehensive approach would be to analyze the phospho-proteome of the cell during infections with SPN and SLO variants. This would contribute to understanding the role of these toxins in cell signaling during an infection. This technique would be more informative than a microarray since many proteins are post-translationally modified and their involvement would not be identified in a microarray. For example, PARP-1 can be modified in several different ways and its activity is not transcriptionally regulated. This is also true of the MAPKs. Using the stable-isotope labeling by amino acids in cell culture or SILAC method would give an overview of how an infection with different SPN modulates the cell. This method was employed in the analysis with *Salmonella* and the SopB effector deletion (8). This method allowed the authors to measure 9500 phosphorylation events and they discovered that over half of the Salmonella induced events were due to the T3SS effector SopB. In our system, SILAC could be employed with various conditions including infections with SPN deletion (which still forms SLO pores), locked SLO and the two NADase variants. This would give us an overview of many processes that occur in the cell in response to *S. pyogenes* and specifically to CMT.

One of the most obvious unanswered questions from this thesis is in regard to the second function of SPN. It is surprising that both SPN are equally cytotoxic when it would seem like the NADase active SPN would have two functions. Between these alleles, there are only typically 9-10 residues that are polymorphic. While we know that 3 of these at the C-terminal are
responsible for the NADase activity and are generally conserved to either the NADase active or inactive, the other six residues are found in both subtypes making these residues unlikely contributors to the second function. However, this same study found a total of 23 residues under positive selection (7) and perhaps these 13 polymorphic residues that do not segregate to either NADase clusters are involved in the second function. Unfortunately for the current system we use, a high throughput screen for SPN mutagenesis is not feasible. SPN and SLO must be expressed from the chromosome of *S. pyogenes* to get secreted into the host cytosol. After creating each mutant plasmid, the native allele would have to be replaced to create mutant strains, then screened, and used to infect cells for a cytotoxicity screen. One method could involve targeting portions of SPN for mutagenesis aside from the terminus involved in NADase activity and verifying these mutants in a cytotoxicity screen. Perhaps one starting point would be to mutate these 13 residues. Though this is not a quick method it would be a start to determine what portion of SPN is responsible for cytotoxicity. The other side of this question is understanding what SPN targeting in the host cell. This also remains an unanswered question since thus far pull-down assays with eukaryotic lysates have given negative results. Perhaps this interaction is transient and cross-linking would be necessary. The previous assays were performed with HeLa lysates and purified protein and perhaps this should be done after an infection where all the necessary components for the interaction would be present.

Finally, determining a role for the NADase inactive SPN *in vivo* would complement these studies. The strain utilized, JRS4, is not virulent in mice however studies involving asymptomatic carriage does not require virulence. SPN and SLO are produced during the exponential phase of bacterial growth and shuts during the stationary phase. During stationary phase, another virulence factor SpeB is made abundantly. This factor can cleave both bacterial
factors like SLO as well as host proteins. Perhaps SPN and SLO contribute to the initial colonization of *S. pyogenes* that is later regulated by SpeB production. Not all strains make SpeB however and perhaps these strains utilize different sets of tools to subvert the host. In either case, it would be interesting to determine exactly how SPN and SLO contribute to an infection in a host. These studies revealed that the inflammatory profiles of the NADase variants differ and this must play a more significant role in the context of other cells especially immune cells.
REFERENCES


