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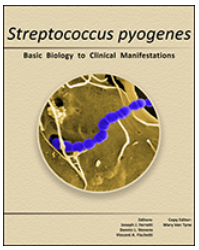

Animal Models of *Streptococcus pyogenes* Infection

Michael E. Watson, Jr., MD, PhD,[1] Melody N. Neely, PhD,[2] and Michael G. Caparon, PhD[3]

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Introduction

An established principle of modern research into bacterial pathogenesis is that no *in vitro*, *in situ*, or *in silico* model can accurately account for the myriad host defense mechanisms and host tissue complexity that a pathogen will encounter in a live animal. This fact was appreciated over 100 years ago by Robert Koch and the early pioneers of pathogenesis research, who recognized that an animal model of infection that mimicked a human disease was a crucial component in establishing a microbial etiology for any given disease (Kaufmann & Schaible, 2005). The postulates described by Koch continue to the present day to be the "gold standard" by which etiology is established (Kaufmann & Schaible, 2005). In the spirit of Koch, the early pioneers of the molecular era of pathogenesis research (most notably Stanley Falkow) have expanded on the principles elaborated by Koch to use animal models to develop our modern understanding of the molecular basis of microbial pathogenesis, in order to establish a functional link between any given gene product of a pathogen and its capacity to cause disease (Falkow, 1988; Falkow, 2004) (Table 1). Thus, the availability of animal models that can faithfully reproduce a human disease continues to be a foundational component of modern microbial pathogenesis research.

For research into the pathogenesis of diseases caused by *Streptococcus pyogenes*, the groundbreaking work of the labs of Joseph Ferretti, Patrick Cleary and June Scott (Ji, McLandsborough, Kondagunta, & Cleary, 1996; Perez-Casal, Price, Maguin, & Scott, 1993; Simon & Ferretti, 1991) established a methodology for the manipulation of the *S. pyogenes* genome, and opened the door for the analysis of pathogenesis that followed the principles elaborated by Falkow. This work spurred the development of new *in vivo* models that could be used to investigate the role of specific virulence factors in *S. pyogenes* pathogenesis. However, for *S. pyogenes*, the development of *in vivo* models has proven to be challenging for a number of reasons: First, *S. pyogenes* is a strictly human pathogen and is exquisitely adapted to its human host to the extent that many of its important virulence factors (for example, its several secreted superantigens and its plasminogen activator streptokinase (Kasper, et al., 2014; Sun, et al., 2004; Reglinski & Sriskandan, 2014)) only have activity against humans cells and

Author Affiliations: 1 Division of Pediatric Infectious Diseases, Department of Pediatrics and Communicable Diseases, University of Michigan Medical School, Ann Arbor, MI 48109-5684. USA.; Email: mewats@med.umich.edu. 2 Department of Immunology and Microbiology, Wayne State University School of Medicine, Detroit, MI 48201. USA.; Email: mneely@med.wayne.edu. 3 Department of Molecular Microbiology, Washington University School of Medicine, St. Louis, MO. 63110-1093. USA.; Email: caparon@wusm.wustl.edu.

✉ Corresponding author.

proteins. The second issue reflects *S. pyogenes*' remarkable versatility as a pathogen, as it is capable of causing diseases that result from very different pathogenic mechanisms. Most of these fall into one of three broad classes (Reglinski & Sriskandan, 2014; Cunningham, 2000; Ralph & Carapetis, 2013; Wong & Stevens, 2013; Cunningham, 2012): first, local, lesional diseases in soft tissue characterized by inflammation, which can result in considerable damage to tissue in more severe manifestations; second, both local and systemic diseases that arise from damage caused by secreted streptococcal toxins; and third, immune dysfunction that results from an inappropriate immune response to streptococcal antigens. The third challenge to model development arises from the range of different tissue compartments that *S. pyogenes* can damage, which ranges from skin and soft tissue to internal organs like the heart and kidneys and to any number of different sites in the skin and other soft tissues. A final major challenge to model development is the population of *S. pyogenes* itself, which has proven to have extensive strain diversity despite its restriction to a human habitat (Bessen, 2009). This means that there is no single strain of *S. pyogenes* that can be considered representative of the population as a whole and also, that relatively few strains have been shown to be virulent in any given animal model.

Despite these challenges, the prior 15 years has seen the development of an impressive number of *in vivo* models in a diversity of animal species, ranging from invertebrates to primates, that have proven useful in the dissection of *S. pyogenes* gene/pathogenesis relationships (Figure 1). In considering these models, it is important to note that there is no single comprehensive model of *S. pyogenes* infection. In fact, there is no single model that can accurately reproduce the authentic pathogenesis of any specific *S. pyogenes* disease. Instead, various models have been developed to model different aspects of various pathogenic mechanisms, and as a result, a thorough understanding of any particular model's strengths and weaknesses is an important consideration for experimental design, for interpretation of results as they apply to understanding pathogenesis in that model system, and for extrapolation to the mechanism by which any *S. pyogenes* gene may contribute to human disease. In the following sections, we will review the salient features of the animal models that have proven particularly useful in modern analyses of *S. pyogenes* pathogenesis, including their utility, strengths, and limitations, as well as some examples of the types of strains and mutants whose pathogenic mechanism a given model has been shown to resolve. For purposes of organization, the various models will be grouped together with the host animal upon which they are based.

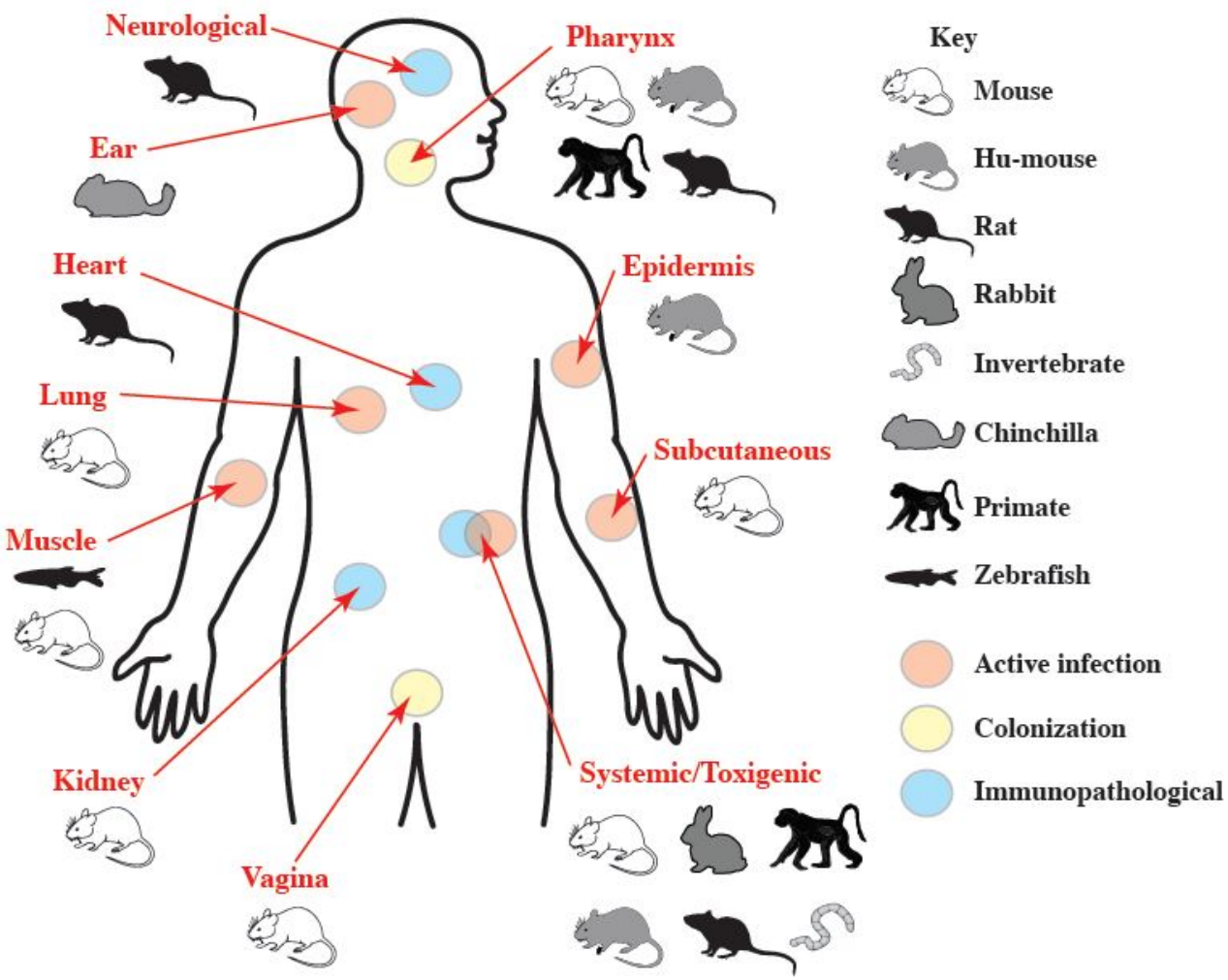

Figure 1. Animal models for analysis of *S. pyogenes* pathogenesis. This figure shows the animal models that have been developed for various human *S. pyogenes* diseases. The models are presented based on the animal species used and the human tissue that is infected, damaged, or colonized in the corresponding human disease. The icons that represent each animal species are defined in the key and the colors show the type of infection that is modeled. The strengths and limitations of each model for analysis of their corresponding human disease are discussed in the text.

Table 1. Molecular Koch's postulates

The phenotype or property under investigation should be associated with pathogenic members of a genus or pathogenic strains of a species.
The gene(s) associated with the supposed virulence trait should be isolated by molecular methods. Specific inactivation or deletion of the gene(s) should lead to a measurable loss in pathogenicity or virulence.
Restoration of pathogenicity should accompany the reintroduction of the wild-type gene(s).

As proposed by Stanley Falkow (Falkow, 1988). Adapted from (Falkow, 2004).

Murine Models

Subcutaneous ulcer/air sac model

One of the most widely used animal models for the analysis of *S. pyogenes* virulence factors is the murine subcutaneous ulcer model and its air sac derivative. This model is particularly attractive because it reproduces

many of the core features that are held in common by a broad range of *S. pyogenes* diseases. These include that the infection results in a highly inflammatory localized lesion in a soft tissue, that there is a robust level of bacterial proliferation, and that the lesion elicits the extensive recruitment of inflammatory cells. Introduced in its modern form by the group of Neil Barg (Bunce, Wheeler, Reed, Musser, & Barg, 1992), it originally involved injecting a mixture of bacteria and cytodex beads into the subcutaneous tissue of a mouse flank. However it was subsequently found that the beads were not necessary to produce an infection (Schrager, Rheinwald, & Wessels, 1996). Typically, 10^6–10^8 CFU of the *S. pyogenes* strain of interest is injected into the tissue, which results in a well-defined area of induration that is apparent by 8–12 hours post-infection. The lesion that forms is characterized by the recruitment of a large number of inflammatory cells, of which the majority are neutrophils that form a loose abscess-like structure that surrounds the area of bacterial growth (Figure 2). Macrophages and the cytokines IL-12, Interferon-γ and TNF-α appear to be important for the formation of this structure, which limits dissemination of bacteria from the site of the lesion (Mishalian, et al., 2011; Raeder, Barker-Merrill, Lester, Boyle, & Metzger, 2000). By 18–24, hours the region of induration ulcerates and develops an eschar, followed by the gradual expansion of the margins of the ulcer to reach a maximum at about Day 3 (Figure 2). At approximately Day 8, the lesion begins to resolve, and by Day 14, it is typically healed. Many different *S. pyogenes* strains have been shown to be virulent in this model, and these fall into two general classes: those that remain localized to the lesion for the duration of the infection (such as those found in (Brenot, King, Janowiak, Griffith, & Caparon, 2004)), and those that have a propensity to disseminate into the vasculature to cause systemic disease after a period of several days (such as those found in (Limbago, Penumali, Weinrick, & Scott, 2000)). Interestingly, mutations have been described, most notably in the CsrR/S (CovR/S) two-component transcription regulator, which can convert a primarily resident strain into a highly invasive strain (Engleberg, Heath, Miller, Rivera, & DiRita, 2001). Many strains of mice are sensitive to this infection, including strains like the BALB/c and C57BL6 strains, as well as their various transgenic and knock-out variants that are commonly used in immunological analyses. However, a commonly used mouse strain is the outbred SKH1 mouse that has a defect in the *Hr* gene located on chromosome 14 that renders it hairless (Benavides, Oberyszyn, VanBuskirk, Reeve, & Kusewitt, 2009), which eliminates the requirement for depilation for injection and observation of the lesions. This model also offers a large number of different infection parameters to assess virulence. A commonly used approach is to quantitate the surface area of the resulting ulceration and how it changes over time. Typically, a conclusion regarding virulence between the wild-type *S. pyogenes* strain and its mutant derivatives is made by a comparison of the lesion area at the time point when the wild-type strain's lesion reaches a maximum area (usually by Day 3). Since the resulting ulcer will have an irregular border, its area is most accurately measured by the use of digital photography and image processing software (Brenot, King, Janowiak, Griffith, & Caparon, 2004). Other parameters that can be assessed include change in weight over time, numbers of colony forming units (CFU) that are recoverable from the lesion, the number of mice that do or do not develop a ulcer following infection, the time to maximal lesion area, and the time to healing of the ulcer. For *S. pyogenes* strains that disseminate from the lesion, additional parameters can be measured, which typically include CFU recovered from the spleen and inguinal lymph nodes and lethality. This model has also proven its utility for the monitoring of the behavior of the *S. pyogenes* transcriptome during growth in a soft-tissue environment. The fact that the bacteria remain confined to a necrotic lesion that is not conducive for maintaining the integrity of host RNAs has allowed the efficient recovery of *S. pyogenes* RNA directly from the infected tissue for analyses of gene expression (Brenot, Weston, & Caparon, 2007; Loughman & Caparon, 2006; Kietzman & Caparon, 2011).

The subcutaneous air sac model is a derivative of the subcutaneous ulcer model. The same subcutaneous tissue compartment is infected in both models. A distinction for the air sac model is that prior to infection, an air pouch is created under the skin by the injection of air into which the bacteria are subsequently injected (Raeder & Boyle, 1993). This method's principal advantage is that it facilitates the recovery of host inflammatory cells, which migrate into the sac lumen and can be recovered by lavage for analyses *in vitro* by FACS and other methods that require a suspension of single cells.

Like any of the animal models for analysis of *S. pyogenes* pathogenesis, the subcutaneous ulcer model has a number of important strengths and limitations that must be taken into account when designing experiments to provide insight into the role of a specific *S. pyogenes* gene product in virulence. As discussed, the model reproduces features of a highly inflammatory infection caused by *S. pyogenes* growing extracellularly in the types of cutaneous tissue that *S. pyogenes* infects in humans. On the other hand, the histopathology of the infection does not closely reproduce that observed for human cutaneous diseases, including impetigo, pyoderma, erysipelas, cellulitis, or necrotizing fasciitis. These observations can be associated with differences in the anatomy of the murine cutaneous tissues as compared to humans, as the mouse has a much higher density of flank skin hair follicles and a much thinner epidermal layer. Also, there is a considerable variation among the virulence of *S. pyogenes* strains in this model, and the virulence of any particular strains in humans is not predictive of its virulence in this model. Certain virulence factors may play a more important role in the mouse rather than the human, since mice lack the human-specific targets of factors, like streptokinase and the superantigens. In association, mutations of some *S. pyogenes* virulence factors do not always result in the same degree of attenuation in different strains. A classic example is the case of the SpeB cysteine protease, whose loss of function in different strains has been associated with significant attenuation or no attenuation at all (Ashbaugh, Warren, Carey, & Wessels, 1998; Lukomski, et al., 1999). This has suggested that different *S. pyogenes* strains may have unique virulence profiles that are comprised of overlapping, redundant and/or combinatorial sets of virulence genes (Saouda, Wu, Conran, & Boyle, 2001; Sierig, Cywes, Wessels, & Ashbaugh, 2003). In examining the immune system, the subcutaneous model has proven valuable for analysis of the role of innate immunity in the host's response to *S. pyogenes* and how specific virulence factors modulate innate immunity. It is less clear that this model will be useful for analyses of the adaptive arm of the immune response, since a protective immune response apparently does not develop after the resolution phase of the infection (Bisno & Gaviria, 1997). However, the limitations of this model are counter-balanced by a number of important strengths. These include its simplicity, its relatively high-throughput and low cost, and the ability to apply the power of mouse genetics to test the role of various host factors-virulence factor relationships (for one example, see (Cusumano, Watson, & Caparon, 2014)). In addition, because the bacteria grow in a defined lesion, it reproduces the dynamic host environment that is remodeled both by host immunity and by microbial metabolism, which consumes host-derived substrates and accumulates metabolic end-products, like organic acids (Loughman & Caparon, 2006). Alterations to the transcriptome that accompanies adaptation to this dynamic environment are closely linked to changes in virulence factor expression (Kreikemeyer, McIver, & Podbielski, 2003). These many advantages have made the subcutaneous ulcer model one of the most versatile and useful models for the analysis of *S. pyogenes* pathogenesis.

Impetigo model

The histological differences between the human and murine epidermal tissues noted above have made the mouse unsuitable for modeling impetigo and other *S. pyogenes* infections of superficial cutaneous tissues. Bessen and colleagues have developed an innovative solution to this problem (Scaramuzzino, McNiff, & Bessen, 2000). Their approach was to create a humanized mouse (hu-mouse) by engrafting human epidermal tissue from neonatal foreskin onto the flanks of the SCID mouse. The absence of an adaptive immune response in this mouse line prevents the rejection of the tissue grafts. Once the grafts are established, they are superficially damaged by cross-wise cuts with a scalpel blade, bacteria are topically applied, and are then occluded with a bandage. Both damage and occlusion were required for establishing the infection, similar to what has been observed in experimental infections conducted in humans (Leyden, Stewart, & Kligman, 1980). This results in an impetigo-like lesion that is characterized by erosion of the stratum corneum, with infiltration of murine polymorphonuclear leukocytes (PMNs) that eventually contribute to the formation of pus, which is surrounded by clumps of proliferating streptococci. Virulence is measured by a semi-quantitative visual assessment of histopathology or by the determination of colony forming units to monitor bacterial growth. The degree of damage generally correlates with the magnitude of streptococcal growth (Scaramuzzino, McNiff, & Bessen,

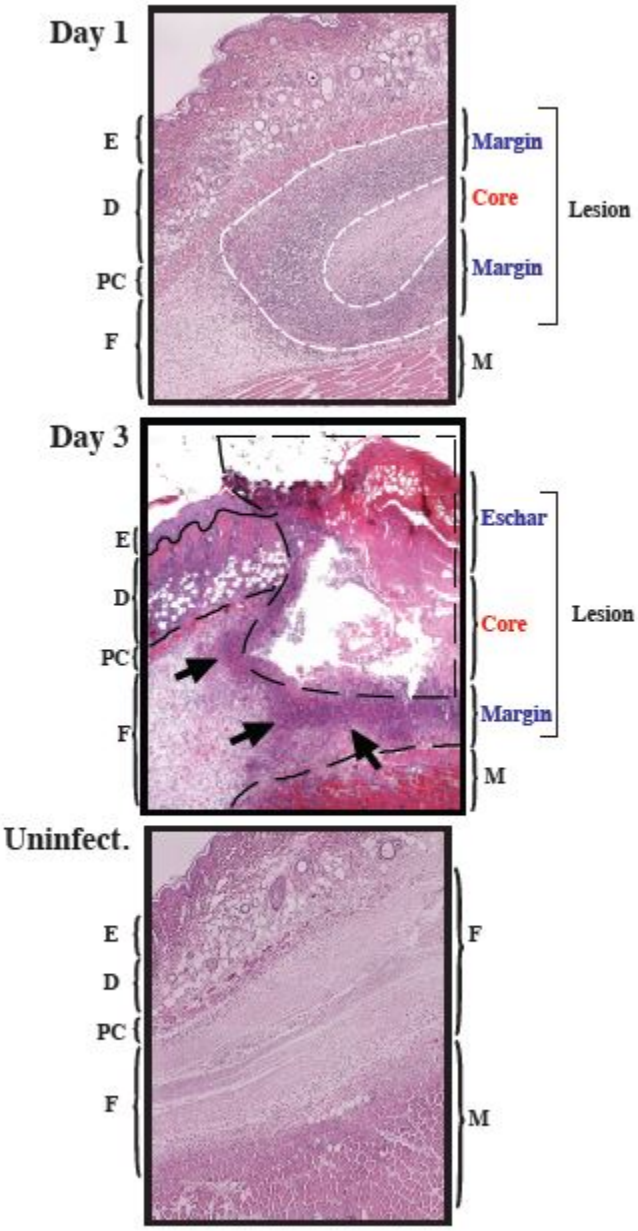

Figure 2. Murine subcutaneous ulcer model. This figure shows the histopathology of infection by *S. pyogenes* HSC5 following the subcutaneous inoculation of 1 x 10^6 CFUs into the flank skin of SKH1 mice. Shown are hematoxylin & eosin (H&E)-stained sections of infected tissues in comparison to uninfected (uninfect.) tissue. By Day 1 post inoculation, a well-defined subcutaneous lesion forms that consists of a necrotic Core that contains replicating streptococci, along with debris from destroyed host cells, surrounded by a Margin that consists of a dense concentration of inflammatory cells, predominantly PMNs, recruited from the vasculature. By Day 3, the lesion has reached its maximal size and the upper margin has eroded through the superficial tissue layers to form an open ulcer with formation of an eschar. Replicating streptococci continue to be confined to the lesion Core by the inflammatory cells in the Margin (shown by arrows). For many strains, including HSC5, the lesion will heal over the course of the next 10 days and the streptococci will be cleared from the tissue (Brenot, King, Janowiak, Griffith, & Caparon, 2004). For other strains and certain mutants (most notably in the CsrR/S [CovR/S] two-component transcription regulator) the infection may progress to systemic disease. Abbreviations: E, epidermis; D, dermis; PC, panniculus carnosus; F, fascia; M, muscle. Magnification, 40X.

2000). Other characteristics that reproduce elements of the human disease include the finding that *S. pyogenes* strains of lineages that are most likely to be associated with impetigo tended to cause more severe tissue damage than those lineages that are more likely to cause pharyngitis (Scaramuzzino, McNiff, & Bessen, 2000), and that

virulence requires known *S. pyogenes* virulence factors, which include the virulence gene transcription regulator Mga (Luo, Lizano, Banik, Zhang, & Bessen, 2008).

Despite these strengths, the hu-mouse model does have some important limitations. It is technically complex and requires both a source of human tissue and surgical skills for reproducible production of the hu-mice themselves. The effort required to generate the hu-mice limits the throughput of the assay, as there is a limited quantity of the animals themselves. Also, although human tissue is infected, the model still lacks the human-specific targets of several important virulence factors, including streptokinase and superantigens, which are the serum protein plasmin and HLA on bone marrow-derived T cells, respectively. The use of the immunocompromised SCID mouse means that the infection proceeds in the absence of adaptive immunity. However, because the model can faithfully reproduce many features of human impetigo, the hu-mouse model is one of the most useful and accurate models available for analysis of *S. pyogenes* infection of the superficial skin.

Models of systemic disease

The common element of the various models of systemic disease is that the major mechanism of damage to the host arises from the presence of streptococci actively multiplying in the bloodstream. With the exception of puerperal sepsis (childbed fever), which is now a rare disease, sepsis is a relatively rare manifestation of infection by *S. pyogenes*, and often occurs after a localized infection in soft tissue (Reglinski & Sriskandan, 2014; Wong & Stevens, 2013; Rantala, Vuopio-Varkila, Vuento, Huhtala, & Syrjänen, 2009). The murine models of systemic infection do have utility for modeling human disease, since growth in human blood *in vitro* is the classic assay for assessment of *S. pyogenes* virulence, for testing its ability to evade phagocytosis, and for testing the protective efficacy of antibodies (Lancefield, 1962). In the mouse, the different types of systemic disease models are distinguished from each other by their route of inoculation, which may occur intravenously (IV), intraperitoneally (IP), intranasally (IN), or intratracheally (IT). The IN route has also been adapted for analysis of oropharyngeal colonization (see below). The IV route introduces the bacteria directly into the bloodstream, while the IN and IT routes require that the streptococci invade across a tissue barrier to reach the vasculature. Each of these routes involves a direct injection or instillation of the bacterial inoculum and is technically simple to perform. It has been proposed that the IN and IT routes may yield more useful information, as they bypass the least number of the steps of infection since they require the bacteria to invade across the lung epithelium in order to reach the bloodstream (Roberts, Scott, Husmann, & Zurawski, 2006). However, to improve upon the upper airway model, Husmann et al. infected mice via IT inoculation to bypass the initial site of upper airway colonization and found that infected mice more quickly developed signs of illness within a few days following infection, as compared to IN infection (Husmann, Dillehay, Jennings, & Scott, 1996). Furthermore, examination of these IT-infected mice found a higher frequency of acute, suppurative bronchopneumonia with two-thirds of these mice developing bacteremia (Husmann, Dillehay, Jennings, & Scott, 1996). Therefore, IT inoculation may be a more efficient mechanism to investigate the role of streptococcal virulence factors in inducing significant airway and systemic disease (Roberts, Scott, Husmann, & Zurawski, 2006; Husmann, Dillehay, Jennings, & Scott, 1996; Husmann, Yung, Hollingshead, & Scott, 1997). Virulence of strains is typically determined by monitoring lethality, by using a single concentration of bacteria to infect a group of mice. Differences in virulence can be tested for significance by the Kaplan-Meier survival probability estimate model (Brenot, King, Janowiak, Griffith, & Caparon, 2004). A typical dose for a strain that is virulent in these assays is approx. 10^5-10^6 CFU and numerous strains have been found to be virulent, although it has long been recognized that there is no correlation between the virulence of strains in humans and in systemic models (Lancefield, 1962). This likely indicates that the mouse and human have a differential sensitivity to certain *S. pyogenes* virulence factors. For those strains that are less virulent, useful information can often be acquired by examination of the kinetics of clearance from the vasculature by enumeration of CFUs recovered from highly perfused organs like the spleen, liver, and lungs. The choice of mouse strain and gender are important considerations, as mice with different genetic backgrounds can have significant differences in sensitivity to systemic infection, with male mice being generally more susceptible (Medina, Goldmann, Rohde, Lengeling, & Chhatwal, 2001). The genes responsible for

these differences are apparently located outside of the MHC locus (Goldmann, et al., 2005), and an absence of adaptive immunity does not alter the course of infection (Medina, Goldmann, Rohde, Lengeling, & Chhatwal, 2001). In addition to existing strains, transgenic technologies have been employed to expand the types of strains that can be useful for the analysis of pathogenesis in systemic models. The most interesting application of this approach has been to overcome the limitation noted above that results from certain virulence factors of *S. pyogenes* having evolved to recognize exclusively human targets. In the case of streptokinase, a transgenic hu-mouse has been developed that expresses human plasminogen (Sun, et al., 2004). Analysis of these mice conclusively establishes streptokinase as virulence factor for systemic infection, as these mice have a markedly increased susceptibility to lethal infection, which is dependent on the expression of human streptokinase (Sun, et al., 2004).

Implanted chamber models

In the models discussed so far, pathology results from streptococci damaging the tissue that are directly associated with or by dissemination through the vasculature. However, many *S. pyogenes* diseases are caused by toxins or other bacterial products that are released by streptococci growing in a localized lesion that then traffic to tissues and organs at a distant site to cause damage (Reglinski & Sriskandan, 2014; Cunningham, 2000; Ralph & Carapetis, 2013; Wong & Stevens, 2013). In some cases, these diseases can be modeled in mice by using an implanted chamber model. The approach is to surgically-implant a "cage" made of steel or Teflon into the subcutaneous tissues on the mouse flank. The incision is allowed to heal over the course of several weeks to allow a fibrous capsule to form around the implant. The infection is initiated by injection of the bacteria through the skin and into the inside of the cage, which allows the influx of nutrients and the release of streptococcal products, but the retention of streptococcal cells. Implanted chambers have been used to produce a glomerulonephritis-like disease that is characterized by deposition of streptokinase and host complement components in the glomerular basement membrane (Nordstrand, Norgren, Ferretti, & Holm, 1998) and to monitor toxin production in an *in vivo* environment (Kazmi, et al., 2001). A similar approach using rabbits has been employed for modeling streptococcal toxic shock syndrome (Schlievert, 2007).

Footpad model

Most animal models of infection quantitatively assess events that are associated with the very late or terminal stages of disease, including bacterial proliferation, damage to tissue and lethality. However, many virulence factors act during the very early stages of infection to help establish a foothold for the bacteria in the infected tissue. Since there are relatively few bacteria present at this stage, and often no gross alterations to tissue, it can be a challenge to observe the streptococcal cells or to measure any parameter of early infection to assess the contribution of specific virulence factors. Recent advances in imaging have begun to open a window into this critically important aspect of *S. pyogenes* pathogenesis. One approach is to use the power of two-photon microscopy, a technology with the resolution to image streptococcal bacteria in live un-fixed tissue at a depth of several millimeters, to examine bacteria over the first hour following their introduction into tissue. In one application, fluorescent dyes were used to label bacterial cells and blood vessels in combination with LysM-eGFP mice, whose macrophages and neutrophils express eGFP. Two-photon microscopy was used to examine the behavior of neutrophils immediately following the injection of wild-type and mutant bacteria into the mouse footpad (Lin, Loughman, Zinselmeyer, Miller, & Caparon, 2009). In addition to positional information both inside and outside of vessels, imaging in real-time allows for the quantitation of neutrophil velocity and meandering for assessment of chemotactic behavior. The application of this method revealed a previously unrecognized role for streptolysin S in altering neutrophil extravasation (Lin, Loughman, Zinselmeyer, Miller, & Caparon, 2009). While this is a promising technique, its general adoption has been slowed by its requirement for expensive and highly specialized equipment.

Oropharyngeal colonization models

A major reservoir for *S. pyogenes* in humans is the posterior oropharynx, especially the mucosal-associated lymphoid tissue known as Waldeyer's ring, which includes the tonsils. This tissue is the site of one of the most common manifestations of *S. pyogenes* infection (pharyngitis), and as a result, there has been considerable interest in developing animal models to attempt to replicate and study this disease process (Cunningham, 2000). However, rodents lack a Waldeyer's ring homologue in the pharynx, and experimental colonization of the rodent oropharynx has been both challenging and limited primarily to only a few select *S. pyogenes* strains. Early experiments by Rebecca Lancefield's group (Hook, Wagner, & Lancefield, 1960) took advantage of a mouse-pathogenic strain of *S. pyogenes*, the M type 50 strain B514/33 that was originally isolated from an epizootic infection of a mouse colony. More recent experiments using this lineage have used strain B514-Sm, a spontaneous streptomycin-resistant derivative of the original strain, whose high level of antibiotic resistance allows direct selection for the determination of recovered CFUs. Strain B514-Sm seems exceptionally capable of colonizing the murine oropharynx, and approximately 60% of inoculated mice remain colonized for over a month. Mice are infected by IN inoculation with 10^7 CFU in a 10 µl normal saline drop that is placed into one nostril and inhaled, which distributes the inoculum throughout the upper airway. Colonization is monitored by throat swabs that are resuspended and diluted in saline and plated onto selective media (THY agar with 1000 µg/ml streptomycin) (Roberts, Scott, Husmann, & Zurawski, 2006). As discussed above, with IN inoculation some mice will develop bronchopneumonia and die; however, the dose-response ratio is non-linear, given the random distribution of the inoculum in the airway. More recently, the group of Alam et al. refined delivery by the IN route to expand the number of strains that can be analyzed in the model, and found that the volume of the inoculum is a critical variable. They found that IN inoculation of a serotype M18 strain in a volume of 2.5 µl to each nare of a mouse resulted in consistent nasopharyngeal deposition without lung involvement or significant nasal clearance (Alam, Turner, Smith, Wiles, & Sriskandan, 2013). In addition, they used bioluminescence imaging of a strain that was engineered to express luciferase as a non-invasive method for monitoring the progress of infection (Alam, Turner, Smith, Wiles, & Sriskandan, 2013). The strengths of these models include the ability to colonize the murine upper airway and oropharynx, which is a site that is highly relevant to human streptococcal disease. This advantage has been used to show that mutation of the CovR/S virulence regulator in a serotype M18 strain attenuates infection of the oropharynx, in contrast to the hypervirulence of mutants in subcutaneous models (Alam, Turner, Smith, Wiles, & Sriskandan, 2013). Some disadvantages of these models include a limited number of *S. pyogenes* strains capable of colonizing the mouse nasopharynx, that strain B514 has an atypical pattern of virulence gene expression (Yung & Hollingshead, 1996), and the significant differences between human and murine nasopharyngeal lymphoid tissue architecture. However, despite these differences, the murine oropharyngeal model has proven to be useful for analysis of both passive and active mucosal immunization using various streptococcal antigens. For example, passive administration of lipoteichoic acid (LTA) or anti-M protein secretory IgA can significantly reduce colonization in the model (Bessen & Fischetti, 1988b; Dale, Baird, Courtney, Hasty, & Bronze, 1994). Active immunization of the nasal mucosa with several surface proteins, including the hemoprotein receptor Shr (Huang, Fisher, Nasrawi, & Eichenbaum, 2011), the fibronectin-binding protein SfbI (Guzmán, Talay, Molinari, Medina, & Chhatwal, 1999), and various M protein-based vaccines, including peptides conjugated to cholera toxin b subunit or diphtheria toxoid (Bessen & Fischetti, 1988a; Olive, et al., 2006; Olive, Clair, Yarwood, & Good, 2002), have proven to be efficacious in reducing mucosal colonization after subsequent IN challenges of vaccinated mice.

Nasopharyngeal-associated lymphatic tissue colonization model

Despite a lack of Waldeyer's ring tissue in the pharynx, mice and other rodents have a pair of lymphoid lobes located along the lateral nasopharyngeal wall, termed nasal-associated lymphoid tissue (NALT), which function analogously to human tonsils (Asanuma, et al., 1997; Koornstra, de Jong, Vlek, Marres, & van Breda Vriesman, 1991). To investigate whether NALT may be a more appropriate model of *S. pyogenes* airway colonization, Pat

Cleary's group infected 8- to 11-week-old BALB/c mice IN with 5 x 10^8 CFU of *S. pyogenes* clinical isolate strain 591 (serotype M49), strain 90-226 (serotype M1), or derivatives (Park, Francis, Yu, & Cleary, 2003; Park, et al., 2004). Their group determined NALT to be a significant site of *S. pyogenes* colonization following intranasal inoculation, and by using strain 591, was able to recover viable CFUs, both intra- and extra-cellularly, for about 8 days post-inoculation (Park, Francis, Yu, & Cleary, 2003). They also introduced bioluminescent imaging as a non-invasive method for the assessment of colonization, using a *S. pyogenes* strain that was modified to express luciferase (Park, Francis, Yu, & Cleary, 2003). An obvious advantage of this model is the ability to directly examine adaptive immune responses following *S. pyogenes* colonization of the NALT tissue, which has proven useful for studies that have investigated potential vaccine candidates. The Cleary group has shown that following intranasal inoculation with *S. pyogenes*, NALT develops a rapid and prolonged activation of $CD4^+$ T cells of the IL-17-producing Th17 subclass, and that antigens of *S. pyogenes*, including sortase A, are capable of inducing an antibody-independent, neutrophil-based immune response that promotes the clearance of NALT colonization (Park, et al., 2004; Wang, et al., 2010; Dileepan, et al., 2011; Fan, et al., 2014). Some disadvantages of this model include a short duration of colonization, a limited number of *S. pyogenes* strains with demonstrated activity in the model, a higher degree of technical difficulty in isolating NALT tissue for experimental analysis, and a requirement for specialized equipment when using bioluminescent imaging.

Vaginal colonization model

Given the limitations of the oropharyngeal and NALT models for carriage, with their limited number of *S. pyogenes* strains capable of causing infection and relatively short duration of infection, a model capable of sustaining a longer period of colonization is required. As a result, the estradiol-primed mouse vaginal colonization model was developed in C57BL/6 and BALB/c mice and was based on previously developed vaginal colonization models for group B streptococcus, *Neisseria gonorrhoeae*, and *Candida albicans* (Watson, Nielsen, Hultgren, & Caparon, 2013; Sheen, et al., 2011; Jerse, 1999; Fidel, Lynch, & Sobel, 1993). While not as prevalent in human female genitourinary disease as some of the other previously mentioned pathogens, *S. pyogenes* is nonetheless a significant cause of bacterial vulvovaginitis and puerperal sepsis (or "childbed fever"), and asymptomatic vaginal carriage has been linked to nosocomial transmission from healthcare workers to patients, causing wound infections (Stamm, Feeley, & Facklam, 1978; Berkelman, et al., 1982; Stricker, Navratil, & Sennhauser, 2003; Anteby, Yagel, Hanoch, Shapiro, & Moses, 1999). In each of the murine vaginal models, pathogen colonization is enhanced and carriage is extended by one or more administrations of up to 0.5 mg ethinyl estradiol dissolved in sterile sesame oil given 24 to 48 hours prior to vaginal inoculation of ~1 x 10^6 CFU *S. pyogenes* in 20 µl of PBS. Estradiol supplementation syncs all of the mice into the estrous phase of the estrous cycle, a period associated with epithelial cell proliferation and minimal inflammatory cell infiltrate; this phase seems to be when mice are the most receptive to colonization with bacterial or fungal pathogens in the vaginal tract, which is likely due to a combination of a hospitable epithelial cell bed and estradiol-induced anti-inflammatory properties. The estrous phase can be monitored by microscopic examination of vaginal lavages with sterile PBS stained with a modified Wright-Giemsa stain and differential counting of epithelial cells and leukocytes (Watson, Nielsen, Hultgren, & Caparon, 2013; Marcondes, Bianchi, & Tanno, 2002). Vaginal lavages can be serially diluted and plated onto selective media to which the *S. pyogenes* strain is resistant (such as Todd Hewitt yeast extract (THY) agar, supplemented with 1000 µg/ml streptomycin); this allows for the determination of streptococcal colony counts over time (Figure 3). With *S. pyogenes*, many strains from a diversity of backgrounds will continue to colonize the murine vagina over a period of several weeks to months, with strains exhibiting variability in the overall rate of clearance from the vaginal mucosa (Watson, Nielsen, Hultgren, & Caparon, 2013) (Figure 3). The significant duration of mucosal carriage this model permits allows for the investigation of the role of streptococcal virulence factors and regulatory mechanisms important for chronic mucosal persistence. The model previously demonstrated attenuated vaginal mucosal carriage of strains that lacked the carbon catabolite repressor, CcpA, and the arginine deiminase (Arc) operon, which suggests these two important streptococcal factors are involved in promoting mucosal survival (Cusumano, Watson, &

Caparon, 2014; Watson, Nielsen, Hultgren, & Caparon, 2013). A major strength of the vaginal model is that carriage is asymptomatic; mice do not exhibit signs of distress or pain and do not lose weight or fur ruffles, among others (Watson, Nielsen, Hultgren, & Caparon, 2013). Furthermore, the ability to sample vaginal lavages for CFU counts, cytokine levels by ELISA, and other inflammatory cell markers implies that fewer mice are required to complete the experiment, given that mice can be sampled serially over time without requiring sacrifice. Disadvantages of this model include the need for a *S. pyogenes* strain carrying a selectable marker (namely, a spontaneous mutation in *rpsL* that produces streptomycin resistance) to eliminate normal vaginal flora, and the requirement for mice to have received prior estrogenization, which may interfere with certain immune responses. Overall, the murine vaginal carriage model should permit investigation of factors that are critical to influencing host-pathogen interactions and persistence at the mucosal surface, a key immunologic barrier to disease.

Rat Models

Oropharyngeal colonization

A variant of the murine models described above has been developed using rats to expand the number of streptococcal strains accessible to investigation. In this model, 8- to 12-week-old Fischer CDF344 rats were treated with streptomycin (1000 µg/mL) in their drinking water to disrupt their normal flora and then inoculated either IN or orally for pharyngeal colonization (Hollingshead, Simecka, & Michalek, 1993). IN infection utilizes 20 µl inocula, while oral inoculations are 50 µl, with both containing ~10^8 CFU. The Fischer rat was found to support colonization with additional strains of *S. pyogenes*, as compared to the mouse oropharynx model. For example, Hollingshead et al. colonized Fischer rats with the M6 serotype strain JRS4, isogenic M-negative mutant derivatives of JRS4, and an additional unrelated M6 serotype strain S43-29R; in these experiments, strains that expressed M protein exhibited significantly longer oropharyngeal carriage than isogenic M-negative strains (Hollingshead, Simecka, & Michalek, 1993). The duration of colonization in the rat model was several weeks for some individual rats, although the numbers of CFUs recovered from throat swabs (which ranged from a few to ~200 CFUs per rat) remained relatively small over the course of the experiment. Mice colonized with the M6 serotype strain developed serum and salivary antibody responses directed against the M protein, which indicates that the model may have utility for the analysis of the development of serotype-specific immunity. A limitation to the model is that due to streptomycin treatment of the rats, it requires that the infecting *S. pyogenes* strain be resistant to streptomycin, although spontaneous streptomycin-resistant derivatives of many *S. pyogenes* strains can readily be obtained with a single-hit frequency in *rpsL*, the gene that encodes the 30S ribosomal protein S12 (Watson, Nielsen, Hultgren, & Caparon, 2013).

Autoimmune carditis

In addition to the many diseases it causes by active multiplication and direct damage to tissues or toxigenic diseases, *S. pyogenes* can also cause a plethora of diseases with an autoimmune character (Cunningham, 2000; Ralph & Carapetis, 2013). One of the most prominent of these is acute rheumatic fever (Cunningham, 2012). The heart is particularly sensitive and damage to it is manifested as carditis and valvulitis. The latter is the cause of most morbidity and mortality that results from mitral valve stenosis and aortic regurgitation. Most evidence indicates that this pathology results from autoimmunity that is triggered by a response against *S. pyogenes* antigens that cross-react with heart tissue. For modeling these diseases in the laboratory, the Lewis rat has been the species of choice. Since the disease is a sequela to an active infection and is driven by adaptive immunity, rats are not infected, but are immunized with *S. pyogenes* antigens that are implicated as cross-reactive with human tissues. Immunization with streptococcal M protein, a surface protein that is the serotype-specific antigen of *S. pyogenes*, results in the development of a myocarditis and a valvulitis. The pathology that develops resembles human rheumatic disease, including the formation of Anitschkow cell-containing granulomas (called Aschoff bodies) and infiltration of CD4+ and CD8+ T cells into the valvular lesions (Gorton, Govan, Olive, & Ketheesan,

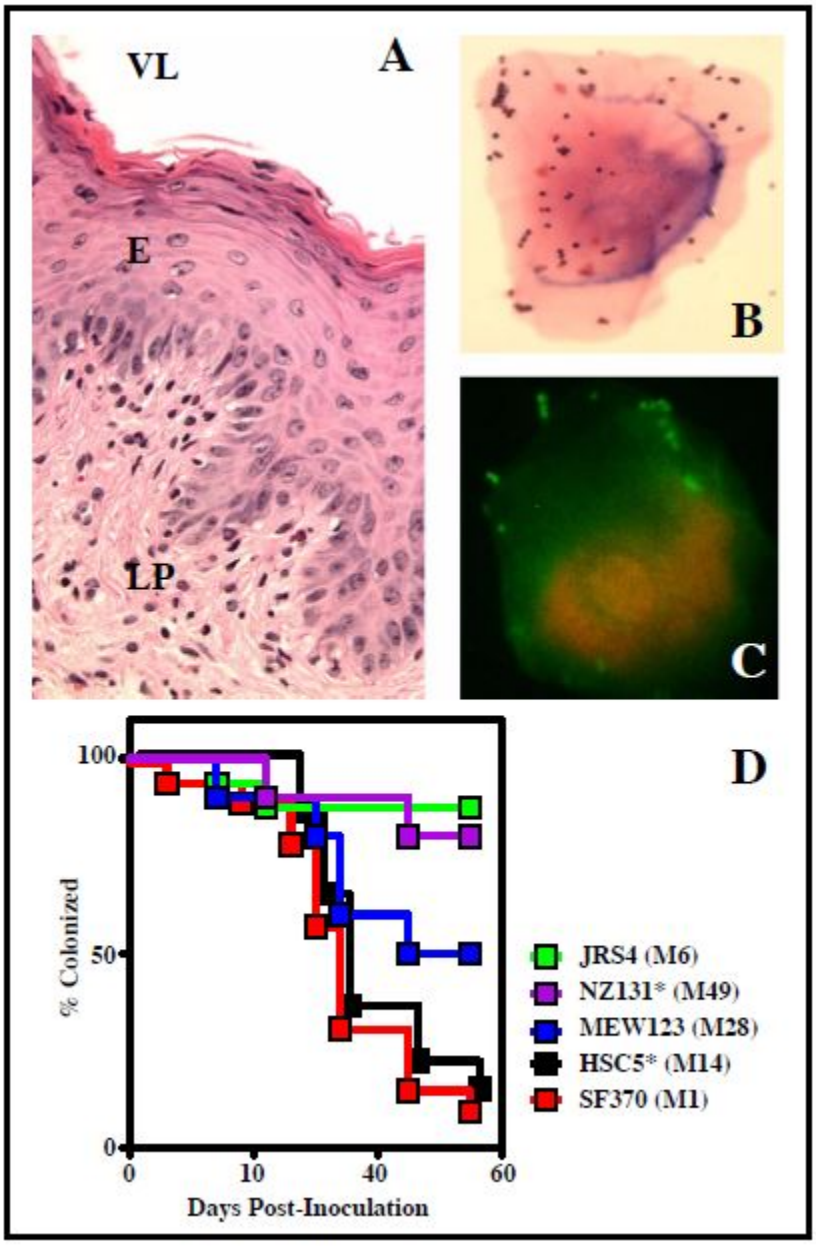

Figure 3. Vaginal model of asymptomatic mucosal carriage. A. Presented is an H&E-stained cross-section of vaginal wall from an estradiol-treated mouse colonized with *S. pyogenes* HSC12 for 8 days (magnification, 40X). Anatomy landmarks include vaginal lumen (VL), epithelium (E), and lamina propria (LP). Note the absence of significant inflammation, which is consistent with the asymptomatic nature of the carriage. **B**. Gram-stained murine vaginal epithelial cell from a vaginal wash. Numerous *S. pyogenes* bacteria are apparent as Gram-positive cocci in pairs and chains attached to the cell surface (magnification, 100X). **C.** Immunofluorescent micrograph of a murine vaginal epithelial cell stained with a FITC-conjugated anti-*S. pyogenes* antibody (ViroStat, Inc., Portland, ME) and counterstained with Evan's Blue (ViroStat) (magnification, 100X). **D.** Multiple *S. pyogenes* strains from diverse M protein serotypes and clinical origin are compatible with the murine vaginal colonization model. Shown are the percentage of mice that are colonized with each strain at the indicated time points, following inoculation of 1 x 10^6 CFU on day 0, as recovered by vaginal washes. The *S. pyogenes* strains are streptomycin-resistant or are a streptomycin-resistant derivative of the listed strain (indicated by "*") to enable direct selection on Todd Hewitt yeast (THY) extract agar supplemented with streptomycin (1000 μg/ml) (Asanuma, et al., 1997).

2006; Kirvan, Galvin, Hilt, Kosanke, & Cunningham, 2014; Quinn, Kosanke, Fischetti, Factor, & Cunningham, 2001). Cross-reactive epitopes have been mapped by using peptides that correspond to defined regions of the M

protein, both by direct immunization and by the adoptive transfer of peptide-reactive T cells to naïve rats to induce valvulitis (Kirvan, Galvin, Hilt, Kosanke, & Cunningham, 2014).

PANDAS

Acute rheumatic fever is only one of several autoimmune-type diseases that are caused by *S. pyogenes* (Cunningham, 2000). Of these, Sydenham's chorea, obsessive-compulsive disorder, Tourette's syndrome, and pediatric autoimmune neuropsychiatric disorders associated with streptococcus (known as PANDAS) all have a prominent neurological or neuropsychiatric component (Esposito, Bianchini, Baggi, Fattizzo, & Rigante, 2014; Macrì, Onori, Roessner, & Laviola, 2013). As with rheumatic fever, it is hypothesized that autoantibodies that react with neuronal tissue in the brain are produced against cross-reactive *S. pyogenes* antigens during an active infection. These antibodies persist, long after the initial streptococcal infection is cleared, to produce chronic symptomatology. The Lewis rat has also been used to model the pathological consequences of streptococcal-induced neuronal autoantibodies (Lotan, et al., 2014). In this model, rats that are immunized with a whole cell lysate of *S. pyogenes* proceed to develop quantifiable alterations to several behavioral and motor functions, as compared to sham-immunized animals. Serum from these animals reacts with brain tissue *in vivo* and to dopamine and serotonin receptors *in vitro*. Purified IgG from these cross-reacting sera could at least partially reproduce symptomatology, following its direct perfusion into the striatum region of the brain (Lotan, et al., 2014). Further analysis using this model may help to identify those neuronal structures that are susceptible to streptococcal-induced autoimmunity.

Chinchilla Models

Model of Otitis Media

Interestingly, while *S. pyogenes* causes less than 10% of the cases of culture-positive acute otitis media (ear infection), it was the most common agent of this disease prior to the 1950s and remains the fourth most common cause of bacterial culture-positive otitis media today (Segal, et al., 2005). Modern analyses have strongly implicated biofilm formation in the pathogenesis of ear infections caused by numerous bacterial species (Post, 2001), and the most powerful analysis of biofilm formation in otitis media has come from the use of the chinchilla ear infection model (Post, 2001; Hong, Pang, West-Barnette, & Swords, 2007). This model has been adapted for analysis of the contribution of biofilm to the pathogenesis of *S. pyogenes* otitis media (Roberts, Connolly, Doern, Holder, & Reid, 2010). In this model, approximately 10^5 CFU of the strain of interest are inoculated via transbullar injection into the ear of a chinchilla. Infection is monitored by otoscopy over the course of 7 days. By 2 days, the majority of ears show evidence of tympanic membrane and inner ear inflammation with an associated serous middle ear effusion. Biofilm formation is apparent as macroscopic structures that contain bacterial microcolonies in localized three-dimensional communities (Roberts, Connolly, Doern, Holder, & Reid, 2010). Numerous parameters can be quantitated, including histopathology, the enumeration of CFUs recovered from the macroscopic structures and from the serous effusion, and by monitoring survival of the animals over the 7-day course of infection. Biofilm ultrastructure can be further assessed by electron and fluorescent microscopic analyses of the recovered macroscopic material (Roberts, Connolly, Doern, Holder, & Reid, 2010). This model has been used to show that the transcription regulator Svr enhances biofilm formation *in vivo* (Roberts, Connolly, Doern, Holder, & Reid, 2010).

Primate Models

Non-human primate model of pharyngitis

Despite the relatively low cost and accessibility of rodent models of *S. pyogenes* pathogenesis, there has long been a desire to develop non-human primate models of disease to more closely mimic the human condition of

pharyngitis. Rodent models have failed to establish significant oropharyngeal colonization with *S. pyogenes* of any sufficient duration and do not develop evidence of symptomatic infection. Non-human primates offer advantages of a more similar development and components of immune responses to humans, as compared to rodents, as well as easier access to the site of infection, larger blood volumes to conduct biochemical analyses, and the ability to use multiple different strains of *S. pyogenes.* Initial reports of primate models for group A streptococci began in the early twentieth century and have evolved over the past 100 years to several different non-human primate species, including the baboon, rhesus monkey, chimpanzee, and the cynomolgus macaque (Taranta, Spagnuolo, Davidson, Goldstein, & Uhr, 1969; Vanace, 1960; Watson, Rothbard, Swift, & de Mello, 1946; Krushak, Zimmerman, & Murphy, 1970; Sumby, Tart, & Musser, 2008). *S. pyogenes* effectively colonizes the oropharynx of these primates, and there is an associated characteristic humoral immune response with type-specific M-protein antibodies detectable in serum (Taranta, Spagnuolo, Davidson, Goldstein, & Uhr, 1969; Vanace, 1960; Watson, Rothbard, Swift, & de Mello, 1946; Krushak, Zimmerman, & Murphy, 1970; Ashbaugh, et al., 2000). For example, in the baboon model, inoculation with ~3 x 10^5 CFU of a serotype M3 *S. pyogenes* strain establishes pharyngeal colonization for at least 6 weeks; in contrast, acapsular derivatives or M-protein–deficient mutants of the parent strain were significantly attenuated for carriage (Ashbaugh, et al., 2000). A hyaluronic acid capsule was further shown to be important for oropharyngeal colonization, as expression of the hyaluronic acid synthase (*has*) operon was significantly upregulated following inoculation into the baboon pharynx (Gryllos, et al., 2001), which likely served to inhibit phagocytosis by approaching leukocytes at sites of inflammation. Transcriptome analysis indicates similar expression profiles of a subset of *S. pyogenes* genes in the macaque pharynx, as compared with the human pharynx (Virtaneva, et al., 2003). Advantages of the primate model include the extended persistence of *S. pyogenes* in the primate pharynx, as compared to rodents, and the subsequent development of type-specific humoral immune responses—both features that are believed to be necessary for the development of rheumatic fever. Limitations of this model include the prohibitive expense and limited availability of primate colonies and the fact that baboon and rhesus monkeys do not develop symptomatic pharyngitis following inoculation. On the other hand, chimpanzees and macaques do develop significant pharyngeal erythema, fever, tonsillar enlargement, and palatal petechiae following infection, which is reminiscent of human *S. pyogenes* pharyngitis (Virtaneva, et al., 2003; Friou, 1950). Given the limited ability of *S. pyogenes* to colonize the rodent oropharynx, the non-human primate has become, and currently remains, the gold-standard model for upper airway and oropharyngeal *S. pyogenes* colonization.

Non-human primate model of sepsis

Hypotension and multi-organ failure are the signature features of streptococcal toxic-shock syndrome. This is a devastating disease with mortality rates that can be as high as 80%, despite the availability of aggressive antibiotic and supportive therapies (Stevens, et al., 1996). The disease has a very rapid time-course and patients often succumb even before an *S. pyogenes* etiology can be ascertained. Thus, a priority for understanding the pathogenesis of this disease is to elucidate the critical early checkpoints that can be exploited to develop therapeutic strategies for blocking the subsequent onset of multi-organ failure. As discussed above, many of the toxins that are critical for the pathogenesis of this disease, including the superantigen toxins, only recognize species-specific targets. Thus, animal models sensitive to these toxins are required for a critical analysis of the pathogenesis of this disease. This requirement has made the non-human primate model of sepsis (Creasey, et al., 1993) particularly attractive for this analysis (Stevens, et al., 1996). In this model, baboons (*Papio cynocephalus cynocephalus*) under light anesthesia are intravenously infused with a high dose of *S. pyogenes* (approx. 10^{10} CFU of log-phase, washed *S. pyogenes* cells) over a 2 hour period (Stevens, et al., 1996). Analyses of physiology and blood chemistry are then conducted over a 10-hour period, which results in a mortality rate that approaches 100% (Stevens, et al., 1996). Parameters of infection assessed include body temperature, heart rate, mean systemic arterial blood pressure and serum chemistry, and cytokine profiles (Stevens, et al., 1996). In this model, blocking the cytokine TNF-α using a therapeutic monoclonal antibody significantly improved both mean arterial blood pressure and survival (Stevens, et al., 1996).

Invertebrate Models

Silkworm and wax worm models

The principal advantages of infection models using easily bred invertebrates include that they are simple to perform, are inexpensive, do not require extensive animal husbandry, and offer a relatively high-throughput. One invertebrate model that has been used to analyze the virulence of human pathogens is the silkworm (*Bombyx mori*). Fatal infections occurred with inoculation of pathogenic pathogens (*S. aureus, P. aeruginosa, V. cholerae*), but not by non-pathogenic strains (Hamamoto, et al., 2004; Kaito, Akimitsu, Watanabe, & Sekimizu, 2002). Inoculation into the silkworm is through injection into the haemolymph (comparable to the bloodstream) of approximately 9 x 10^8 CFU of *S. pyogenes*, followed by monitoring survival for 5 days at 27°C (Kaito, et al., 2005). A similar model uses the wax worm (*Galleria mellonela*), whose principal advantage is that unlike for other invertebrates, infections can be conducted at 37°C (Loh, Adenwalla, Wiles, & Proft, 2013; Olsen, et al., 2010). Both of these models have proven sensitive to known *S. pyogenes* virulence factors and have been used to identify novel factors (Kaito, et al., 2005; Loh, Adenwalla, Wiles, & Proft, 2013; Olsen, et al., 2010).

C. elegans model

The well-known *Caenorhabditis elegans* invertebrate animal model has also been used to analyze *S. pyogenes* virulence (Bolm, Jansen, Schnabel, & Chhatwal, 2004; Jansen, Bolm, Balling, Chhatwal, & Schnabel, 2002). While most *C. elegans* virulence assays involve growing the worms on a plate inoculated with the pathogen, a liquid assay was developed to increase reproducibility. Two hundred sterile worms were added to ~2 x 10^7 CFU of *S. pyogenes* in a 24-well plate and monitored every 24 hours. More than 80% of the *C. elegans* organisms were killed by *S. pyogenes* in under 24 hours. However, colonization of the intestine does not occur with *S. pyogenes*, as when the worms are infected with *Enterococcus faecalis* strains. Interestingly, killing is not toxin-mediated, as strains with mutations in the genes encoding SLO and SpeB did not inhibit killing. Further analysis determined that killing was mediated by the production of hydrogen peroxide by *S. pyogenes*, which could be abrogated by the addition of catalase to the cultures (Jansen, Bolm, Balling, Chhatwal, & Schnabel, 2002).

Zebrafish Model

Myonecrosis model

The zebrafish (*Danio rerio*) infectious disease model has been particularly successful for analysis of *S. pyogenes* acute, deep tissue, myonecrotic infections. The symptoms observed mimic those reported for *S. pyogenes* deep tissue infections in humans, including lack of inflammatory cell infiltrates, large aggregates of bacteria dissecting along tissue planes, and extensive areas of necrotic tissue damage. While *S. pyogenes* does disseminate to the spleen, its systemic spread to other organs is not observed (Neely, Pfeifer, & Caparon, 2002). Experimental evidence suggests that fatality results from toxic shock, and most likely occurs through the systemic dissemination of streptococcal toxins (Benavides, Oberyszyn, VanBuskirk, Reeve, & Kusewitt, 2009; Rosch, Vega, Beyer, Lin, & Caparon, 2008). Inoculation is usually by IM injection into the dorsal muscle, although IP injections have also been analyzed (Phelps, Runft, & Neely, 2009). The IP LD_{50} for a serotype M14 strain of *S. pyogenes* in adult zebrafish was 2.5 x 10^2 CFU, while the IM route resulted in an LD_{50} of 3 x 10^4 CFU (Neely, Pfeifer, & Caparon, 2002). Over the course of 24 hours, an IM injection results in the formation of a hypo-pigmented lesion at the site of injection; this is indicative of extensive muscle necrosis that is apparent when the tissues are examined by histology (Figure 4). The lesions continue to enlarge until the death of the animal at 36 to 96 hours post injection. When analyzing pathogenesis with strains that carry mutations in known or putative virulence genes, a dose of 10 to 100 times the LD_{50} is used to determine survival, in comparison to fish injected with the wild-type strain using the Kaplan-Meier survival probability estimate model to test for significant differences (Neely, Pfeifer, & Caparon, 2002). Colonization can also be quantified over time by euthanization and

dissection of the muscle tissue to determine replication and/or clearance of the pathogen at the site of injection. Dissemination to the spleen can be determined by aseptic dissection of the organ, followed by serial dilution plating (Phelps, Runft, & Neely, 2009).

Multiple strains of *S. pyogenes* have been successfully used in the zebrafish model, including M59 (Bates, Toukoki, Neely, & Eichenbaum, 2005; Fisher, et al., 2008; Montañez, Neely, & Eichenbaum, 2005), M14 (Brenot, King, Janowiak, Griffith, & Caparon, 2004; Benavides, Oberyszyn, VanBuskirk, Reeve, & Kusewitt, 2009; Rosch, Vega, Beyer, Lin, & Caparon, 2008; Neely, Lyon, Runft, & Caparon, 2003), and M1 strains (Neely M. , 2015). Analyses of these strains have been used to examine multiple characteristics of *S. pyogenes* pathogenesis, including nutrient acquisition (Bates, Toukoki, Neely, & Eichenbaum, 2005; Fisher, et al., 2008; Montañez, Neely, & Eichenbaum, 2005), virulence gene regulation (Neely, Lyon, Runft, & Caparon, 2003), protein secretion (Rosch, Vega, Beyer, Lin, & Caparon, 2008), tissue-specific gene expression (Cho & Caparon, 2005) and toxin-mediated inhibition of host responses (Benavides, Oberyszyn, VanBuskirk, Reeve, & Kusewitt, 2009; Lin, Loughman, Zinselmeyer, Miller, & Caparon, 2009; Rosch, Vega, Beyer, Lin, & Caparon, 2008; Phelps & Neely, 2007) (Figure 4). However, like any animal model, inoculation with some strains does not cause disease and a notable example is the commonly used M6 strain JRS4 (Neely, unpublished research).

One of the major advantages of this model is that the zebrafish is one of the smallest animals that has both an adaptive and an innate immune system. In fact, analysis of its completely sequenced genome (Genome Research Limited, 2015) reveals a high degree of synteny with the human genome (Postlethwait, Amores, Force, & Yan, 1999) and similarity to the mammalian immune system (for further review, see (Hsu, et al., 2004)). This includes homologs to all of the mammalian Toll-like receptors (Jault, Pichon, & Chluba, 2004; Meijer, et al., 2004) and the complement system (Hsu, et al., 2004). The small size and fecundity of the zebrafish and the relatively low cost and simplicity of natural breeding allows for the production and housing of thousands of animals in a small space. This facilitates large-scale screens, including pathogen-centric signature tagged transposon mutagenesis screens (Kizy & Neely, 2009), and host-centric genetic or small molecule-based screens using embryos or larvae arrayed in 96-well plates or via automated microinjection (Wang, Liu, Gelinas, Ciruna, & Sun, 2007). There is an extensive methodology available for analysis of different life stages of zebrafish, and several of these have been successfully employed for analysis of the effect of *S. pyogenes* toxins on immune cell infiltration (Lin, Loughman, Zinselmeyer, Miller, & Caparon, 2009). Zebrafish genes can be selectively and transiently knocked-down using morpholino technology (Nasevicius & Ekker, 2000) and extensive collections of modified and mutant zebrafish lines are available. These include lines that are transparent (Ren, McCarthy, Zhang, Adolph, & Li, 2002; White, et al., 2008) and lines where cells of the myeloid lineage express GFP (for one example, see (Gray, et al., 2011)). The Zebrafish International Resource Center (ZIRC) (The Zebrafish International Resource Center, 2006) maintains a searchable database for finding zebrafish strains with specific mutations. Recent developments in genetic technology has made creating transgenic zebrafish with specific genetic knock-outs highly successful, including the use of Zinc-finger nucleases, TALENs, and the CRISPR/Cas9 systems (for further review, see (Schulte-Merker & Stainier, 2014)).

As with other models, the zebrafish model is not without its weaknesses. These include that the development of reagents, like monoclonal antibodies for cells and markers of interest, lags behind those available for murine models, and it has been difficult to establish zebrafish-specific cell cultures *in vitro*. While zebrafish do have an immune system, some important differences remain. For example, one interesting difference is a lack of discernable lymph nodes, although evidence of a zebrafish lymphatic system was recently reported (Küchler, et al., 2006; Yaniv, et al., 2006). Lastly, the optimal temperature for the incubation of zebrafish is 27–29^{o}C, which is problematic for those *S. pyogenes* virulence genes that may be temperature-regulated.

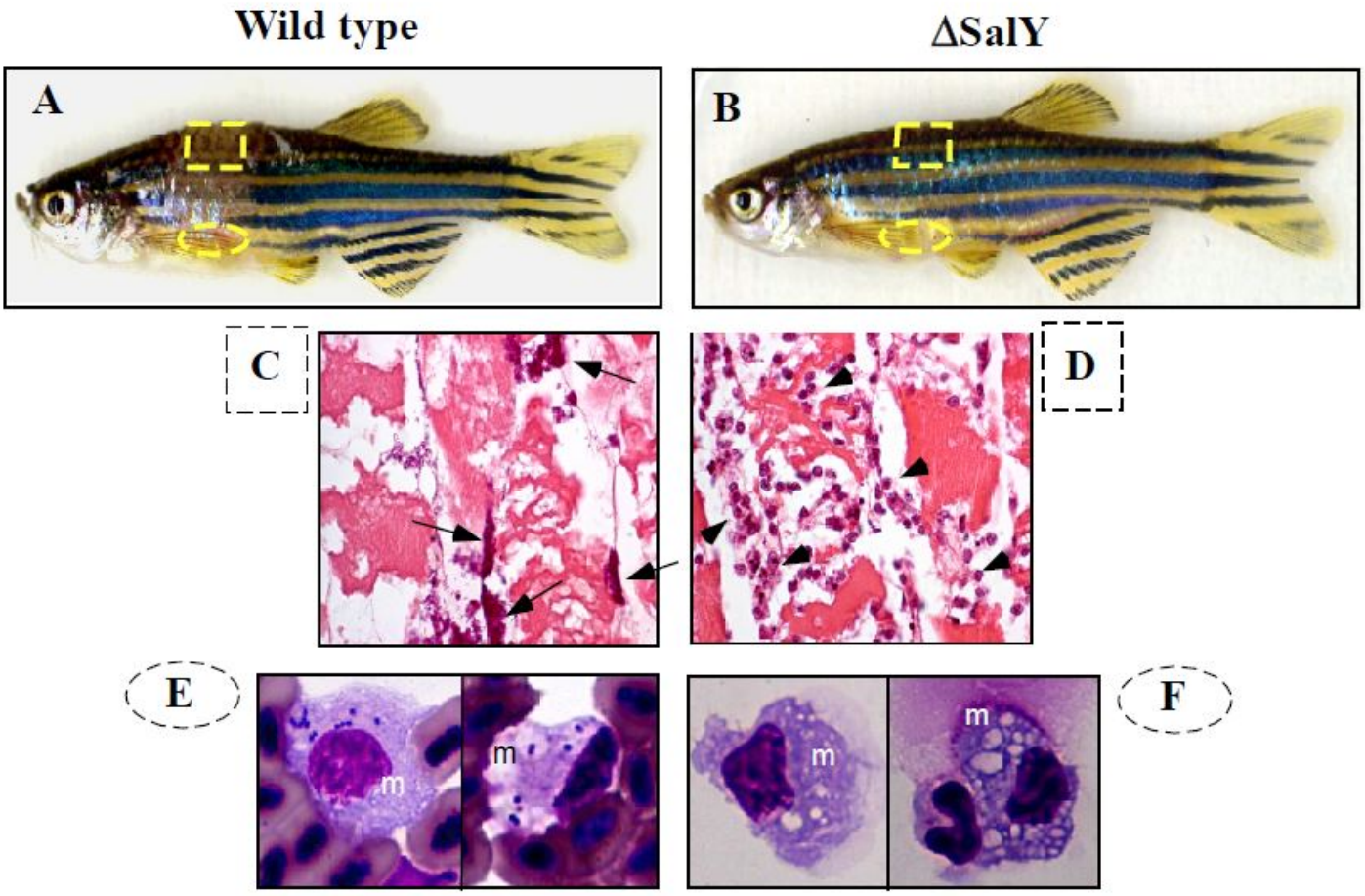

Figure 4. Zebrafish myonecrosis model. Zebrafish-infected IM with 10^5 CFU of wild type *S. pyogenes* strain HSC5 and a HSC5 *salY* mutant (ΔSalY), as indicated. At 24 hours post-infection, fish are euthanized and further processed as described (98). SalY is a component of a lantibiotic ABC transporter that is required for the survival of *S. pyogenes* in zebrafish macrophages (Rosch, Vega, Beyer, Lin, & Caparon, 2008). Comparison of intact zebrafish reveals that a large hypo-pigmented lesion in the dorsal muscle has formed around the site of injection of the wild-type strain (outlined by the dashed-box) (**A**), but that no lesion is apparent in the ΔSalY mutant (**B**). Examination of H&E-stained paraffin embedded sections of skeletal muscles reveals that the hypo-pigmented lesion caused by the wild-type strain is accompanied by large aggregates of streptococcal bacteria in the muscle (black arrows) and no inflammatory cell inflammation (**C**). In contrast, muscle infected by the ΔSalY mutant contains few cocci and a large infiltration of inflammatory cells (black arrowheads) (**D**). Macrophages (m) recovered from a spleen homogenate from wild type-infected zebrafish contain intracellular cocci (**E**) that are not observed in splenic macrophages of the ΔSalY mutant, which instead contain empty vacuoles (**F**). The location of the spleen is shown by the dashed oval.

Conclusion

In his commentary on the fifteenth anniversary of the publication of his "molecular Koch's postulates," Stanley Falkow made the observation that his experimental approach for the identification of pathogenicity genes remains relevant even in an age where the complete genomes of pathogens are readily available, noting that "Genomes do not necessarily reveal their secrets about pathogenic traits by simple visual inspection or by sophisticated bioinformatics analysis" (Falkow, 2004). Thus, the various animal models described in this chapter will continue to play an important role in the analysis of *S. pyogenes* gene/pathogenesis relationships for the foreseeable future.

When applying the Falkow strategy (Table 1), there are some important considerations to be made when reaching conclusions about the role of any *S. pyogenes* gene in an *in vivo* model. First, it is always important to

determine the growth rate of a mutant as compared to its wild type parent using an *in vitro* medium. This is not because genes that promote growth are less interesting or unimportant, but because the pathogenesis in virtually all of the models described in this chapter extends from the ability of *S. pyogenes* to rapidly proliferate in tissue. Thus, a decrease in growth rate will almost always result in attenuation. The choice of an *in vitro* medium is an important consideration, given the complexities of the tissue environment. As a result, strategies have been developed for the comparison of *in vivo* and *in vitro* growth conditions (Loughman & Caparon, 2006; Cho & Caparon, 2005). Secondly, as described by Falkow in his postulates, it is important to establish that re-introduction of the wild-type gene is accompanied by a restoration of virulence (Table 1). In cases where plasmid-based complementation is used for *S. pyogenes*, it is always important to determine that the complementing plasmid is stably maintained in an *in vivo* environment in the absence of antibiotic selection. If the plasmid is not stable, an alternative is to restore the wild-type gene at its resident locus on the chromosome (for an example, see (Watson, Nielsen, Hultgren, & Caparon, 2013)).

Finally, it is important to keep in mind that the various models described here each have important limitations, and have all been designed to model specific aspects of the various and varied *S. pyogenes* diseases. Thus, care should be taken when concluding that a particular gene may not be a virulence factor, when a more likely explanation is that the particular animal model used cannot resolve its contribution. This often occurs because the step of virulence process it is required for is bypassed in the model. Modern genomics and genetic technology, combined with the many creative *in vivo* models described here, have opened a golden era of research into the pathogenesis of *S. pyogenes*.

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