TprK Variation and Syphilis Pathogenesis

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Abstract

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Treponema pallidum subsp. *pallidum* is the etiologic agent of syphilis, a multistage disease that poses a global health problem. Despite the availability of curative therapy and ongoing public health efforts to eradicate syphilis, the disease is re-emerging in developed countries and remains prevalent in other locations. Calculating the basic reproductive number (R_o) of syphilis allows for the estimation of the number of people infected by a single infectious person. R_o is proportional to the duration of infectiousness, efficiency of transmission, and the number of new susceptible contacts. An infectious disease is expected to increase prevalence in a population when $R_o>1$, and will die out if $R_o< 1$. It is known that antibodies directed to exposed surface antigens are critical for opsonophagocytosis and clearance of this pathogen from lesions. TprK is a *T. pallidum* antigen that undergoes antigenic variation during infection. Evidence shows that antibodies are directed to the variable regions of the protein and TprK sequence changes abrogate antibody binding, thus allowing immune escape. This dissertation describes TprK's effect on two of the three parameters used to calculate R_o. The first is the role of TprK variation in duration of infectiousness, exemplified by the association found between TprK variants and secondary lesions. Length of the infectious period is increased by facilitating development of the second stage of syphilis in the face of developing opsonic antibody to TprK. The second

component of R_o impacted by TprK variation is the number of new susceptible contacts an infected individual encounters. TprK variation contributes to *T. pallidum's* ability to reinfect or superinfect individuals that have been previously exposed by altering opsonic antibody epitopes such that newly infecting treponemes escape the established immune response.

In summary, antigenic variation of TprK is associated with the ability of the spirochete to re-infect previously exposed individuals, and increase the duration of infectiousness by facilitating persistence into the second stage of syphilis. Antigenic variation of the outer membrane protein, TprK, accounts for a larger estimated R_o value for syphilis, and thus may be associated with its centuries of success as a devastating disease.

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I. Background and Introduction

Historical Perspectives

Syphilis has been known by many names over the centuries. From the French disease, the Spanish disease, and the Great Pox, this venereal disease is associated with a rich history. Additionally, there is much debate over the initial origins of the syphilis associated with the great pandemic in Europe during the late 15th century. The Columbian theory postulates that Columbus and his crew brought a treponemal disease back to Spain from the New World (Haiti). The pre Columbian theory conversely argues that syphilis was already present and circulating in Europe before Columbus, but that the disease was simply confused with other diseases (e.g. leprosy) common at the time (1). Indeed, syphilis is known as the Great Imitator, as the signs and symptoms of disease throughout its complex natural history can resemble multiple disease processes. Currently, some genetic evidence supports the Columbian hypothesis of acquisition of a yaws-like disease from the new world and introduction into Europe (2-4). A major limitation in these genetic studies is the use of very few single nucleotide polymorphisms (some located in known variable genes) to draw conclusions about the phylogenetic relatedness of treponemal subspecies (5). Recent evidence highlights the possibility of recombination amongst Treponema subspecies and the ability to horizontally transfer virulence factors (6). Some reports suggest that the syphilis that was circulating in Europe in the 15th and 16th centuries was far more virulent (7), and often fatal, compared to the chronic disease known today, suggesting introduction into a completely treponematosis-naïve population (8). In contrast, there are many differing viewpoints on the origins of the disease we know now as syphilis, some even theorizing that treponematoses were endemic in both the old

and new worlds and that the reports of severely virulent and fatal syphilis infection were simply misidentification of the disease and/or media exaggeration to instill fear for what was viewed as a biblical pestilence (1).

The disease was first described in European literature at the end of the 15th century, particularly in the first major book on syphilis by Francisco Lopez de Villalobos in 1498. Later, a long line of influential physicians and scientists studied the natural history and pathogenesis of this disease. These include John Hunter (notable for reported self-experimentation with gonococcal pus unknowingly contaminated with *T. pallidum*) (9) and Philippe Ricord who dispelled confusion about the etiologic agent of syphilis based on over 2500 human auto-inoculations in 1838 (10, 11). Lastly, it was William Wallace who showed that the lesions of secondary syphilis (once thought to be non-infectious) were in fact infectious, utilizing inoculation of healthy subjects with exudate from the syphilis exanthema (12).

Syphilis has had a great impact on medicine in many ways. Sir William Osler, the father of modern medicine, is often quoted as saying that “he who knows syphilis, knows medicine” (13, 14). This is testament to the idea that syphilis can mimic many disease processes and can present to the physician a multitude of signs and symptoms. In addition to the impact clinical syphilis has had on our understanding of medicine, studies of the natural history of syphilis in the context of the Tuskegee experiment (15, 16) have greatly impacted regulation of studying human subjects and the reluctance of some demographic groups to enroll in research studies.

Although it is not clear if there was a true dramatic and sudden decrease in disease severity, there have been reductions in syphilis prevalence from the epidemic proportions reported in 16th century Europe. In 1937 prevalence was estimated to be as high as 10% of the population in the US and Europe (17). But with the discovery and availability of penicillin, there

were great reductions in prevalence. Penicillin was an important game changer for many reasons. It was a highly effective cure for syphilis as well as gonorrhea at that time, and was a much better tolerated treatment than the predecessor syphilis treatment, Salvarsan (18). Importantly, when it became clear that penicillin alone would not be sufficient to control syphilis in the US, the government launched an extensive campaign to increase awareness and testing for the disease (19). In different regions of the country, there were troughs in incidence, down to 3.9 cases per 100,000 reported in 1956 (20) but there were resurgences of syphilis incidence in approximately 10 year intervals associated with the sexual revolution and homosexual liberation of the 1960's and 1970's (20) and outbreaks in the 1980s in minority populations associated with the crack cocaine epidemic (21). Implementation of new education programs in response to the AIDS epidemic, and the resultant behavior change, led to new lows in prevalence (22, 23).

Grassly *et al.* postulated that the cyclical nature of these rises in syphilis incidence every decade or so, suggesting patterns of waning of immunity in different demographic groups, at different times (20, 21, 24). Grassly's view is debated by Breban *et al.* who suggest that the 8-11 year cycles of syphilis epidemics simply represent disease outbreaks associated with exogenous factors such as changing sexual practices and changes in intensity of public health interventions, as opposed to the endogenous factor, immunity (25). Additionally, Fenton *et al.* suggested that endemicity (and discrete outbreaks) are seen in groups that have poor access to healthcare, are socially marginalized, are of low socioeconomic status, and have high rates of partner change (26). Recently there were reports of serious outbreaks of syphilis, particularly among men who have sex with men, in many cities including Seattle (27-29) which may be due in part to HIV sero-sorting, as well as HIV treatment optimism leading to reductions in safe sex practices. Although penicillin remains a highly effective therapy in the treatment of syphilis

(unlike gonorrhea) (30-33), the disease has not been tightly controlled or eradicated. This further emphasizes the role public health efforts (such as The National Plan to Eliminate Syphilis from the United States) have in controlling STD's (34). This recent program, which began in 1999, succeeded in reducing rates of syphilis in the target populations: women (thus reducing congenital syphilis) and African Americans. However, there were still overall increases in incidence of syphilis, particularly in Caucasian men who have sex with men (MSM), leading to a redesign of the elimination effort (34).

Epidemiology

Despite great strides in reducing the prevalence of disease, syphilis is still a public health problem in many parts of the world. In 2001, the World Health Organization estimated that 12 million new cases of syphilis occur each year, with current increases in incidence in many populations in the world (34-36). in 2005 the WHO reported that the greatest prevalence of disease was found in SE Asia, South America, and Africa; globally, there were an estimated 36 million people infected (36). Around the world, we see drastically increasing rates of syphilis, notably in China (37-39) where syphilis is the most commonly reported infectious disease (40), although the disease was once nearly eradicated (41). After the dissolution of the Soviet Union, there were great increases in syphilis incidence. For example, in the Russian Federation, there was a 48 fold increase in syphilis rates from 1989 to 1996 (42) and incidence remains high (43). Other countries where syphilis is on the rise include Ireland (44, 45), Australia (46), and areas of the US and Europe (47, 48). In the US and Europe, increases in incidence have been seen mostly in MSM populations in large cities such as San Francisco, Los Angeles (23), New York, Chicago, and Houston (49). In the Northwest US, there is a current resurgence of syphilis with

increasing incidence amongst MSM (22, 27, 29, 50, 51). Across the entire US, syphilis rates have increased among men from 5.1 in 2005 to 9.8 in 2013, accounting for 91.1% of all early syphilis cases in 2013. Encouragingly, there has been steady decline in incidence among African American women since 2008, a sign that public health efforts directed towards this group continue to be successful (52).

In the study of malaria transmission, Ronald Ross and later Macdonald *et al.* adapted an ecological/demographic tool to understand the epidemiology of infectious diseases (53). Later, Anderson and May applied this equation to understand the transmission dynamics of HIV infection (54). The basic reproductive rate (or ratio) (R_o) of infectious diseases are often estimated using the following equation (55, 56):

$$R_o = \beta cD$$

This simple mathematic model allows for the indirect estimation of the number of secondary infections resulting from a single infected individual. The three transmission dynamics in this equation are β (which represents the efficiency of transmission of infection per contact), C (the number of susceptible contacts per unit time), and D (the duration of the infective period of the disease). It is important to note that an individual infected with syphilis is infectious only during the early stages of disease (primary and secondary syphilis); with the exceptions of congenital transmission and transfusion/tissue transplantation, the decades long asymptomatic latent stages is not infectious. So, although certain aspects of pathogenesis may allow the treponeme to survive in the host for decades, the period of infectiousness is shorter than the total duration of infection. While the duration of infectiousness is impacted by the ability of the biologic agent to evade the host immune response and persist, and the number of susceptible contacts is dependent on the ability of an agent to infect immunologically naïve

individuals or to reinfect individuals with prior infection, the efficiency of transmission of a microbe is innate and is a characteristic of that specific infectious agent and its route of infection.

Syphilis is transmitted by direct (usually sexual) contact with a primary or secondary lesion, as these are the most infectious clinical stages. Alexander *et al.* observed that the percentage of individuals who reported contact with a primary syphilis lesions acquired infection (62.7%) was roughly the same as those who had contact with secondary syphilis lesions (61.8%) (57). These data show that persistence to the second stage of syphilis extends the period of infectivity and preserves the rate of transmission. Schrober *et al.* describes 42% of people exposed to dark field positive primary chancre failed to be infected, highlighting the inefficiency of syphilis transmission (58), potentially due to the natural protection of intact epithelial barriers. For syphilis infection, it was determined from experimental studies that the mean infective dose (ID50) for intradermal inoculation is 57 organisms in humans (59) and 10-23 organisms in rabbits (60). The doubling time for the syphilis spirochete during early infection was determined experimentally in rabbits to be between 30-33 hours (61). For naturally occurring infections, a primary chancre (an indurated, ulcerating yet painless lesion) will form at the site of initial contact after about 4-6 weeks. An incubation time of 4-6 weeks suggests an inoculum of very few treponemes doubling every 30-33 hours during natural infection.

As may be expected for a sexually transmitted disease, primary ulcers are most commonly found in the anogenital region but may be found on any mucus membrane (such as in the mouth and rectum) and anywhere a micro abrasion may allow bacteria to breach keratinized epithelial barriers. Infection most frequently occurs after sexual encounters or other contact with an infectious syphilis lesion. Primary chancres are described as painless open sores with a clean base and indurated raised edges, usually occurring as a single or very few

ulcers. This is in contrast to the clinical presentation of other genital ulcerating diseases such as chancroid (*Haemophilus ducreyi*) in which lesions are described as multiple, soft, and painful, and herpes simplex virus in which multiple small lesions develop that are described to be very painful (62). Less commonly, donovanosis and lymphogranuloma venereum also account for genital ulcers. Although there are distinguishing characteristics amongst these, it is often difficult to attribute a single etiology to a genital ulcer based solely on clinical appearance, and co infections do occur (63). As a genital ulcer disease, syphilis may also increase the transmission and acquisition of HIV. In fact, risk of HIV transmission is 2-5 times greater in persons infected with syphilis (64-66). Biologically this makes sense as any infectious process that 1) breaks down the epithelial barrier (open genital ulcers) and 2) increases inflammation and recruitment of HIV susceptible target immune cells (62) aids in HIV virus entry and replication (67).

Natural History

Generally, the primary chancre develops 4-6 weeks after inoculation (often in the vagina, rectum, and on the penis) and heals spontaneously after 4-6 weeks. This spontaneous healing is thought to occur by opsonophagocytosis of treponemes by activated macrophages within the lesion. Spirochetes begin to disseminate throughout the body soon after initial exposure (68). During early syphilis, treponemes can disseminate to multiple tissues in the body, including the central nervous system (69), liver (70), chambers of the eye, and the gestating fetus to name a few (71). Some of these disseminated bacteria localize in the skin and mucus membranes, causing secondary syphilis which may manifest as a macular/papular skin rash, most notably on the palms of the hands and soles of the feet but also involving mucus membranes. Other

clinical features of secondary syphilis include fever, patchy alopecia, painless lymphadenopathy and condylomata lata. Secondary lesions occur in 90% of untreated patients, contain bacteria, and are infectious (12, 72). Interestingly, the rash of secondary syphilis usually develops after healing of the primary chancre, in which the host immune response directed against treponemes is clearing the bacteria. Despite the presence of a targeted anti-treponeme response, secondary lesions arise at sites distant to the chancre (concurrent with chancre in 15% of patients (72), can persist for weeks, then heal spontaneously. Despite spontaneous healing of the secondary rash of syphilis, secondary lesions can recrudesce in multiple cycles. We believe that this pattern of pathology is due to subpopulations of treponemes escaping clearance at each stage where there is complete healing and resolution of the active lesions. During the pre-antibiotic era, such recurrent secondary syphilis rashes were seen in about 24% of patients (72). Considering coincident antibiotic therapy and the difficulty in distinguishing recrudescence of untreated syphilis, treatment failure, and reinfection, few modern examples of recurrent secondary syphilis are reported (73).

Following healing of the rash, individuals enter early latency (up to 1 year post infection) with no further clinical signs or symptoms. After a year post infection, untreated individuals enter the late latent stage of syphilis, again characterized by the lack of clinical signs and symptoms. During latent syphilis, treponemes are difficult to demonstrate in the infected individual, but serologic tests show evidence of infection. A small proportion of patients develop tertiary syphilis that may involve granulomatous destruction of soft tissues such as vital organs and skin as well as bone or cartilage (called gummas), late neurologic complications, and cardiovascular syphilis (72). Before antibiotic therapy was widely available, tertiary complications of syphilis infection were more common. Cardiovascular syphilis accounted for 40-60% of syphilis-associated deaths (15). Neurologic manifestations of the disease may

appear during secondary syphilis and beyond, and range from asymptomatic cellular changes in the CSF to iritis, uveitis, deafness, and confusion (17, 74). Late neurologic diseases include demyelination of the dorsal columns of the spine (tabes dorsalis), optic nerve blindness, and progressive dementia.

Vertical transmission to the fetus may happen at any time during infection but is most common during the first year of infection and less likely during latency in the pregnant woman (75). Congenital infection can cause abortion, still birth, deformities of the fetus, and disease or neonatal death in the infant (76). Worldwide, congenital syphilis affects more fetuses and newborns than any other infectious disease (77) including HIV (78, 79). Congenital syphilis is recognized as such a serious problem that the World Health Organization has a campaign in effect for the elimination of congenital syphilis (80, 81). Aside from miscarriage and still birth, classic signs of early congenital syphilis infection include snuffles (chronic rhinitis), enlargement of the liver and spleen, pneumonia, and skin lesions are notable during the first two years of life. Late signs such as saber shins (periostitis) leading to saddle nose, Hutchinson teeth, and neurosyphilis can manifest throughout life (82-85). Despite adequate treatment, some of the symptoms due to gummatous destruction may still progress (86, 87).

The relationship between infected infants and their mothers led to important observations regarding reinfection and “chancre” immunity. Abraham Colle’s law states that a symptom-less mother cannot be infected by nursing her infected infant, even though the same child is capable of infecting other individuals including wet nurses. Conversely, Giuseppe Profeta’s law states that a symptom-free infant cannot be infected by its infectious mother. Colle’s and Profeta’s laws both pre-date serologic testing and assume that the asymptomatic individual (mother and child, respectively) is free from infection, but somehow developed immunity (88, 89). We now know that the asymptomatically (latent) infected mother and child

developed some immunity to homologous reinfection during their current infection. Kassowitz observed that the greater the duration between maternal infection and pregnancy, the better the outcome for the offspring. This is likely due to a higher spirochetemia during early syphilis, with subsequent reduction in treponeme load over time, and thus reduced spirochete dissemination to the developing fetus. So, children born soon after maternal infection (during the first few years of infection) are more likely to suffer the sequelae of congenital syphilis compared to children born in successive years (90). In light of our current knowledge of the natural history of syphilis, these observations made during the 19th century provide profound examples of the vertical transmissibility of syphilis, and the development of protective immunity to reinfection. Unfortunately, despite knowledge of the infectiousness of congenital syphilis (presumably via oral mucosal lesions), physicians and wealthy families actively deceived wet nurses to care for infected infants, resulting in further spread of syphilis to other families (91).

Much of the natural history of untreated syphilis (Figure I-1) was discerned in a retrospective evaluation of untreated syphilis in the Oslo study, as well as in the prospective Tuskegee study. The former study was performed in the pre-antibiotic era in Oslo, Norway. The principal investigator, Boeck, sought to determine whether the natural course of syphilis was more benign than the fairly toxic available treatments of that time. From this study we learned that 23.6% of patients with secondary syphilis develop multiple bouts of “secondary relapse”, most within the first year post infection and 85% had lesions involving mucosal surfaces. Among the study subjects, syphilis accounted for 11% of deaths;14% of men and 8% of women suffered cardiovascular syphilis; while 9% of men and 5% of women had symptomatic neurosyphilis (72). The major objective of the Tuskegee experiment was to study the natural history of syphilis in African Americans in Macon County; a resource-poor community with a high prevalence of the disease. Investigators sought to compare the course of naturally

acquired syphilis infection, histologically and clinically in African American men to that reported for Caucasian's in previous studies. The prevailing thought was that, because syphilis was endemic in African American populations, the natural course of infection was potentially less severe than the disease in Caucasian-Americans. The subjects of the longitudinal Tuskegee study (408 men enrolled from 1932-1972) were actively denied antibiotic treatment even though it became available during the study (15, 16, 92). Thirty percent of the 92 men followed to autopsy were found to have syphilitic involvement of the cardiovascular or nervous system that caused their deaths (93). Another abhorrent example of questionable ethics in human research is exemplified by the recently publicized Guatemala experiments, in which researchers intentionally infected healthy individuals with syphilis and gonorrhea to study the efficacy of recently discovered penicillin for these infections. These studies involved sex workers, prisoners, soldiers and the mentally ill; none of the findings from this study were published (94).

The Sing Sing prison study of syphilis infection and reinfection, on the other hand, involved informed consent of the volunteer prisoners [arguably still a vulnerable population, not free from coercion (95)] who participated and yielded value information about immunity and reinfection after previous syphilis infection.(59). From this study we learned that partial to full immunity to reinfection is acquired over the course of infection. Subjects with untreated latent syphilis were unable to be re-infected following intradermal challenge (0/5), while 82% (9/11) of subjects treated during early syphilis, 80% (4/5) of those with congenital syphilis, and only 38% (10/26) of those treated during late latency were susceptible to reinfection with the Nichols strain. These data suggest that immunity to reinfection increases over the duration of untreated, naturally acquired infection into late latency, by a prolonged interaction between the immune system and viable treponemes. As such, antibiotic therapy early in the disease course stops this accumulation of immunologic memory, which may then wane after treatment. These

findings are significant in considering the current syphilis epidemic. Many outbreaks are confined to specific demographic groups, such as MSM in urban centers (22, 51, 96-100), in which re-exposure to infection is common. While immediate treatment of all syphilis cases is the necessary and appropriate standard of care, curative treatment of early syphilis decreases the likelihood that a person's immunity will be protective against reinfection.

The phenomenon of repeated infections, that may or may not be clinically obvious, is immediately relevant to the epidemiology, treatment, and management of patients with known previous syphilis. Repeated infections can occur in two ways: a reinfection after a patient is treated and cured of their initial syphilis infection; and superinfection in which a patient has not been diagnosed and treated for an initial infection prior to re-exposure. Grimes *et al.* recently utilized a new typing method to examine the introduction of strains over time in a high-risk population in Seattle, WA (29). Figure I-2 exemplifies how one strain type (14d/g in blue, for example) enters a population and replaces the formerly predominant strain over time. A similar example of the replacement of the 14d/f strain type by 14d/g along with reinfection by a heterologous strain was described in Paris, France (101). Because individuals in this study may have had multiple episodes of syphilis, it is likely that the new strain was able to overcome pre-existing immune responses directed against the initial strain type in order establish infections in the population. It is known that some of the people in this cohort with the new strain were re-infected after prior treatment, and it is possible that some were superinfected (28, 29).

With the advent of molecular typing methods for *T. pallidum*, it is possible to recognize the introduction of new strains into a community where people have already had, or currently have, syphilis infection. This scenario can be recapitulated in rabbits that are infected with an initial strain, acquire homologous immunity to symptomatic repeat-infection, but may develop clinical lesions following challenge with some (but not all) heterologous strains (102). It is

unclear which molecular determinants are relevant for establishment of a new infection in an individual who has already developed partial immunity to previously infecting strains. Syphilis is most efficiently spread during symptomatic infection, so a superinfecting strain that can produce lesions has a greater likelihood of being transmitted in a population with some syphilis immunity (103), thus having the effect of enlarging the susceptible pool (c in $R_o = \beta cD$).

Early researchers in the 19th century noted that infected individuals with a primary chancre were unable to be reinfected with syphilis. In these studies, subjects were challenged by inoculating the skin with material containing treponemes from that same subject, i.e. homologous treponemes(10, 11). Additional studies showed that this "chancre immunity", or refractoriness to reinfection, was maintained in individuals provided that the original infection was of sufficient duration (59, 104). Previous work reported in 1942 by Turner and McLeod (102) describes cross-immunity between some, but not all, strains of *T. pallidum* in the rabbit model. Specifically, once an animal has become refractory to superinfection upon challenge with the same strain (~3 months post infection), only a subset of heterologous strains are able to produce clinically evident superinfection (apparent by the development of skin lesions) (102). The strains examined in this older study have all been lost except for the Nichols strain, and so cannot be further investigated.

Attachment and Invasion

At the site of entry (typically mucosal surfaces and microscopic breaks in the skin), treponemes initially begin to divide unchecked by the immune system. It is generally believed that small breaks in epithelial barriers are necessary for transmission of syphilis. This may help explain the relatively low transmission rates of 30% cited in studies (31), although it has been reported to be up to 60% (57, 105).

As described previously, syphilis chancres are indurated lesions that are usually painless, contain many treponemes, and ulcerate. The clinically evident induration is due to immune cell infiltrate to the area of infection. Tissue damage is hypothesized to be due to ischemic necrosis caused by vasculitis and endothelial swelling in small vessels (106). The central necrosis seen in primary chancres and other ulcerating lesions is due to occlusion of blood vessels by red blood cells and fibrin (107). Although tissue destruction can be due to pathogen-derived toxins [for example, *Clostridium perfringens* alpha-toxin (108)], *T. pallidum* has no known toxins, and tissue damage is thought to be mediated by the activated immune cells described below.

Treponemes multiply at the initial focus of inoculation, but also disseminate widely throughout the body. The kinetics of early dissemination were investigated in the experimental rabbit model of infection, in which investigators described treponemes in the blood minutes after infection (109). During needle inoculation of treponemes, it is possible to inadvertently deliver the bacteria into small blood vessels, which may account for early detection of treponemes in the blood. While it is not completely clear whether treponemes invade the circulation in the first minutes after infection, we know that the bacteria are circulating and disseminate throughout the body, well before clinical lesions appear. In humans, treponemes are detectable in the blood of patients with primary and secondary syphilis (110); additionally, it is known that treponemes circulate in the blood of patients with incubating syphilis, before lesion development (111). After inoculation, there is rapid dissemination of treponemes into the blood and lymphatics (during the first 24 hours) and multiplication of bacteria in the primary chancre. There appears to be a greater burden of bacteremia detected during the second stage of disease, suggesting that as treponemes are cleared from primary lesions, there is multiplication of disseminating treponemes (110, 112).

A possible virulence attribute of treponemes is their ability to bind components of the extracellular matrix (ECM) (113). Binding of host ECM components is essential for virulence in many pathogens [for example *Borreliaburgdorferi*, the related spirochete etiologic agent of Lyme disease (114)] by facilitating adherence to cells and invasion into host tissues. Recently, predicted outer membrane proteins have been shown to bind and degrade components of the ECM. These include Tp0136 (along with Tp0483 and Tp0155), which are known to bind fibronectin (113, 115) and Tp0751 which not only binds laminin and fibrinogen but also degrades these ECM components (116). Pallilysin (Tp0751) was most recently shown to complex with Tp0750 (another degrader of fibrin that interacts with host proteins involved in fibrinolysis), which may help target *T. pallidum* to vascular endothelial cell to facilitate dissemination (117). Most striking is the discovery that, when animals are immunized against recombinant Tp0751 and challenged with live treponemes, there is a significant reduction in the bacteria's ability to disseminate throughout the rabbit, as evidenced by the failure of transferred lymph nodes from challenged animals to cause infections in naïve animals (personal communication, Caroline Cameron). Nonpathogenic *Treponema phagedenis* does not bind laminin (a component of the ECM) but, after transformation and heterologous expression of Tp0751, laminin binding was observed (118). Treponemes are often seen in a perivascular pattern histologically (119) and are believed to invade the vascular space by traversing tight junctions between endothelial cells to gain access to the circulation (120). Treponemes are able to invade the blood brain barrier and infect the cerebral spinal fluid as well as the meninges and brain parenchyma. In 1913, Noguchi was the first to definitively show treponemes in the brains of patients who suffered general paresis (69). *T. pallidum* is also known to invade and disseminate through the placenta and multiple other organs and tissues, presumably using similar mechanisms.

Immunology

In the location where treponemes begin multiplying, it is believed that innate immune signaling by Toll like receptor 2 (TLR 2) serves as the initial trigger to begin the inflammatory reaction. TLR 2 is associated with the outer membrane of host cells, particularly monocytes/macrophages, dendritic cells, and B cells. Sensing of bacterial pathogen associated molecular patterns (PAMPS) by this family of pattern recognition receptors triggers cytokine production by the sensing cell. In the case of treponeme infection, lipoproteins (such as TpN47 and TpN17) have been proposed to be the major PAMP signaling through TLR2 (121). Blister fluid raised at the injection site of TpN47 and 17 is highly enriched for macrophages/monocytes, dendritic cells, and T cells similar to what is seen in primary and secondary lesions, suggesting that lipoproteins are responsible for the stereotypic treponemal immune response (122).

In a new study of the relationship between TLRs and syphilis, Marra *et al.* demonstrated significant associations between specific TLR single nucleotide polymorphisms and increased risk of neurosyphilis (123). TLR2 can heterodimerize with TLR1 to detect triacyl lipoproteins as well as TLR6 to detect diacyl lipoproteins. The cytokines produced in response to this signaling are MyD88/Mal dependent through TLR2 and include pro inflammatory cytokines such as TNF-alpha, interleukin (IL)-8 (both neutrophil attracters), and lL-12. IL-8 production is directly involved in increases vascular endothelial cell permeability by down regulation of tight junction components, and may facilitate treponemal dissemination (124). TLR2 signaling also triggers phagocytosis of bound bacterial molecules by antigen presenting cells (APC). These APC's then travel by lymphatics from the area of initial inoculation to a draining lymph node where the treponemal antigen is presented to naïve T cells in the context of major histocompatibility

complex class II (MHC II). In the context of IL-12, there is differentiation of T cells into the T Helper 1 (TH1) subset. The predominance of TH1 phenotype is seen not only in experimental syphilis infection (125) but also in both human primary and secondary lesions (126), but is not seen systemically (127).

Delayed type hypersensitivity (DTH), a strong cellular immune response, is associated with the high level of inflammation in lesions and subsequent clearance of treponemes in early syphilis; but this reaction is also associated with the highly destructive gummatous lesions of tertiary syphilis. DTH is mediated by CD4+ T cells, TH1 cytokines, and macrophages at the site of persistent antigens. An accumulation of antigen in the presence of sensitized antigen-specific T cells leads to an over-exuberant and chronic inflammatory response resulting in plasma cell infiltration (secondary lymphoid organ), granuloma formation, as well as tissue destruction (128). Similar to the balance between DTH and antibody responses in the disease manifestations of leprosy, some believe that an insufficient or weakening DTH response leads to the appearance of secondary and tertiary syphilis disease (128). Interestingly, TH1 cytokines and CD4+ T cells are repeatedly described in the lesions of secondary syphilis, as well as interferon-γ (IFNγ)-producing CD8+ T cells, suggesting an intact DTH response during this stage (126, 127, 129).

Prototypically, in the draining lymph node, antigen presenting cells such as dendritic cells expose naïve T lymphocytes to antigen in the context of MHC II, the antigen receptor that detects external pathogens, often leading to TH2 polarization of the immune response to attack extracellular pathogens. It is therefore somewhat paradoxical that the immune response to *T. pallidum* (an extracellular pathogen) is polarized to the TH1 cellular response typically associated with clearance of intracellular pathogens. The TH1 phenotype consistently observed in infectious syphilis lesions is described as involving IL-2, IFNγ, and IL-12. IL-2 is necessary

for growth and differentiation of CD4+ cells, while IL-12 is responsible for differentiation into TH1 effecter cells. These provide cytokine help and activate CD8+T cells to produce IFNγ and cytotoxic proteins. IFNγ is a critical activator of macrophages, greatly facilitating phagocytosis and destruction of pathogens. Lukehart *et al.* showed that activated macrophages are indeed the immune effecter cells responsible for treponemal clearance and destruction within a lesion (130, 131).

It also appears that IFNγ within primary and secondary lesions originates primarily from CD8+ cells, suggesting that the role of these cytotoxic cells is to activate infiltrating macrophages to clear treponemes by opsonophagocytosis (132). The cytotoxic effector molecules of CD8+ cells (granzyme and perforin) may then contribute to the tissue destruction seen within lesions. Natural Killer (NK) cells, similar to CD8+ cells, are cytotoxic and may also be involved in tissue destruction. NK cells are enriched in secondary lesions and may also provide IFNγ activation of macrophages to facilitate opsonophagocytosis (127). NK cell influx to lesion sites is expected as treponemal lipoproteins signal through TLR2 on resident phagocytic cells, which respond by producing IL-2 and -12, both of which activate NK cells.

Another possible mechanism for the induction of the TH1 response seen in syphilis lesions is the cross presentation of extracellular antigens by MHC I, which would lead to differentiation of naïve CD4+ T cells into TH1 effecter cells. Additionally, it was postulated by Stary *et al.* that CD8+ cells could be directly activated by TLR2 signals (132). Although the syphilis spirochete is considered an extracellular pathogen, there are reports that suggest that an intracellular niche might serve as a protected reservoir for the pathogen; in which case, an effective CD8+ response would be appropriate to clear the infected cells. The finding that CD8+ cells are co-expressing IFNγ with IL-17 suggests that, within lesions, there is a role for TH17

cells, which are important in both anti-microbial immunity and in pathology. Additionally, signaling via TLR2 results in IL-6 production, another contributor to TH17 cell differentiation.

Although the immune response is directed primarily to a TH1 type of cellular immunity, there are still TH2 effectors which presumably support the differentiation of antigen specific B cells to plasma cells. This is supported by studies demonstrating a limited TH2 cytokine production within lesions (125), the presence of plasma cells in granulomatous tissue, and the high titers of antibody to treponemal and non-treponemal antigens. Azar *et al.* described phagocytosis of treponemes by plasma cells as a potential route of plasma cell activation triggering production of antibody (106). Further, the presence of antibodies specific for outer membrane antigens is key in efficient opsonophagocytosis of treponemes (133). Roles for these anti-treponemal antibodies are also evident by passive transfer of partial immunity in rabbits (134), *in vitro* neutralization of treponemes in experimental infection (135), as well as immobilization of treponemes in the presence of complement (136).

Diagnostic Tools

Because treponemes are very thin and cannot be visualized by light microscopy, special silver stains as well as immunohistochemistry are used to detect treponemes in tissue samples. Direct tests for *T. pallidum* include darkfield microscopy, histological staining, the rabbit infectivity test, and PCR (110, 137-139). One rapid and inexpensive specific test for syphilis infection is the visualization of the bacterium by darkfield microscopy. This is best accomplished by studying exudate samples obtained from active chancres and condylomata lata, as these lesions contain the greatest concentration of bacteria (140). In addition to darkfield microscopy, histologic staining of tissue samples by silver staining protocols such as

the Dieterle stain may also reveal the bacterium (141), but can result in staining artifacts that can be misidentified as treponemes. Silver stains are also used to stain other atypical bacteria (colorless after gram staining) such as Mycobacterium, Helicobacter, other spirochetes, and Mycoplasma (142, 143). An improvement upon the silver stain is immunohistochemistry staining using polyclonal or monoclonal antibodies to the syphilis bacterium (144, 145).

The rabbit infectivity test (RIT) is often reported to be the gold standard for detection of infectious treponemes and is used to measure the sensitivity of other tests. RIT is also highly specific, allowing treponeme numbers to be amplified from biologic samples (138, 146). This is of great value in latent disease where there are no active lesions in which to search for treponemes, but live organisms may still be present in blood and lymph nodes. Although highly specific and sensitive, the RIT is impractical to be used for routine diagnosis. PCR tests are also very specific but perhaps less sensitive, may not be accessible to all patients, and are suitable primarily during the early stages of disease or in pathology samples (110) although it is possible to detect treponemal DNA by PCR in earlobe scrapings of patients with latent syphilis (147). In addition to the direct tests described above, serologic tests, which measure antibodies induced by infection, are of great importance for screening in public health and clinical settings because they require only a serum sample, allow detection of latent disease, and provide fast results depending upon the test used.

Non-treponemal tests detect the presence of antibodies to lipids such as cardiolipin-cholesterol-lecithin antigens and become positive 1-4 weeks after a primary chancre appears. These tests are said to be “non-treponemal” because the antigens they target are not specific for the syphilis bacterium, but were initially thought to be released from damaged host cells. As such, false positive reactions are possible, especially in autoimmune disease where antibodies to cardiolipin and lecithin may be found. There is now evidence, however, that host lipids are

incorporated into the outer membrane of *T. pallidum*, perhaps directly stimulating the production of antibodies (148, 149). The first serologic test for syphilis was a complement fixation test, the Wassermann test, named after its inventor (150). Subsequent refinements are no longer in use today because they have been replaced by flocculation tests such as the Rapid Plasma Reagin (RPR) (151-153) and Venereal Disease Research Laboratory (VDRL) test (154, 155). The flocculation tests are of particular importance because a quantitative test can be performed and monitored after treatment; declines in VDRL or RPR titer indicate effective treatment (156). And because antibody titers decrease substantially in latency or with adequate treatment, they are useful in detecting subsequent reinfections by revealing increases in antibody titers suggestive of a new infection (140). Prozone reactions, where high antibody titers produce grainy or rough flocculation tests, are rarely seen but can lead a serologist to report a false negative result (140). It is therefore critical to properly follow up atypical flocculation test results after diluting the serum sample to ensure detection of infection and appropriate timely treatment (157).

Because the non-treponemal tests are often used in the setting of mass screening and have known false positives, reactive results should be followed by a confirmatory treponemal test. Such assays detect anti-treponemal antibodies and include fluorescent treponemal antibody absorption (FTA ABS), *Treponema pallidum* hemagglutination (TPHA), *T. pallidum* particle agglutination (TPPA), and enzyme immuno assay (EIA). Although the treponemal tests become positive earlier during infection than the non-treponemal tests, they cannot be used to monitor treatment because most individuals maintain reactivity even after treatment (140). The current use of EIA in “reverse sequence screening” is a growing concern as the test is less specific than the previously recommended non treponemal test when used as the first screening tool. This leads to discordant results in which more than half of EIA positive results are RPR negative, and 30% of those RPR negative samples are also TPPA or FTA ABS negative (158-

161). The thought is that these cases of EIA positive results that are negative by all confirmatory studies are examples of false positives. This may be due to the antigens commonly used in EIA platforms. Tp15, Tp17 and Tp47 possess homologues in other Treponemal species, particularly the many (largely uncultivable) treponemes associated with periodontal disease. Potential cross reactivity of antibodies with EIA antigens may account for false positives "syphilis" results in the clinical setting of periodontal disease (162). False positive EIA results gathered in reverse sequence screening can increase health care costs by requiring extensive follow up of individuals who are not infected. This may lead to unnecessary treatment, unnecessary psychological trauma, and social/marital repercussions.

Treatment

Although current treatments for syphilis are well tolerated, this was not always the case in treating this potentially life threatening and disfiguring disease. Previous therapies included mercury (163), bismuth (164), malaria-induced fever (165-167), and arsenic (18). Since penicillin was found highly effective in the treatment of syphilis in 1943 (30, 168), it has remained the first line treatment for syphilis, with very few reports of suspected treatment failure in the literature (73, 86, 169). Almost immediately following initiation of bactericidal treatment, patients with syphilis may develop on immunologic phenomena known as the Jarisch-Herxheimer reaction (JHR). This reaction was shown to be relatively rare [1.4% in China (170)] though older reports cite rates of 9-80% (171, 172) and may present as a worsening of skin lesions and constitutional symptoms such as fever, tachycardia, myalgia. JHR may be observed in patients with sero-negative incubating syphilis, sero-positive primary and secondary syphilis as well as tertiary syphilis, but is generally not seen during treatment of latent cases of

syphilis. It is believed that release of inflammatory lipoproteins from lysed spirochetes leads to the self-limited pathology of JHR (173).

In addition to penicillin, other broad spectrum antibiotics such as tetracyclines (including doxycycline) and macrolides (azithromycin, clarithromycin, and erythromycin) have been used to successfully treat the infection. The macrolides (specifically azithromycin) are attractive in treating syphilis in an outbreak setting because 1) they have some activity against other STI's 2) one-time dosage for syphilis eliminates inadequate treatment due to losing patients to follow-up 3) oral therapy is amendable to partner-delivered prophylaxis 4) pills may be more stable to deliver care in resource-limited settings and require less training compared to delivering injectables (33, 174-177). Unfortunately, there is reported resistance in *T. pallidum* to macrolide therapy, associated with two discrete mutations in the 23s ribosomal RNA gene (178-181). These mutations conferring resistance have become more prevalent and highlight again that penicillin therapy is still the preferred treatment regimen (29, 50, 182-185). Benzathine penicillin G (BPG), 2.4 million units in a single IM injection, is used to treat primary, secondary and early latent syphilis. The same dose delivered once a week for three weeks is the recommended treatment for latent and late disease. BPG treatment does not penetrate the central nervous system in measurable quantities, so to ensure that the antibiotic reaches the CNS to clear treponemes, different treatment protocols are necessary. Neurosyphilis requires IV delivered aqueous crystalline penicillin G 18-24 million units per day as 3-4 million units every 4 hours or as a continuous infusion, for 10-14 days. Additionally, neurosyphilis can be treated with a daily intramuscular injection of aqueous procaine penicillin G and oral probenecid 4 times a day, both for 10 - 14 days.

Biology of the Etiologic Agent

Treponema pallidum subsp. *pallidum* is a gram negative spirochete. Its long fragile body (6–15 μm long by ~0.2 μm in diameter) and minuscule genome (1.14 Mb) make it a surprising candidate for the causative organism of the complex disease, syphilis (186). The order Spirochaetales is populated by gram negative, thin spiral shaped organisms that often require special stains for visualization and include the genera Leptospira, Brachyspira, Borrelia, and Treponema. The genus Treponema includes treponemes associated with termite guts, and bovine digital dermatitis (187) and Treponema human pathogens include those associated with periodontal disease (188). Due to its lack its lack of biosynthetic genes, *T. pallidum* has yet to be maintained continuously in culture (189), although many advances in characterizing treponemes have come from studies of the cultivable treponemes which include the Reiter treponeme (*Treponema phagedenis*) (190) and *Treponema denticola* (191). The most closely related bacteria to *T. pallidum* subsp*. pallidum* are the agents of yaws (subspecies *pertenue*), pinta (*Treponema carateum*), bejel (subspecies *endemicum*), rabbit syphilis (*Treponema paraluiscuniculi*), as well as the simian strain (Fribourg-Blanc) (192, 193). These species and subspecies are serologically and morphologically indistinguishable from the syphilis spirochete *Treponema pallidum subspecies pallidum*. These spirochetes are also thought to be less invasive than *T. pallidum* subsp*. pallidum*, and thus not associated with neurologic disease and vertical transmission to the fetus, though there is some evidence that the endemic treponematoses (particularly *T. pallidum* subsp. *pertenue*) exhibit invasiveness (194, 195).

Treponemes are particularly interesting due to their unique cell wall structure compared to typical gram negative bacteria. The bacteria have an outer membrane, a cell membrane, and a periplasmic space. This periplasm is the home of 1-8 periplasmic flagella (responsible for the bacterium's motility and a peptidoglycan layer overlying the cell membrane. The cell

membrane- associated peptidoglycan layer and the lack of LPS in the outer membrane set Treponema apart from conventional gram negative bacteria (187). The location of the peptidoglycan layer may help to explain the fragility of the outer membrane and ease in which it is disrupted during experimental manipulation such as washing and centrifugation.

From the first published *T. pallidum* genome sequence (186), we have learned a great deal about the organism. An early report stated that *T. pallidum* contained a single small extrachromosomal plasmid (196), but this finding was not confirmed by later genome analysis; so the current opinion is that all *T. pallidum* genes reside on a single circular chromosome of about 1.14 Mb. *T. pallidum's* relatively small genome lacks genes for the TCA cycle, electron transport, amino acid synthesis, purine and pyrimidine synthesis, fatty acid and LPS synthesis, and restriction modification systems (186). In order to acquire necessary nutrients it is unable to synthesize, *T. pallidum* must rely on transporters. From the *T. pallidum* initial genome sequence we know that there are 18 distinct transporters utilized by the bacteria (186). This may explain the difficulty in supplying the bacterium with its metabolic requirements *in vitro* and the long doubling time observed *in vivo* (61). Understanding the limited metabolic capabilities of this bacterium aids in explaining the fastidiousness of this organism, our inability to sustain *in vitro* cultures, and the need to propagate it in a laboratory animal.

Animal Models

Multiple animal models have been examined for the study of syphilis. These include mice, hamsters, guinea pigs, rabbits, and non-human primates. Of these animal models, only rabbits and non-human primates develop disease that resembles human disease such as skin lesions. While treponemes do replicate in mice and hamsters, these animals are less

susceptible to symptomatic infection and require larger inocula than needed to infect rabbits. Interestingly, hamsters are more susceptible to symptomatic *T. pallidum* subsp. *pertenue* than *pallidum,* even though these bacteria are very closely related. Although immunocompetent guinea pigs develop lesions after intradermal (ID) injection with large inocula, they do not develop a Th1 response during infection; mice fail to develop any clinically obvious pathology following infection (197-199). A newly described mouse model attempts to address the need in the field for an inbred animal model of infection, and although experimental infection in this model illustrates the critical role TLR signaling plays in syphilis pathogenesis, neither wild type nor the Myd88-deficient mouse displays pathology comparable to human and rabbit infection (200).

The rabbit model of infection, on the other hand, recapitulates disease processes found in human infection. In addition to the similar clinical and histologic appearance of rabbit lesions, ID_{50} (61), and immune responses (125) are nearly identical to humans. Lastly, rabbits develop a chronic syphilis infection characterized by early dissemination to blood, other organs, as well as CNS infiltration (201), making this small animal model of infection the most relevant to human disease (102). Although there are no inbred strains of rabbits and just a few commercially available immunologic tools, this is the animal model of choice in studying syphilis infection. Because rabbits are the natural host of a related treponematosis (*T. paraluiscuniculi*), serologically positive animals must be omitted from experiments to avoid the confounding effects of cross-immunity between the agents of rabbit syphilis and human syphilis (102).

Although humans are the only natural host for syphilis, the bacterium is successfully maintained by serial intratesticular inoculation of the rabbit, as it cannot be maintained *in vitro.* At the site of experimental intradermal inoculation, the animal develops a painless indurated lesion that ulcerates and spontaneously heals, typical of a primary chancre in humans.

Following infection by various routes (intratesticular, intradermal, and intravenous), rabbits may develop disseminated skin lesions on areas kept free of fur. Disseminated skin lesions that develop after intratesticular and intradermal infection are analogous to secondary lesions seen during the second stage of human infection because they appear during and after healing of a primary lesion (orchitis lesion or skin chancre). In contrast, the disseminated skin lesions that arise after intravenous inoculation are considered primary lesions because they the first lesions to develop (102). During experimental infection, rabbits have an immune response comparable to that seen in humans, including Th1 CD4+ and CD8+ cellular infiltration at the site of infection and development of specific antibodies to the same antigens, suggesting that expression of antigens is the same in rabbits as in humans (125, 202). Additionally, there is a clear primary stage of disease (including chancre development and ulceration in inoculated skin) that resolves spontaneously. During and after resolution of the primary lesions (intradermal as well as intratesticular) a secondary stage of disease can manifest as disseminated skin lesions in areas where the animal is kept free of fur (102). Older reports also suggested that rabbits may develop tertiary gummatous disease of the cardiovascular system; a sequelae described in human syphilis as well (72, 203, 204).

Non-human primates are also susceptible to experimental infection. Interestingly, both rabbits and non-human primates are reported to have their own endemic treponematosis, suggesting a natural susceptibility to pathogenic treponemes. *Treponema paraluiscuniculi* causes rabbit venereal syphilis lesions (205, 206) and an unclassified treponeme, the Fribourg-Blanc strain, was isolated from a baboon (193, 207-209). Both of these bacteria are highly related to *T. pallidum subspecies pertenue* and some believe that the non-human primate may have serve as a reservoir for human infection (193, 210).

Vaccines

As described above, there have been efforts past and present, to control syphilis infections in many different populations. Although curative therapy is available in the form of penicillin, and public health campaigns to increase awareness and testing have proved effective in reducing syphilis incidence, this disease still reemerges periodically and is a public health problem around the world. This is partially due to the lack of protective immunity acquired prior to treatment for the early stages of infection, resulting in susceptibility to repeated infections. This means that, in a sexual network, there is maintenance of a large proportion of people who are susceptible to a given circulating strain. Furthermore, lack of access to diagnosis and treatment in many communities results in high incidence and prevalence of this infection. The best tool to stably control this disease is successful and broad-based vaccination. In 1973, James Miller showed proof of principle that sterilizing immunity to homologous infection can be achieved by immunizing rabbits with gamma irradiated treponemes (211, 212). The immunization regimen provides hope that a protective vaccination is attainable, though the frequent IV administration (60 doses over 37 weeks) of attenuated treponemes is not practical. Additionally, the preparation would be contaminated with rabbit proteins, particularly testicular antigens that may sensitize individuals leading to allergic response and testicular damage. Lastly, this vaccine was composed of whole treponemes, which elicited the same post-infection antibody responses detected by the VDRL, FTA-ABS, and TPI tests, thus eliminating the usefulness of these tests in diagnosis of real infections should infection occur. Previous studies describe the importance of opsonophagocytosis in clearance of treponemes in infected hosts, as such, a vaccine should elicit antibodies to outer membrane proteins that are opsonic and elicit strong delayed type hypersensitivity response based on cellular immunity (128). Despite reports that the outer membrane is deficient in integral membrane proteins compared to other

gram negative bacteria (213), these rare surface exposed proteins are likely key candidates for immunization. There is much interest in the field to identify and describe these elusive protective OMPs in an effort to address the need for an effective syphilis vaccine.

Outer Membrane Proteins

Much of what has been discovered about treponemal cell architecture was from early freeze fracture visualization of bacteria (213-215). These studies revealed that the outer membrane of *T. pallidum*, compared to other bacteria such as *Escherichia coli* and *Treponema denticola*, was almost devoid of integral membrane proteins. This conclusion, however, is difficult to reconcile with the multiple observations that sera from infected individuals greatly enhance opsonophagocytosis, suggesting a number of opsonic targets on the bacterial surface. Despite these data, however, the identity of outer membrane-associated proteins is controversial in the field, highlighting the fact that the identification of surface exposed proteins in *T. pallidum* is difficult.

As already discussed, freeze fracture studies suggest that the outer membrane of *T. pallidum* has 2 to 3 orders of magnitude fewer integral membrane proteins than *E. coli* (213-215). Several approaches have been used to identify potential outer membrane proteins (OMPs) and many of these candidate proteins have been further examined. Bioinformatic approaches have yielded a number of predicted OMPs (216, 217) . For example, Tp0453 was initially identified by bioinformatics and later proposed to be a lipoprotein that inserts into the inner leaflet of the outer membrane, increasing the permeability of the membrane to nutrients by undergoing conformational changes (218, 219). Expression library screening with highly characterized opsonic antiserum has also been used to identify potential OMPs (220).

Candidate OMPs have also been suggested by isolating and studying outer membranes from treponemal samples (Tp0163, Tp0663), though subsequent data indicate that Tp0163 is likely associated with the periplasm (221). Although Cameron *et al.* demonstrated ECM binding by Tp0483 and Tp0155, and thus provided functional, indirect evidence of their outer membrane location (113), Thomson *et al.* failed to support its inferred outer membrane location by indirect immunofluorescence (222). Similarly, Tp0136 was shown to participate in fibronectin binding but the authors additionally demonstrated outer membrane labeling by anti-Tp0136 antibodies in an indirect immunofluorescence assay described below (115). As previously discussed, the *T. pallidum* proteins associated with ECM binding are thought to facilitate treponeme attachment and, in some cases, dissemination by directly interacting with host proteins. Studies of Tp0751 finally provided the field with a convincing example of a true outer membrane protein supported by multiple lines of functional data (opsonophagocytosis, gain of function (ECM binding) by heterologous expression in a non-adherent treponeme, and enzymatic activity against host proteins (118).

Assays that allow direct probing of the intact treponemal outer membrane are attractive in the pursuit of T. pallidum OMPs, but the fragility of the outer membrane makes most protocols ineffectual due to damage of the membrane during washing and centrifugation. Gel microdroplet assays attempt to stabilize the outer membrane of the spirochete by surrounding it with porous agarose. This allows for incubating and washing steps required for indirect immunofluorescence to take place without damaging the outer membrane and exposing components of the periplasm or cell membrane (223). Live bacteria are first incubated with primary antibodies directed to the OMP in question, and then with a secondary antibody to amplify the signal. Utilizing this method of detection, Brinkman *et al.* illustrated the outer membrane location of Tp0136, the only predicted OMP to be labeled in this way (115).

Although this method of detection of OMPs is promising in the field, there are multiple examples of conflicting results using the agarose gel micro droplet technology concerning the likely cellular location of OMPs [specifically, Tp0136, Tp0326 (BamA), and Tp0897 (TprK)] (115, 217, 222). Because there is no OMP to serve as a reliable positive control in this assay, it is unclear if this method is sufficiently sensitive to detect treponemal OMPs, which likely exist in the outer membrane at a very low density.

Because outer membrane proteins are exposed to the host milieu, one might expect the bacteria to evolve mechanisms to change its surface antigens to impart specific tissue tropisms and to evade immune responses directed to exposed epitopes. Currently, characterization of Tp0136 sequence variability amongst strains is underway and may have significant implications for the relative abilities of different strains of *T. pallidum* to bind host fibronectin (personal communication, Wujian Ke). Sequence variation in *tp0548*, an putative OMP predicted to participate in lipid transport [based on structural homology to *E. coli* FadL (224)], is currently being utilized in an enhanced strain typing mechanism, along with members of the *T. pallidum* repeat (Tpr) family containing some predicted OMPs (28). In addition to Tp0136 and Tp0548, the sequences of other predicted OMPs are known to vary amongst strains.

The 12 member Tpr family of proteins orthologous to the major outer sheath protein (MSP) in *T. denticola,* was identified by subtractive hybridization and screening of opsonic antibody (225) and genetic analysis (186). The *tpr* genes account for 2% of the *T. pallidum* genome and are divided into three subfamilies: Subfamily I includes TprC, TprD, TprF, and TprI; Subfamily II includes TprE, TprG, and TprJ; and Subfamily III includes TprA, TprB, TprH, TprK, and TprL (225). Furthermore, some members of the Tpr family were identified as potential OMPs in a computer-predicted treponemal OMPeome (217, 225, 226) (all except TprG and H) and shown to possess Potra signal sequences to target them for outer membrane insertion by

Tp0326, the BamA (beta barrel assembly machinery protein A) orthologue (227) (all except Tpr's A, F, G, and H). BamA is predicted to be essential in the biogenesis of the outer membrane by facilitating insertion of beta barrel containing OMPs into the membrane (227). The treponeme orthologue of BamA is also known to induce opsonic antibodies upon immunization and partial protection after challenge (228).

In addition to representing a class of elusive potential outer membrane proteins, sequence variability in the Tpr's can distinguish subspecies and some strains and has been hypothesized to be associated with tissue tropism and differential pathologies amongst the treponematoses (229-232). Giacani *et al*. reported the transcription levels of the *tpr's,* revealing that *tprK* is preferentially transcribed throughout infection (226, 233). Phase variation by alternating the number of G-repeats in promoter regions was demonstrated for subfamily II Tpr's (234) and is suspected for *TprK* due to the observation of differing numbers of A- repeats in its promoter region (235). In 2013 Anand *et al.* showed, using similar methods in both studies, that *T. denticola* MSP and TprC are integral membrane proteins that trimerize to form pores, but that the N-termini are located in the periplasm (236, 237). These data are perplexing considering the earlier demonstration that antibody raised to Subfamily I conserved N-terminus is protective and opsonic, thus suggesting that the N-termini of subfamily I (including TprC) are located on treponeme surfaces and accessible to opsonizing antibody (238). This issue has yet to be resolved.

Treponema pallidum repeat Protein K (TprK)

Many of the Tprs are predicted to be outer membrane proteins, and there is evidence that opsonic antibody is directed against several of them (unpublished). TprK, the most

extensively studied member of the Tpr family, is predicted to be a typical beta-barrel OMP, induces opsonic antibody, and induces significant protection following immunization (225), although one group maintains that the protein is periplasmic (239). Hazlett *et al.* described their inability to demonstrate anti-TprK antibody binding to intact treponemes by indirect immunofluorescence (by gel microdroplet) and minimal accumulation of sequence diversity after challenge of TprK immunization and naïve infection with the Nichols Farmington strain (239). Prior to this report, Centurion *et al.* had shown enhanced opsonophagocytosis with anti-TprK antibodies (225), and subsequently demonstrated the accumulation of *tprK* sequence diversity in varied *T. pallidum* strains during infection and after immune selection (73, 240-243) and protection following homologous challenge (244). Demonstration that the *tprK* sequence varies slowly in the Nichols strain of syphilis (isolated in 1912) compared to other strains, highlights the difficulty in detecting sequence change over the course of short infections (~10 days) compared to Nichols infections of longer duration (slower passage in rabbits) (242). *TprK* is surface exposed and has high sequence variability within and among strains. All sequence variation is confined to 7 discrete variable regions that are flanked by terminal repeats. This sequence variability is seen during human, as well as rabbit, infection (73, 242). Variable region sequences change by gene conversion between donor sequences located elsewhere on the chromosome and the *tprK* expression site (theoretically ranging from a few base pairs to whole donor sites). This segmental gene conversion mechanism is thought to be facilitated by internal repeats, as well as V region-specific terminal repeats, and is capable of yielding millions of possible *tprK* variants (245) (Figure I-3. Model of TprK variation by gene conversion). This system of variation is conserved among *Treponema pallidum* subspecies and related treponemes (246) and if left untreated, these bacteria can all cause chronic disease.

In addition to the sequence analysis data showing variation limited to discrete variable regions, immunological studies add additional support to the *in silico* prediction of TprK's OM location and orientation such that the seven variable regions form exposed loops on the bacterium's surface (Figure I-4). Variants are positively selected during the active immune response (247), and B cell epitopes are confined to variable regions during latent infection, while T cell epitopes are found in the conserved regions of the protein (248). It was shown that antibody raised to recombinant TprK is opsonic and that, when immunized with the N-terminus, rabbits developed highly attenuated lesions and were partially protected from challenge (244). The degree of protection correlates to specific antibody against variable regions (249), further providing functional evidence that the variable regions are the portions of the protein that are exposed to the immune milieu.

Antigenic Variation and Pathogenesis

All of the previous studies suggest a role for antigenic variation of *TprK* in syphilis disease progression by providing a mechanism of immune escape and persistence. (103, 225, 250). Antigenic variation is a mechanism of immune evasion that involves changing the antigenic epitopes of surface exposed proteins to abrogate antibody binding. So while the host is mounting an antibody-mediated immune response to a particular antigen, any bacterial cells expressing an altered antigen are able to avoid opsonophagocytosis-mediated clearance. And again, as the host responds to the new antigen, newly variant bacteria will avoid detection again in a molecular arms race in which the bacteria establish a persistent infection. The waves of spirochetemia and fever in tick-borne relapsing fever are due to antigenic variation of the variable membrane proteins (VMPs) of *Borrelia hermsii* (251), and are reminiscent of the

successive episodes of pathology seen during syphilis infection. Antigenic variation is a common method of pathogenesis employed not only by *T. pallidum*, but also by *Neisseria gonorrhoeae* (252, 253), *Borrelia burgdorferi* (254), *Mycoplasma genitalium* (255) and *Anaplasma marginale* (256, 257). In each of these systems of antigenic variation, sequences from pseudogenes are recombined into an expression site (*pilE*, *vlsE*, *mgpB* and *C*, and *msp2* respectively). For *T. pallidum,* the equivalent pseudogenes for variation of the *tprK* expression site are called “donor sites” and are located at the 3’ and 5’ flanking regions of the *tprD* gene.

Syphilis exhibits complex pathogenesis by appearing in multiple successive clinical stages. If treponemes can be cleared from primary lesions after the development of a robust humoral and cellular immune response, how are treponemes able to survive opsonophagocytosis and cause a clinically evident secondary stage of disease? There is ample evidence that TprK undergoes sequence changes during infection and Giacani *et al.* showed that the host immune response selects for *tprK* variants, implying that *TprK* variation is important for immune evasion (247). Elucidating the relationship between antigenic variation and the natural history of syphilis will shed light on the importance of *tprK* in persistence and success as a pathogen (258). Specifically, by investigating the diversification of TprK in the inoculum and secondary lesions following infection, Chapter 2 will show that antigenic variation is directly relevant to the progression of syphilis infection in its host.

In addition to being able to evade an active host immune response, the syphilis treponeme is able to overcome existing immunity during repeat-infections. None of the published studies were carried out with the benefit of modern molecular biology to identify the treponemal determinants responsible for repeated infection. *Anaplasma marginale*, a gram negative bacterium that is thought to vary an important surface antigen MSP2 by a gene conversion mechanism, is able to establish superinfections only if the second infecting strain

has a distinct pseudogene repertoire compared to the initial infecting strain (257, 259). TprK in *T. pallidum* is thought to undergo variation by a similar gene conversion mechanism in which sequences located in donor sites (analogous to the pseudogenes in *A. marginale*) are incorporated into variable regions in the expression site (Figure I-3). Multiple donor cassettes exist for each variable region and combine to form new mosaic expressed variable regions. Differences in the number of donor sites alter a strain's TprK repertoire and, presumably, immune escape potential. The role of TprK's donor site repertoire as a molecular determinant of superinfection will be explored in Chapter 3 along with the role of antigenic variation of *tprK* in repeated infections similar to antigenic variation of *pilE* of Neisseria (260-262), allowing new variants to infect an individual with immunologic experience with that organism.

An understanding of the R_o for syphilis is important for understanding syphilis epidemiology and the success of *T. pallidum* as a pathogen. An association between TprK variation and *T. pallidum's* ability to persist and spread in a population will be discussed in the following chapters. Antigenic variation during infection allows persistence by facilitating immune evasion similar to that seen in tick-borne relapsing fever. The ability to re-infect hosts is also facilitated by antigenic variation, and exemplified by *pilE*'s role in gonorrhea and MSP2's role in *A. marginale* reinfections. By increasing the time in which an infected person is infectious and increasing the ability to cause new infections in partially-immune individuals, TprK increases the R_o of syphilis, leading to higher levels of endemicity and contributing to disease outbreaks.

Figures

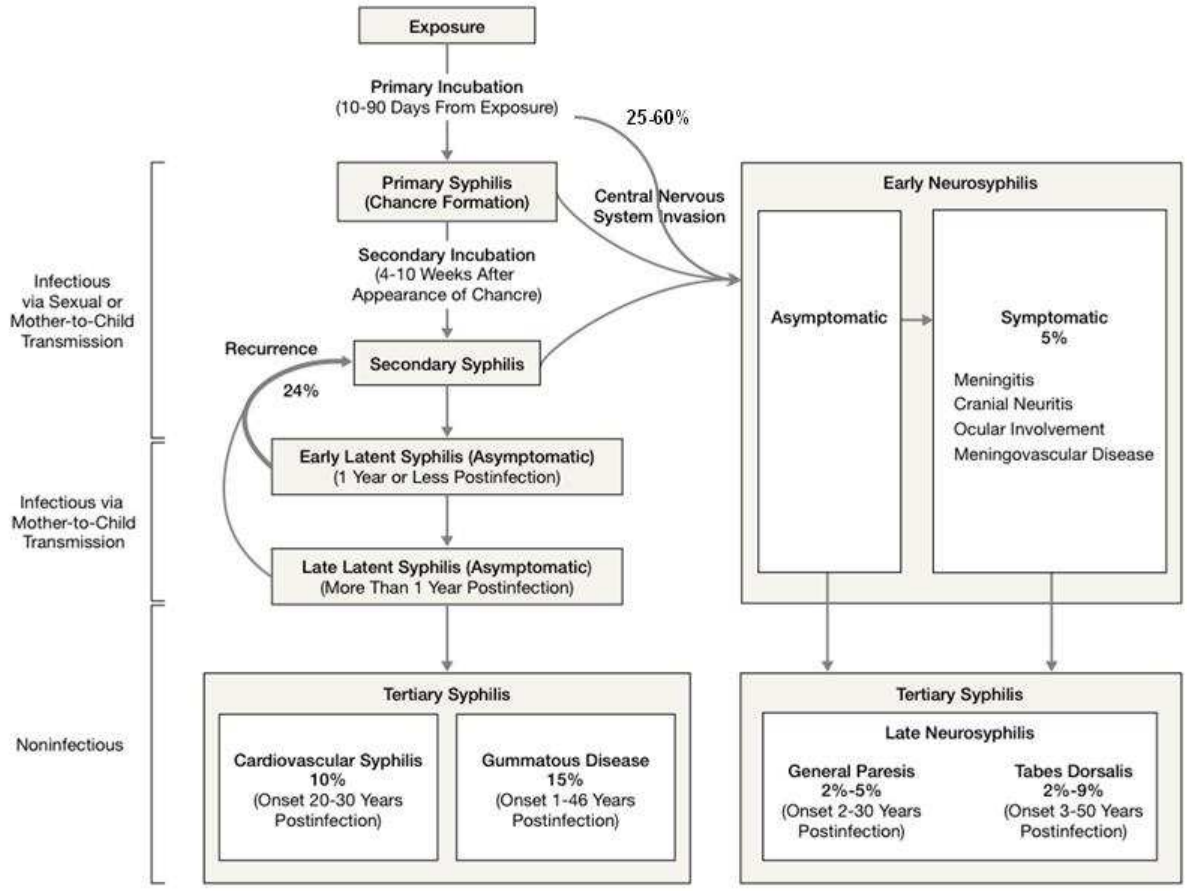

Figure I-1. Syphilis natural history

Summary of the natural history of syphilis infection modified from Golden *et al.* (17). Based on information from references (72, 74, 263).

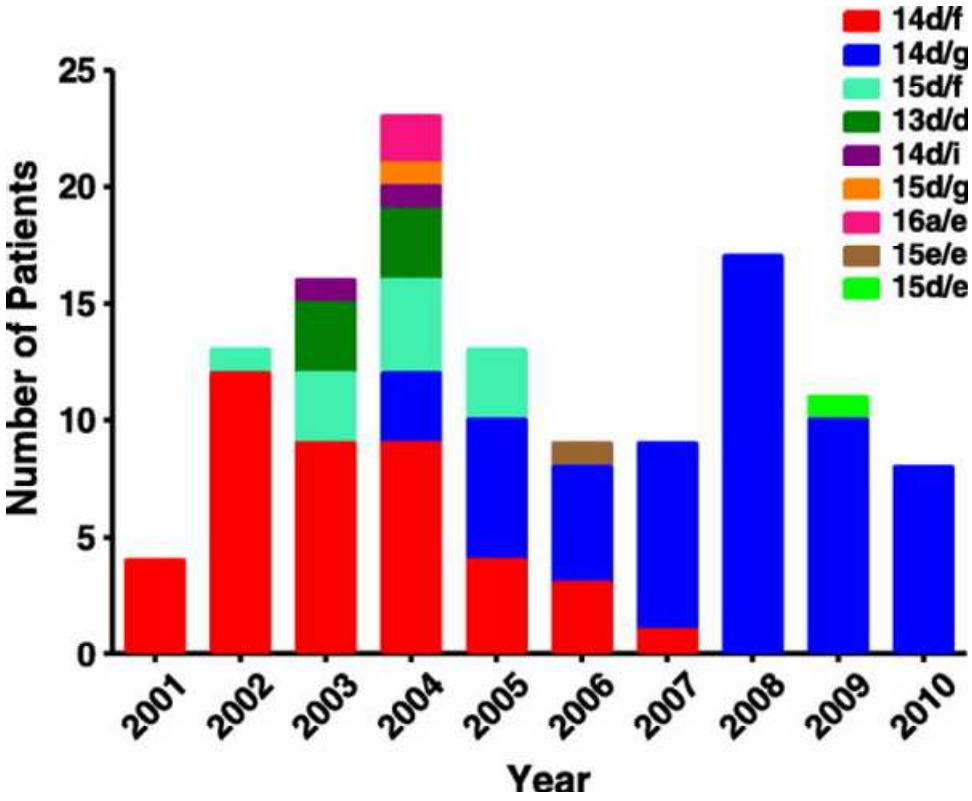

Figure I-2. Prevalence of strain types in Seattle

Utilizing a newly described typing mechanism, Grimes *et al.* and Marra *et al.* described the introduction of new strains into a community (28, 29). Note the decreasing prevalence of the 14d/f type (red) and replacement with the 14d/g type (dark blue).

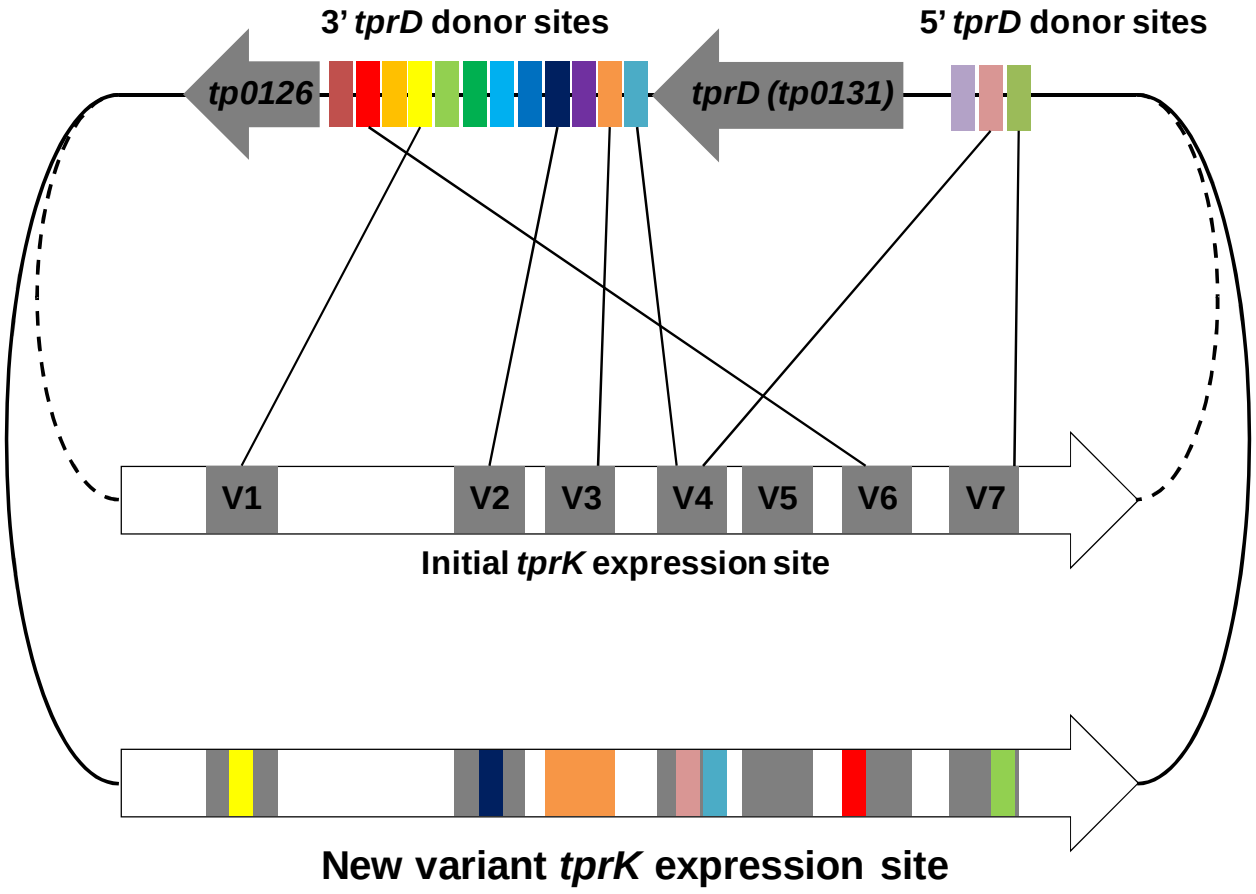

Figure I-3. Model of TprK variation by gene conversion

In this illustration of tprK gene conversion, there are seven variable regions in which portions of the original sequence can be replaced by segments of multiple donor sites located on the bacterial chromosome. Alternatively, there are variable regions that may be replaced in their entirety by a new donor site (V3). This mosaic pattern of gene conversion across seven variable regions has the potential to produce millions of new TprK antigens. The depiction of genes is not to scale.

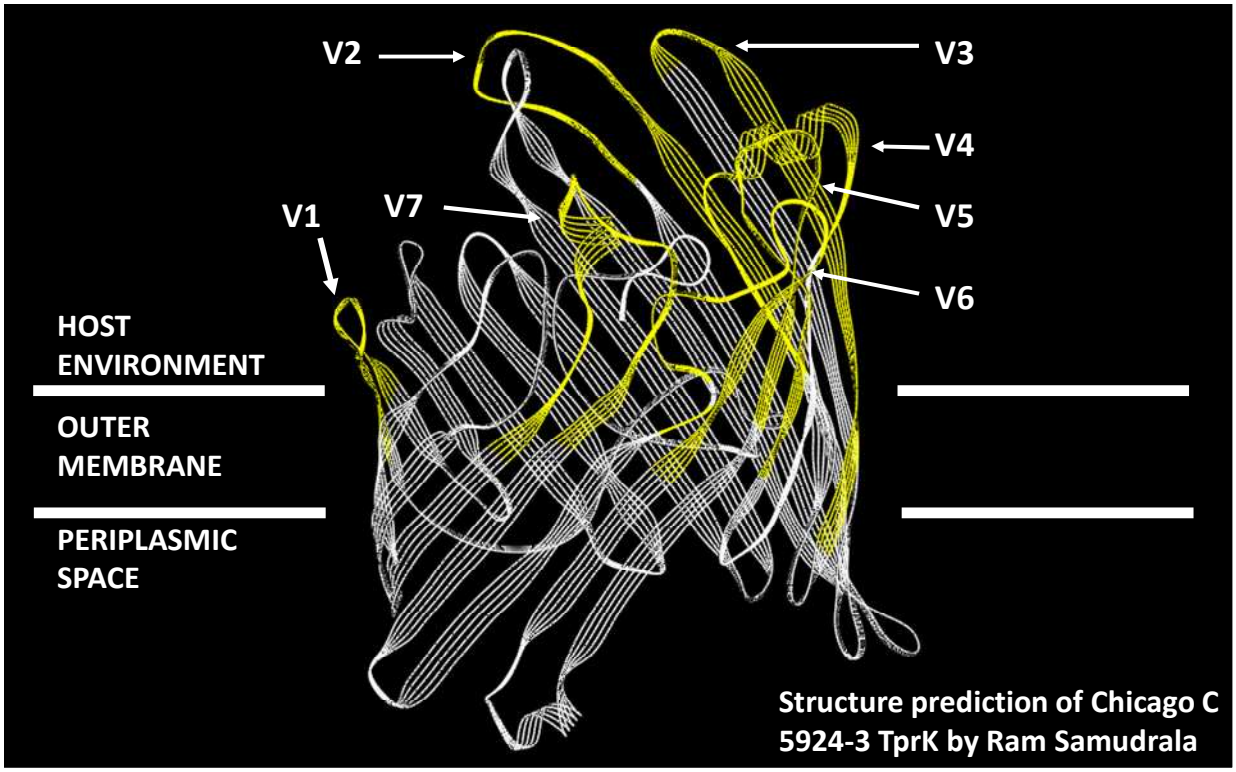

Figure I-4. Predicted structure of TprK

Predicted TprK structure and orientation in the *T. pallidum* outer membrane. TprK is predicted to form a beta barrel structure (common among OMPs) and is oriented such that the variable regions form loops exposed to the host environment.

II. Antigenic Variation of TprK Facilitates Development of Secondary Syphilis

Abstract

Although primary syphilis lesions heal spontaneously, the infection is chronic with subsequent clinical stages. Healing of the primary chancre occurs as antibodies against outer membrane antigens facilitate opsonophagocytosis of the bacteria by activated macrophages. TprK is an outer membrane protein that undergoes antigenic variation at seven variable regions, and variants are selected by immune pressure. We hypothesized that individual TprK variants escape immune clearance and seed new disseminated lesions to cause secondary syphilis. As in human syphilis, infected rabbits may develop disseminated secondary skin lesions. This study explores the nature of secondary syphilis, specifically the contribution of antigenic variation to development of secondary lesions. Our data from the rabbit model show that secondary lesions are significantly more likely to have been seeded by *tprK* escape variants, compared to disseminated lesions that arise after primary intravenous infection [27/43 (63%) vs. 17/50 (34%); p= 0.007)] and that 96% of TprK variant secondary lesions are seeded by single treponemes. Analysis of antibody responses demonstrates high antibody titers to *tprK* variable region sequences found in the inoculum, but a lack of reactivity to *tprK* variant sequences found in newly arising secondary lesions. This suggests that *tprK* variants are escaping the initial immune response raised against the V regions expressed in the inoculum. These data further support a role for TprK in immune evasion, and suggest that the ability of TprK variants to persist despite a robust immune response is instrumental in the development of later stages of syphilis.

Introduction

Syphilis, caused by *Treponema pallidum,* is a complex disease that manifests as multiple clinical stages. Infection begins with development of a primary chancre at the site of inoculation. The primary chancre heals as opsonic antibodies against surface-exposed antigens facilitate opsonophagocytosis and clearance by activated macrophages (130). Despite the presence of circulating antibodies that are functionally opsonic (127, 131, 225, 228, 236, 264), the second stage of syphilis appears as disseminated treponemes multiply and establish new infectious lesions throughout the body. These new lesions may develop as a macular or papular rash on the skin, as well as mucosal lesions. 90% of untreated patients develop secondary syphilis and 22.5% of these have recurrent secondary lesions, that is, multiple successive bouts of this disseminated infectious rash (72). The infected individual will then enter latency and may, after years, develop tertiary manifestations (72).

The development of secondary syphilis is puzzling because the immune response has already been quite effective in healing the primary chancre, and high titers of circulating antibodies are present (265). This pattern of successive episodes of clinical disease during infection is reminiscent of other diseases in which antigenic variation of the infectious agent accounts for repeated cycles of pathology. For example, the waves of spirochetemia and fever in tick-borne relapsing fever are due to antigenic variation of the variable membrane proteins (VMPs) of *Borrelia hermsii* (251). Antigenic variation of outer membrane antigens is a hallmark of many chronic multi-stage infectious diseases including Lyme disease [vmp-like sequence E (VlsE) in *Borrelia burgdorferi*] (254), anaplasmosis [major surface protein 2 (MSP2) in *Anaplasma marginale*] (256), and trypanosomiasis [variant surface glycoprotein (VSG) in *Trypanosoma brucei*] (266). We sought to elucidate the mechanisms by which *Treponema pallidum* are able to evade the early immune response to cause the second stage of syphilis.

Antigenic variation of TprK is hypothesized to be central to *T. pallidum's* ability to escape antibody binding and opsonophagocytosis, thus permitting it to persist in the host (247, 248). Multiple lines of evidence support this hypothesis. TprK is predicted to be located in the outer membrane and oriented such that each of its 7 variable (V) regions is exposed and accessible to host antibodies (267). Sequence variation is restricted to these 7 variable regions (243, 248), and variation occurs by segmental gene conversion, with multiple donor sites located distant from the single *tprK* expression site (245). Infection–induced antibodies are focused against V regions where sequence variation abrogates binding of antibodies raised to different V regions (243). Lastly, selection of TprK variants is dependent on an intact acquired immune response, as shown in the rabbit model (247).

Treponema pallidum organisms are routinely passaged and studied in the rabbit model, which closely recapitulates the disease in humans. During or following resolution of primary chancres in rabbits (i.e. after intradermal [ID] inoculation), generalized secondary syphilis may appear in infected rabbits, and lesions are readily visible on the skin if the fur is kept clipped (102, 268). These lesions are considered to be true secondary lesions because they appear in the face of an effective immune response, just as in human infection. Rabbits may also develop disseminated skin lesions as their initial clinical manifestation after intravenous (IV) infection (201). In this case, the initial immune response is just developing as these disseminated primary lesions appear. Because *T. pallidum* cannot be grown *in vitro*, we utilize disseminated primary infection in the rabbit model to "clone" *T. pallidum*: that is, to obtain isogenic strains of *T. pallidum* with near perfect identity at the *tprK* locus (269), suggesting that each disseminated primary lesion is seeded by a single treponemal cell. Such isogenic strains have allowed us to monitor development of antigenic variation and immune selection of variants during the course of infection (245).

In this study, we sought to address the questions of 1) whether secondary lesions are seeded by single treponemes and 2) how treponemes are able to persist to cause the second stage of syphilis despite the presence of an immune response that actively clears treponemes from the primary chancre. We tested our hypothesis that, as treponemes are cleared from the healing primary lesion, single escape variants with unique *tprK* sequences seed skin sites leading to the new disseminated lesions of secondary syphilis. Herein we demonstrate that treponemes expressing variant TprK escape the immune response and seed new secondary lesions.

Methods

Animal experiments

Male New Zealand white rabbits were used for propagating strains and for the experiments described in this study. Animals were fed an antibiotic-free diet and housed at 16-18℃. All protocols involving animals were approved in advance by the University of Washington Institutional Animal Care and Use Committee.

Chicago C strain isolation

The Chicago strain of *T. pallidum* was isolated from a chancre in 1951 (102). As described previously by us, the Chicago C isolate was obtained after IV inoculation of a naïve rabbit and harvesting of the resulting disseminated skin lesions (269) and has been used extensively in studies of TprK antigenic variation (243, 245-247, 249, 270). Because the Chicago C strain is known to have a high rate of *tprK* variation, special care was taken to ensure each inoculum was as isogenic at *tprK* as possible. In this study, prior to both ID and IV infections, the Chicago

C isolate was re-isolated as described previously (243, 269) with the following modification. After taking biopsies of discrete skin lesions and mincing the tissue in 500 µl of sterile normal rabbit serum (NRS), the serum/treponeme suspension was removed from the residual skin material, gently mixed with 500 µl of sterile glycerol, and then immediately frozen in a dry ice-ethanol slurry to maintain the viability of the bacteria. The remaining material from the same skin biopsy was homogenized in lysis buffer [10 mM Tris (pH 8), 0.1 M EDTA, 0.5% SDS] and also frozen in a dry ice-ethanol slurry.

Subsequently, DNA was extracted from the lysis buffer sample and *tprK* sequences from multiple lesions were analyzed for isogenicity at *tprK*. After identifying lesions that appeared to have the most homogenous *tprK* sequences, the corresponding viable treponemes frozen in glycerol were recovered by adding 1 ml of sterile NRS to thaw the sample, and injecting the mixture intratesticularly (IT) into a naïve rabbit. At peak orchitis (3-4 weeks post infection), the isogenic population was expanded once by passaging in naïve animals (1-2 weeks). Because some *tprK* sequence variation can occur during these propagation steps, we retained a sample of the experimental inocula so that sequence variation that occurred during propagation could be analyzed.

Experimental infection

Intradermal inoculation to cause the primary stage of syphilis and development of secondary syphilis

Rabbits were infected intradermally (ID) with 0.1 ml of a 10^7 treponeme/ml suspension at 10 marked sites along the rabbit's back. The rabbits' backs were meticulously kept free of fur by daily clipping throughout the experiment. As primary lesions appeared, progressed, and healed, measurements of erythema, induration, and ulceration were noted. When secondary lesions

began to develop, they were biopsied as quickly as possible after appearance to prevent development of additional *tprK* variation within the lesion. In selecting lesions for biopsy, a distinction was made between true secondary lesions and satellite lesions. The latter develop in an annular pattern at the leading edge of primary lesions; true secondary lesions develop as diffuse single macular, then papular, lesions at least one centimeter from the edge of a primary lesion. At this early stage of development of secondary lesions, the full lesion was easily encompassed by a 4mm diameter biopsy punch. Animals were monitored for lesion development for 100 days.

Intravenous inoculation to obtain disseminated primary skin lesions

For comparison with secondary lesions, we also examined disseminated primary lesions. Rabbits were inoculated with 10^8 total treponemes intravenously into the marginal ear vein, under general anesthesia. Again, fur on the animals' backs was clipped daily to prevent hair accumulation. As above for secondary lesions, disseminated primary lesions were biopsied as quickly as possible after they appeared (~4 weeks post infection). Unlike secondary lesions, large numbers of disseminated primary lesions developed nearly simultaneously in a given rabbit, so the animals were euthanized to permit biopsy of a large number of lesions.

Biopsy

Full skin thickness 4mm biopsies were taken, after euthanasia or with local lidocaine anesthesia, using sterile biopsy punches. Each biopsy sample was transferred to a sterile dish and minced thoroughly utilizing sterile forceps and scalpel before homogenizing in a solution of 400 µl of lysis buffer. The entire 400 µl of the biopsy preparation was first incubated with proteinase K (100mg/ml) for 8-16 hours at 56℃, the n DNA was isolated using the QIAamp DNA

Mini Kit (Qiagen, Chatsworth, CA), with the addition of a second wash step with 500 µl of AW1 solution. DNA was eluted in 200 µl of nuclease free water.

DNA analysis

TprK sequences in secondary and disseminated primary lesions were analyzed with a two-pronged approach. First, single variable region sequences were analyzed by Fragment Length Analysis (FLA) to determine the relative proportions of size-variants within a sample (247). FLA is a very sensitive measure of V region sequences that differ in size, but cannot distinguish V region amplicons that are the same size but different sequence. Secondly, *tprK* was amplified directly from lesions and the resulting amplicons were sequenced. The addition of amplicon sequence analysis addresses the limitations of FLA mentioned above.

From DNA samples, *tprK* was amplified using primers that encompass the entire open reading frame (Table II-1) as previously described (267). *tprK* amplicons were then sequenced directly. Products of a second *tprK* amplification from both inocula (ID and IV) and from DNA extracted from secondary lesions were cloned into a sequencing vector (Invitrogen, Grand Island, NY) and 10 *tprK* clones containing inserts were sequenced per sample as previously described (241). Sanger sequencing was performed at the UW Biochemistry Core Facility (http://dnaseq.bchem.washington.edu/bdsf/) or GeneWiz (South Plainfield, NJ). Sequences obtained were analyzed using BioEdit software (www.mbio.ncsu.edu/BioEdit/bioedit.html). GenBank accession numbers are listed in Table II-3.

Individual V regions were amplified for FLA, as previously described (247) with the following modification: PIG-tailed primers (listed in Table II-1) were used to nullify Taq adenosine-addition artifacts (271). Because of low DNA concentrations in a few lesions, a

nested PCR was used to obtain some FLA data; this nesting step did not alter the FLA size ratio values compared to non-nested samples (data not shown).

Quantitative real-time PCR (qPCR) was used to probe the inoculum for specific V region sequences to determine whether specific V region sequences found in secondary lesions had also been present in low quantity in the inoculum. Primers and probes (Table II-1) for individual V regions were designed using Primer3Plus (http://www.primer3plus.com) and PrimerExpress (Applied Biosystems Grand Island, NY). Probes were 5'FAM-labeled and quenched with 3'TAMRA (Eurofins, Huntsville, AL). Briefly, HPLC purified primers and probes were used at 0.4uM and 0.2uM concentrations, respectively, with 2.5 µl of template DNA and 2x Taqman Fast Advance (Applied Biosystems, Grand Island, NY) in a 10 µl reaction. Reactions were performed in triplicate and analyzed on a Viia7 Real-time PCR system (Applied Biosystems), with the following cycling conditions: two hold cycles each at 50℃ for 2 min and 95℃ for 20 sec; then 50 cycles of 95℃ for 1 sec and 62℃ for 20 sec. P CR reactions for V4 were run with an annealing temperature of 64℃ instead of 62℃. Eac h primer/probe set was assessed for its specificity to the corresponding target DNA sequence (data not shown). Copy numbers were extrapolated from standard curves constructed using 10-fold serial dilutions of plasmids containing full-length *tprK* sequences from lesion and inoculum sequences. A primer/probe set complementary to the conserved region between V1 and V2 was used as a positive control (data not shown). The proportion of any variant sequences found in the inoculum was calculated as copies of variant 1 or 2 divided by total *tprK* copies detected, with (variant 1+variant 2 + the inoculum sequence = total *tprK* copies detected).

Antibody reactivity

After initial infection, sera were collected weekly for ten weeks from each rabbit, and stored at -80℃ until tested. Prior to use, serum samples wer e heat-inactivated at 56℃ for 30 minutes. Synthetic peptides, designed to reflect individual V region sequences found in the inoculum and in variant *T. pallidum* from secondary lesions (Table II-2), were produced to >70% purity (GenScript Corporation, Piscataway, NJ) and used as antigens. Sera were tested at a 1/20 dilution for their ability to bind V region peptides by ELISA as previously described (243, 248) with the following modification: washing steps (4x30 second washes with 350 µl PBS 0.05% Tween 20) were carried out on a MultiWash Advantage plate washer (TriContinent, Grass Valley, CA). Each sample was tested in triplicate.

Phylogenetic analysis

DNA alignments were constructed using ClustalW and adjusted manually in BioEdit software (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). Phylip version 3.695 (http://evolution.genetics.washington.edu/phylip.html) was used to construct bootstrapped (100 x) maximum parsimony trees. Consensus trees were visualized with Figtree (http://tree.bio.ed.ac.uk/software/figtree/).

Statistical Analysis

The Fisher two-tailed test was used to determine significance of the comparison of the proportion of *tprK* variants in secondary lesions vs. disseminated primary lesions. $P<0.05$ was considered significant. Bootstrap support of >70/100 was considered significant for the phylogenetic analyses (272).

Results

Rabbits can develop disseminated skin lesions after development and healing of primary ID infection (secondary lesions) and after initial IV infection (disseminated primary lesions). Secondary lesions arise in the presence of an active immune response, while disseminated skin lesions that develop after IV infection are in fact disseminated primary lesions that form before and during the onset of the early immune response. We sought to understand how secondary lesions arise during infection and examined the role of antigenic variation of TprK in this process.

Secondary lesions are seeded by single treponemes

In order to obtain isogenic "clones" *of Treponema pallidum* for these studies, we collected individual disseminated skin lesions from IV infected animals. These cloning experiments led us to conclude that each disseminated skin lesion is seeded by a single treponeme because each lesion contains a nearly identical *tprK* sequence. We sought to determine how secondary lesions arise: whether they are an expansion of a diverse collection of treponemes, or clonal expansion of a single treponemal cell. To address this question, we sequenced 10 clones of the *tprK* amplicon from each disseminated secondary skin lesion. All sequence changes preserved the *tprK* expression site open reading frame; there were no early terminations or changes in reading frame. Examples of V7 sequences are shown in Figure II-1. Insertions, deletions and non-synonymous substitutions distinguish each of the 4 variants shown in the figure from the inoculum sequences. Additionally, there is visually obvious "blocking" of sequence changes contained in each secondary lesion.

We scored a lesion as having arisen from a single *tprK* variant treponeme if ≥70% of its sequenced clones were identical and different from the inoculum in at least one V region. Because our inoculum was nearly homogeneous in sequence at *tprK*, we cannot determine the number of treponemes that may have seeded lesions containing primarily inoculum sequences. The 70% cutoff was chosen because, as *T. pallidum* divides, some sequence change can occur. By the time a lesion becomes clinically evident, many divisions have occurred and some V region change is inevitable, even without immune selection. Using this definition, we found that 96% (26/27) of *tprK* variant secondary lesions were seeded by single treponemes. These data support our previous hypothesis that the vast majority of disseminated skin lesions in our rabbit model of secondary syphilis are indeed seeded by single treponemes.

As a more stringent assessment, we used the data obtained from sequencing clones from these lesions to construct maximum parsimony phylogenetic trees of the full-length *tprK* ORFs. For all 27 *tprK*-variant secondary lesions studied, representing 8 rabbits, we found a clustering pattern in which the majority of sequences from a single lesion cluster tightly together and each lesion is separate from the others. A representative example of 4 lesions arising in rabbit 7901 is shown in Figure II-2, demonstrating tight clustering of sequences within each secondary lesion; these lesions are separate from each other, with significant bootstrap support. These data further support our hypothesis that secondary lesions are seeded by single treponemes.

TprK variants cause secondary lesions

In addition to demonstrating the tight clustering of *tprK* clone sequences, Figure II-2 also exemplifies the dispersion of variant sequence clusters away from the cluster of inoculum sequences. This pattern of branching is evidence of new secondary lesions being founded by

tprK variant treponemes during infection. If *tprK* sequence variation abrogates antibody binding to variant bacterial cells during the primary stage of syphilis, then we expect that TprK variant treponemes would escape killing by the host immune response and could seed new lesions (i.e. the secondary stage of syphilis).

We hypothesized that the treponemes multiplying in secondary lesions are doing so in the presence of an active immune response that is raised against the antigens expressed by the inoculum and is responsible for healing the primary lesions. To compare the proportion of disseminated lesions containing variant *tprK* treponemes vs. inoculum-like sequences in the presence and absence of an effective immune response, we performed a similar analysis in disseminated primary lesions (developing after IV infection) and compared the data to those obtained from secondary lesions. Lesions with *tprK* variants were defined as those in which, by FLA, at least one V region contains fewer than 30% inoculum sequence. Sequences obtained by direct amplicon sequencing were used to identify variant sequences when FLA sizes were the same as those found in the inoculum. Careful analysis, by individual V region, of the secondary and disseminated primary lesions revealed the proportions of lesions arising from variant treponemes; these are shown by rabbit, in Figure II-3A and II-3B, respectively. Of the ten rabbits in the secondary syphilis study, two animals did not develop any secondary lesions, and one animal (rabbit 7909) produced a very large number of secondary lesions. The number of secondary lesions per rabbit varied from 0-24 (median=3; mean= 4.3), and they appeared 17 to 73 days post infection (median=39; mean= 40.5). In contrast, the disseminated primary lesions appeared on all rabbits between days 27 and 31 post infection, and totaled more than 100 lesions on 5 rabbits. In rabbit 7909, which had an unusually high number of secondary lesions, only 42% (10/24) of lesions carried variant sequences. In contrast, in the 7 remaining rabbits that developed secondary lesions, 89% (17/19; p=0.0016) of lesions contained only

variant sequences. In 5 of these 7 rabbits, all secondary lesions that developed contained variant sequences. Overall, as defined by FLA and amplicon sequence analysis, *tprK* variants seeded 63% (27/43) of all secondary lesions, compared to 34% (17/50; p= 0.007) of disseminated primary lesions (Figure II-3C).

tprK variants found in secondary lesions develop during infection

tprK variants were defined in this study as those sequences that were not found in our initial sequence analysis (full ORF sequencing of 10 clones) of the inoculum. Our initial sequencing did reveal some minor populations with different V regions in the inoculum, and this information was considered when identifying and calculating the contribution of variants to secondary lesions above. It is quite possible, however, that there were very small populations with different *tprK* sequences that went undetected by our initial sequence analysis, and that some of the sequences that we found in secondary lesions may in fact have been present in the initial inoculum. It would be likely that any minority sequences present in the inoculum might be seen in lesions from multiple rabbits. To examine this possibility, we chose to investigate our secondary lesion inoculum by PCR for V region sequences that had been found in secondary lesions from multiple animals.

To interrogate our inoculum, we performed a targeted PCR-based search for 7 individual V3, V4, V5, V6 and V7 sequences (no variants were found at V1 and only single lesions contained V2 variants). DNA extracted from the inoculum was probed with specific Taqman probes that were complementary to the *tprK* variant as well as known inoculum sequences. None of the tested variants for V4, V5, V6, and V7 were detectable in the inoculum. In contrast, two V3 variants that had been seen in secondary lesions from multiple rabbits were found in the inoculum, and were estimated to be present in the inoculum at 8.17% and 1.29% of *tprK* copies

detected. These two V3 variants (different from the inoculum only at V3) were solely responsible for identifying 15% (4/27) of secondary lesions as *tprK* variant lesions and we acknowledge that they came from the inoculum. Even though these minor variants were present in the inoculum, we examined whether they may have survived to cause secondary lesions because they were able to escape immune detection.

TprK variants escape humoral immunity

Giacani *et al.* demonstrated that, in the face of an active immune response, treponemes expressing variant TprK sequences are positively selected (247). To test whether new TprK variants have a selective advantage over the inoculum treponemes in our study, we examined whether antibodies recognizing the secondary lesion variant sequences were present in rabbits at the time that secondary lesions developed. This was accomplished by ELISA using synthetic peptides based on inoculum and *tprK* variant sequences. High levels of antibody were detected against the V2, V4, V5, V6, and V7 peptide sequences of the majority population contained in the inoculum in all 10 rabbits in the secondary syphilis group; a representative example is shown in Figure II-4A, blue bars. In contrast, those same sera collected at the time that secondary lesions arose, had little to no detectable antibody reactivity to the minority populations that were present in the inoculum (also blue bars) or to the variant V2, V4, V6, and V7 sequences found in those secondary lesions (Figure II-4A, red and yellow bars). Similar findings were seen for all 10 rabbits in the secondary syphilis study. These data suggest that antibodies to V2, V4, V6, and V7 select against the inoculum majority treponemes by facilitating bacterial clearance from primary lesions. TprK variants, even those present as minority populations in the inoculum, can escape clearance because there is no antibody against the specific TprK variant protein being expressed. These organisms subsequently evade the immune response and are able to cause secondary lesions.

In the rabbits infected IV to establish disseminated primary lesions, little to no antibody was detectable, even to majority inoculum TprK V region sequences, at the time that disseminated lesions developed (Figure II-4B). These findings are consistent with the concept that treponemes can survive to cause lesions when specific anti-V region antibodies are not present.

Discussion

There are no known non-human reservoirs for venereal syphilis, making this disease a potential candidate for vaccine eradication, but infection-induced immunity is not complete (59) and reinfections occur (273). Efforts to understand the treponeme-host interface have focused on characterizing outer membrane proteins (274) of *T. pallidum*. This is especially difficult considering the treponemes cannot be cultured *in vitro*, and *in vitro* manipulations of the spirochete easily damage the fragile outer membrane. It is known that, during infection, a small population of treponemes develop that are resistant to opsonization and thus escape immune clearance, presumably due to alterations in their outer membrane proteins (265). Clinically this makes sense as, despite a robust immune response that clears most treponemes and heals primary chancres, later episodes of infectious skin lesions (secondary syphilis) and chronic infection occur.

To understand *T. pallidum's* ability to evade opsonophagocytosis, an understanding of the spirochete's reported "paucity of outer membrane proteins" is needed (213). Few *T. pallidum* candidate OMPs have been studied in detail (113, 227, 236, 264, 275-277), but included among these are the Tpr's, specifically TprK. TprK is the only *T. pallidum* antigen known to undergo sequence variation during the course of infection (242, 243, 247), and is

hypothesized to be central to immune escape and persistence (267). Sequence variation is confined to 7 discrete variable regions and occurs by a gene conversion mechanism (225, 245). The TprK variation mechanism is found not only in the syphilis subspecies (*pallidum*), but also in the other subspecies of *Treponema pallidum (endemicum, pertenue*, and the Fribourg-Blanc isolate) and the agent of rabbit syphilis *Treponema paraluiscuniculi (246)*. All of these treponemes can cause chronic infection and have intact TprK variation and expression systems, with V region donor sites (246). Additionally, *in silico* modeling predicts a beta barrel structure for TprK that places each variable region on exposed external loops, where antibody binding may drive selection of new variants. Indeed, TprK variants are selected by the acquired immune response (247), and B cell epitopes are restricted to the variable regions (248) during persistent infection. Although *tprK* variation itself has been well described, its relationship to the discrete clinical stages of syphilis had not yet been demonstrated. This report demonstrates an association between *tprK* variation and syphilis disease progression from the primary to the secondary stage in the rabbit model.

In our study of secondary syphilis, there was some clinical variability among the rabbits. Eight of the 10 rabbits infected intradermally subsequently developed secondary lesions during the 100 day observation period in our studies. Seven of these 8 rabbits developed a small number of lesions (1 to 4), and the majority of these lesions (17/19; 89%) were seeded by single TprK variants. The two animals that failed to develop secondary lesions had comparable primary lesion progression, as well as typical serological responses as measured by VDRL titers, and ELISA reactivity to the conserved lipoprotein p47 recombinant antigen (data not shown). Interestingly, these two animals had 2-5 fold greater antibody reactivity to a TprC epitope, when compared to the other 8 rabbits in this group ($p<0.001$, data not shown), which is noteworthy in light of the recent characterization of TprC as an outer membrane protein (236).

Rabbit 7909 was unusual in that he developed many lesions at once, similar to that observed in the rabbits with disseminated primary lesions after IV infection. Further, the ratio of variant TprK sequences to the inoculum sequence in lesions in rabbit 7909 approached the ratio observed after IV infection. We therefore sought, but did not uncover, an explanation for the unusual course in this single rabbit. Antibody titers (e.g. VDRL, anti-V region) for 7909 were not different from the remaining 9 rabbits. There was slight variation in primary lesion progression, including time to ulceration and diameter of lesions among the 10 animals, but 7909 was not an outlier in this regard. Thus, there was no obvious evidence of an inadequate immune response in this rabbit. We were unable to monitor the time to lesion healing in rabbit 7909, however, because he was euthanized early for collection of many lesion biopsies.

In analyzing the sequence data from each secondary lesion, it was apparent that certain *tprK* escape variant sequences (especially for V3) were found in multiple lesions, and in different rabbits. This led us to ask whether these "TprK escape variants" developed *de novo* during the experimental infection, or whether they were represented in the inoculum at a low frequency that was not detected in the initial sequencing of the inoculum (10 clones). Our careful examination of the inoculum by qPCR demonstrates, however, that the vast majority of the *tprK* variants found in secondary lesions developed during infection as opposed to being small subpopulations already present in the inoculum.

In our examination of antibody responses to TprK, strong antibody reactivity was detected in all rabbits to the V2, V4, V6, and V7 sequences expressed by the majority population in the inoculum. Based upon our ELISA data, however, no antibody reactivity was detected to V3 peptides, even to the inoculum V3 sequence. It was therefore puzzling that four secondary lesions had *tprK* sequences differing from the inoculum only at V3, perhaps suggesting that variation at V3 may be sufficient for immune escape. It is possible that V3 may

be part of an important conformational epitope, and that anti-V3 antibodies fail to bind the short linear peptides in our ELISA studies.

Our studies require that we use as an inoculum a *T. pallidum* clone that has nearly identical sequences in the *tprK* expression site so that we can clearly identify variants that develop. We have shown that, despite our efforts to maintain a *tprK*-clonal population, some low-level diversity inevitably accumulates during expansion of the treponemes prior to inoculation. Examples of this diversity are found at V3 (minority populations detected by qPCR), as well as at V5 and V6 (minority populations detectable by sequencing 10 clones). In our studies, we demonstrate that a negligible antibody response develops to minority TprK sequences expressed in the inoculum. LaFond *et al.* showed that, following infection with a *T. pallidum* suspension that contains diverse *tprK* sequences, antibodies are developed to TprK V regions sequences from multiple inoculum subpopulations, but the frequencies of these minority populations in the inoculum were not provided (243). From our data, it appears that minority populations that account for less than 10% of the inoculum (calculated from qPCR data or 1/10 inoculum clones) do not elicit antibodies to their V regions (Figure II-4). This suggests that the antibodies formed to the majority population in the inoculum (and not the infrequent minority population), positively select for the minority inoculum populations as well as new variants that develop during infection.

In natural human infection, people are infected with populations of treponemes that are heterogeneous at *tprK* (241). This higher TprK diversity of the inoculum in human infections is important in pathogenesis of syphilis because a diverse inoculum with multiple minority populations may serve as a source of scattered and early disseminating *tprK* variants (e.g. to the central nervous system and other organs) that are not recognized by the antibodies induced by the growth of the majority populations remaining at the site of the developing primary

chancre. Our demonstration that secondary lesions are seeded by immune escape variants, regardless of whether they develop during primary infection or are present as minority populations in the inoculum, supports a critical role for TprK in the pathogenesis of syphilis and in the progression of the infection through multiple clinical stages.

The clinical variability described above for our outbred rabbits replicates the wide range in clinical presentation of syphilis infection in humans. For example, the rash of secondary syphilis is variable in appearance: it may be quite florid in some patients, while symptoms go un-noticed in others (278, 279). In addition to heterogeneity in host responses, part of the variability in human clinical presentation may be associated with *T. pallidum* strain-specific differences and with inoculum size (59, 104), both of which were controlled in our experimental model. We know that some strains of *T. pallidum* vary the sequence in the *tprK* expression site more readily than other strains (242). Such strains might be expected to cause more apparent clinical disease such as, for example, more florid or recurrent secondary lesions, but this has not yet been explored in this the experimental model. It is also possible that treponemes can actively modulate the amount of TprK protein on cell surfaces, potentially eliminating the target of some opsonic antibodies. Strain-specific differences in quantity of *tprK* mRNA may suggest a mechanism of *tprK* transcriptional regulation (246, 280), although there is evidence that *tprK* is expressed throughout the course of primary intradermal infection (247). There are no data on *tprK* expression during the latent stage in which down-regulation of opsonic targets might be most advantageous to the persistent organisms.

During early infection, *T. pallidum* infection elicits a robust humoral and cellular immune response (248) clearing the primary chancre and then a small subpopulation effectively evades host opsonophagocytosis (265). A similar mechanism of clearance and immune evasion likely

occurs during healing of the secondary stage, leaving persistent organisms that may remain quiescent during latency or escape to proliferate at sites of tertiary manifestations.

TprK's clear role in facilitating immune evasion and persistence has important implications for understanding not only the natural history but also the epidemiology of syphilis infection. The basic reproductive rate (R_o) of an infectious disease predicts whether that disease can spread through a population and is directly proportional to the duration of the infectiousness (54, 55). Evading host opsonophagocytosis may greatly increase the duration of the infectious period of syphilis in at least two possible ways: 1) by increasing the duration of the primary stage, as TprK variants locally maintain the chancre as they replace the initial infecting phenotype to which the initial immune response was generated; and 2) by facilitating the development of the widespread infectious lesions of the secondary stage of syphilis and, potentially the recurrence of secondary lesions in some infected persons (72). We propose that TprK antigenic variation increases *Treponema pallidum'*s infectious period by facilitating immune escape and persistence to cause later stages *in vivo*, thus increasing the basic reproductive rate of syphilis in a population and contributing to *T. pallidum*'s success as a pathogen.

Tables and Figures

Table II-1. Oligonucleotides used in Chapter II

Primer	**Sequence**	**Use**[a]
KII1-S	TCCCCCAGTTGCAGCACTAT	1
KIr1-As	TCGCGGTAGTCAACAATACCA	1
M13F-S	CAGGAAACAGCTATGAC	1,2
M13R-As	GTAAAACGACGGCCAG	1,2
9V-S	GATATTGAAGGCTATGCGGAGCTG	2
9V-As	CCTCAAGGAAAGAAGTATCAGG	2
***tprK*internal-S**	TGGCGTGCAGGAATACATTA	2
V1-S	GTGGGCTCAGGTTTCGTTC	3
V1-As	GTTTCTTCGCATAGACATTCCCCTCAC	3
V2-S	GGGGCTCACGTTTGATATTG	3
V2-As	GTTTCTTCCGGTGAGCTCCACTTTAAT	3
V3-S	GAGCGTACGCGTGAAGATG	3
V3-As	GTTTCTTTAGCAGCCAGAGCACACAGA	3
V4-S	CTTTGGGGTCTGTGTGCTCT	3
V4-As	GTTTCTTAACGATACCCCAACGTCAAC	3
V5-S	TTGGGGTATCGTTGGTTCTC	3
V5-As	GTTTCTTCCCAAATCAAGACCCTCAAG	3
V6-S	AAACCAAGGGGTCTGATCCT	3
V6-As	GTTTCTTTAGACGATACGAACCCCAGA	2,3
V7-S	TGGGTGAGTATGGTTGGGTTA	3
V7-As	GTTTCTTGCCGAATCTCCACCTTCTCT	3
qV1-S	CCCGGATATTGAAGGCTATGC	4
qV1-As	CACAATGGGGAACACAATCTTAA	4
qV2-S	TTTGCGTCTAACACCGACTG	4
qV2-As	CCCCCCAATCCATACTTGCT	4
qV3-S	AACTCCACACTGTCTAGCGGCTAT	4

qV3-As	CCCCGACATCCCATAAGATG	4
qV4-S	TGTGTGCTCTGGCTGCTACAG	4
qV4-As	CCAACGTCAACAACGCATCT	4
qV5-S	TTCTCGGCGGGAGGATATTT	4
qV5-As	TCGAGCTTAATATAGGCAGCACAGT	4
qV6-S	GGGTGTTGATGTGCGTACGTA	4
qV6-As	ACTTTTCCATACACCGGGAAGTAG	4
qV7-S	AAACTTGTACGGCGGTACGAA	4
qV7-As	CCCACACTCGTAATACCCACAAT	4
V1-V2cons-S	ACTTTGCTCCACCATTTGACTC	4
V1-V2cons-As	TGTTCCCCATTGTGGCAA	4
V1 inoc[b]	CATATGAAGATGGTAGCGCCGGAAACC	5
V2 inoc[b]	CAAACGGCAACGTCCCAGCAGG	5
V2 7909K[b]	CAAAGACAGTCAGGGCAAGGCCCC	5
V2 7912D[b]	ACAGTCAGGGCAACGTCCCAGCAG	5
V3inoc[b]	CACAGCCCGAGCCGGAGCC	5
V3 7903C[b]	CCCAAGCAGCCGGAGCCG	5
V3 7909W[b]	CCACAGCCCAACCCCCAGCC	5
V4inoc[b]	AATGCAGCGAACGTCAATGGCACC	5
V4 7912c[b]	AAACGGAGCGAACGTCAATGGCAC	5
V5inoc[b]	TTTCTCACCACACCCATGCAGAAGGAC	5
V5 7909Jminor[b]	TATTTCGCATCGAAGGCCAGCAATG	5
V5 7909L[b]	TTTCTCACCGATACCACACCCATGCA	5
V6 inocmajor[b]	AGCCGCAGTGCCAGCCGC	5
V6 7912 C/D[b]	ATGCCCGTCTATTACTTCACCCAAGCCC	5
V7 inoc[b]	AAGCTGCTGCTGCAGTTCCTGCTACG	5
V7 7909K[b]	AGAAAAACGATGCTCCTGCTACGAAGTGGA	5
V7 7909N[b]	AAAAAGGCCACGCCCCCTGCT	5
***tprK*V1-V2**[b]	ACCGCGGTGAGGGGAATGTCTATG	5

[a] 1, PCR Amplification; 2, Sequencing; 3, Fragment Length Analysis; 4, Quantitative Real Time PCR; 5, Probe

[b] Labeled with 5' FAM and 3' TAMRA

Table II-2. Oligopeptides used in Chapter II

Peptide	Sequence	Size
V1[a]	GIAYEDGSAGNLKH	14
V2[a]	WEGKPNGNVPARVTPSKYGLG	21
V3[a]	GYATARAGADILWDVGAKVSM	21
V4[a]	TDVGHKKENAANVNGTVGAD	20
V5[a]	YFASQASNVFQGVFLTTPMQKDDCAAYI	28
V5-2[a]	YFASQASNVFQGVFLTNNMLQHDCAAYI	28
V6[a]	MPVHYKVLKARARAGAAVPAAADDIYFPV	29
V6-2[a]	PVHYKVLKARALPAAAVPAAADDIYFP	27
V6-3[a]	PVHYKVLKAQARARAAVPAAADDIYFP	27
V7[a]	YGGTNKQAAAAVPATKWKAEYCGYY	25
V2[b]	WEGKDSQGNVPAGVTPSKYGL	21
V2-2[b]	WEGKDSQGKAPAGTPSKYGLG	21
V3[b]	GYATAQPPANILWDVGAKVSM	21
V3-2[b]	YAQAAGADILWDVGAKVSMKLWGL	24
V4[b]	TDVGHKKNGANVNGTVGAD	19
V5[b]	YFASQASNVFQGVFLTDTTPMQKDDCAAYI	30
V5-2[b]	YFASKASNVFQGVFLTTPMQKDDCAAYI	28
V6[b]	PVHWKARARAGAAVPAAADDIYFP	24
V6-2[b]	PVHYKVLKARALPGAPVPAIYFP	23
V6-3[b]	PVHYKVLKARARAGAAAGVPALPGAAADD	29
V6-4[b]	PVYYFTQARALPGAPVPAIYFP	22
V7[b]	YGGTNKKAAAAAPAPGTKWKAEYCGYYE	28
V7-2[b]	YGGTNKQAAAAVPATKWSKEYCGYY	25
V7-3[b]	YGGTNKQAAAVPGVVPGAVPATKWKAEYCGYY	32
V7-4[b]	YGGTNKKATPPAAPAAPTKWKAEYCGYY	28
V7-5[b]	YGGTNKKNDAPATEWKAEYCGYY	23

[a] Inoculum Sequence

[b] Variant Sequence

Table II-3. GenBank accession numbers

Rabbit	Sample	PCR	Clones
7888	Inoculum	KM015619	KM015633 KM015634 KM015635 KM015636 KM015637 KM015638 KM015639 KM015640 KM015641 KM015642
7898	C	KM015620	KM015643 KM015644 KM015645 KM015646 KM015647 KM015648 KM015649 KM015650 KM015651 KM015652
	D	*	KM015653 KM015654 KM015655 KM015656 KM015657 KM015658 KM015659 KM015660 KM015661 KM015662
	E	KM015621	KM015663 KM015664 KM015665 KM015666 KM015667 KM015668 KM015669 KM015670 KM015671 KM015672
7901	A	*	KM015703 KM015704 KM015705 KM015706 KM015707 KM015708 KM015709 KM015710 KM015711 KM015712
	C	KM015622	KM015713 KM015714 KM015715 KM015716 KM015717 KM015718 KM015719 KM015720 KM015721 KM015722
	D	KM015623	KM015723 KM015724 KM015725 KM015726 KM015727 KM015728 KM015729 KM015730 KM015731 KM015732
	E	KM015624	KM015733 KM015734 KM015735 KM015736 KM015737 KM015738 KM015739 KM015740 KM015741 KM015742
7902	A	KM015625	KM015673 KM015674 KM015675 KM015676 KM015677 KM015678 KM015679 KM015680 KM015681 KM015682
	B	KM015626	KM015683 KM015684 KM015685 KM015686 KM015687 KM015688 KM015689 KM015690 KM015691 KM015692
	C	KM015627	KM015693 KM015694 KM015695 KM015696 KM015697 KM015698 KM015699 KM015700 KM015701 KM015702
7903	A	KM015628	KM015743 KM015744 KM015745 KM015746 KM015747 KM015748 KM015749 KM015750 KM015751 KM015752
	B	KM015629	KM015753 KM015754 KM015755 KM015756 KM015757 KM015758 KM015759 KM015760 KM015761 KM015762
	C	KM015630	KM015763 KM015764 KM015765 KM015766 KM015767 KM015768 KM015769 KM015770 KM015771 KM015772
7909	B	KM015592	KM015844 KM015845 KM015846 KM015847 KM015848 KM015849 KM015850 KM015851 KM015852 KM015853
	C	KM015593	KM015854 KM015855 KM015856 KM015857 KM015858 KM015859 KM015860 KM015861 KM015862 KM015863
	D	KM015594	KM015864 KM015865 KM015866 KM015867 KM015868 KM015869 KM015870 KM015871 KM015872 KM015873

	E	KM015595	KM015874 KM015875 KM015876 KM015877 KM015878 KM015879 KM015880 KM015881 KM015882 KM015883
	F	KM015596	KM015884 KM015885 KM015886 KM015887 KM015888 KM015889 KM015890 KM015891 KM015892 KM015893
	G	KM015597	KM015894 KM015895 KM015896 KM015897 KM015898 KM015899 KM015900 KM015901 KM015902 KM015903
	H	KM015598	KM015904 KM015905 KM015906 KM015907 KM015908 KM015909 KM015910 KM015911 KM015912 KM015913
	I	KM015599	KM015914 KM015915 KM015916 KM015917 KM015918 KM015919 KM015920 KM015921 KM015922 KM015923
	J	KM015600	KM015924 KM015925 KM015926 KM015927 KM015928 KM015929 KM015930 KM015931 KM015932 KM015933
	K	KM015601	KM015934 KM015935 KM015936 KM015937 KM015938 KM015939 KM015940 KM015941 KM015942 KM015943
	L	KM015602	KM015944 KM015945 KM015946 KM015947 KM015948 KM015949 KM015950 KM015951 KM015952 KM015953
	M	KM015603	KM015954 KM015955 KM015956 KM015957 KM015958 KM015959 KM015960 KM015961 KM015962 KM015963
	N	KM015604	KM015964 KM015965 KM015966 KM015967 KM015968 KM015969 KM015970 KM015971 KM015972 KM015973
	O	KM015605	KM015833 KM015974 KM015975 KM015976 KM015977 KM015978 KM015979 KM015980 KM015981 KM015982
	P	KM015606	KM015983 KM015984 KM015985 KM015986 KM015987 KM015988 KM015989 KM015990 KM015991 KM015992
	Q	KM015607	KM015834 KM015835 KM015836 KM015837 KM015838 KM015839 KM015840 KM015841 KM015842 KM015843
	R	KM015608	KM015993 KM015994 KM015995 KM015996 KM015997 KM015998 KM015999 KM016000 KM016001 KM016002
	S	KM015609	KM016003 KM016004 KM016005 KM016006 KM016007 KM016008 KM016009 KM016010 KM016011 KM016012
	T	KM015610	KM016013 KM016014 KM016015 KM016016 KM016017 KM016018 KM016019 KM016020 KM016021 KM016022
	U	KM015611	KM016023 KM016024 KM016025 KM016026 KM016027 KM016028 KM016029 KM016030 KM016031 KM016032
	V	KM015612	KM016063 KM016064 KM016065 KM016066 KM016067 KM016068 KM016069 KM016070 KM016071 KM016072
	W	KM015613	KM016033 KM016034 KM016035 KM016036 KM016037 KM016038 KM016039 KM016040 KM016041 KM016042

	X	KM015614	KM016043 KM016044 KM016045 KM016046 KM016047 KM016048 KM016049 KM016050 KM016051 KM016052
	Y	KM015615	KM016053 KM016054 KM016055 KM016056 KM016057 KM016058 KM016059 KM016060 KM016061 KM016062
7912	A	KM015616	KM015803 KM015804 KM015805 KM015806 KM015807 KM015808 KM015809 KM015810 KM015811 KM015812
	B	KM015617	KM015793 KM015794 KM015795 KM015796 KM015797 KM015798 KM015799 KM015800 KM015801 KM015802
	C	*	KM015813 KM015814 KM015815 KM015816 KM015817 KM015818 KM015819 KM015820 KM015821 KM015822
	D	KM015618	KM015823 KM015824 KM015825 KM015826 KM015827 KM015828 KM015829 KM015830 KM015831 KM015832
7919	A	KM015631	KM015773 KM015774 KM015775 KM015776 KM015777 KM015778 KM015779 KM015780 KM015781 KM015782
7934	A	KM015632	KM015783 KM015784 KM015785 KM015786 KM015787 KM015788 KM015789 KM015790 KM015791 KM015792
8692	Inoculum	nd	KM015572 KM015573 KM015574 KM015575 KM015576 KM015577 KM015578 KM015579 KM015580 KM015581
8693	Inoculum	nd	KM015582 KM015583 KM015584 KM015585 KM015586 KM015587 KM015588 KM015589 KM015590 KM015591
8694	A	KM015562	
	B	KM015563	
	C	KM015564	
	D	KM015565	
	E	KM015566	
	F	KM015567	
	G	KM015568	
	H	KM015569	
	I	KM015570	
	J	KM015571	
8696	B	KM015552	
	C	KM015553	
	D	KM015554	
	E	KM015555	
	F	KM015556	
	I	KM015557	
	J	KM015558	
	K	KM015559	
	L	KM015560	
	M	KM015561	

8697	A	KM015542	
	B	KM015543	
	C	KM015544	
	D	KM015545	
	E	KM015546	
	F	KM015547	
	G	KM015548	
	H	KM015549	
	I	KM015550	
	J	KM015551	
8699	A	KM015532	
	B	KM015533	
	C	KM015534	
	D	KM015535	
	E	KM015536	
	F	KM015537	
	G	KM015538	
	H	KM015539	
	J	KM015540	
	K	KM015541	
8700	B	KM015522	
	C	KM015523	
	D	KM015524	
	E	KM015525	
	F	KM015526	
	G	KM015527	
	H	KM015528	
	I	KM015529	
	J	KM015530	
	K	KM015531	

*PCR did not yield enough amplicon for direct sequencing

nd=not done

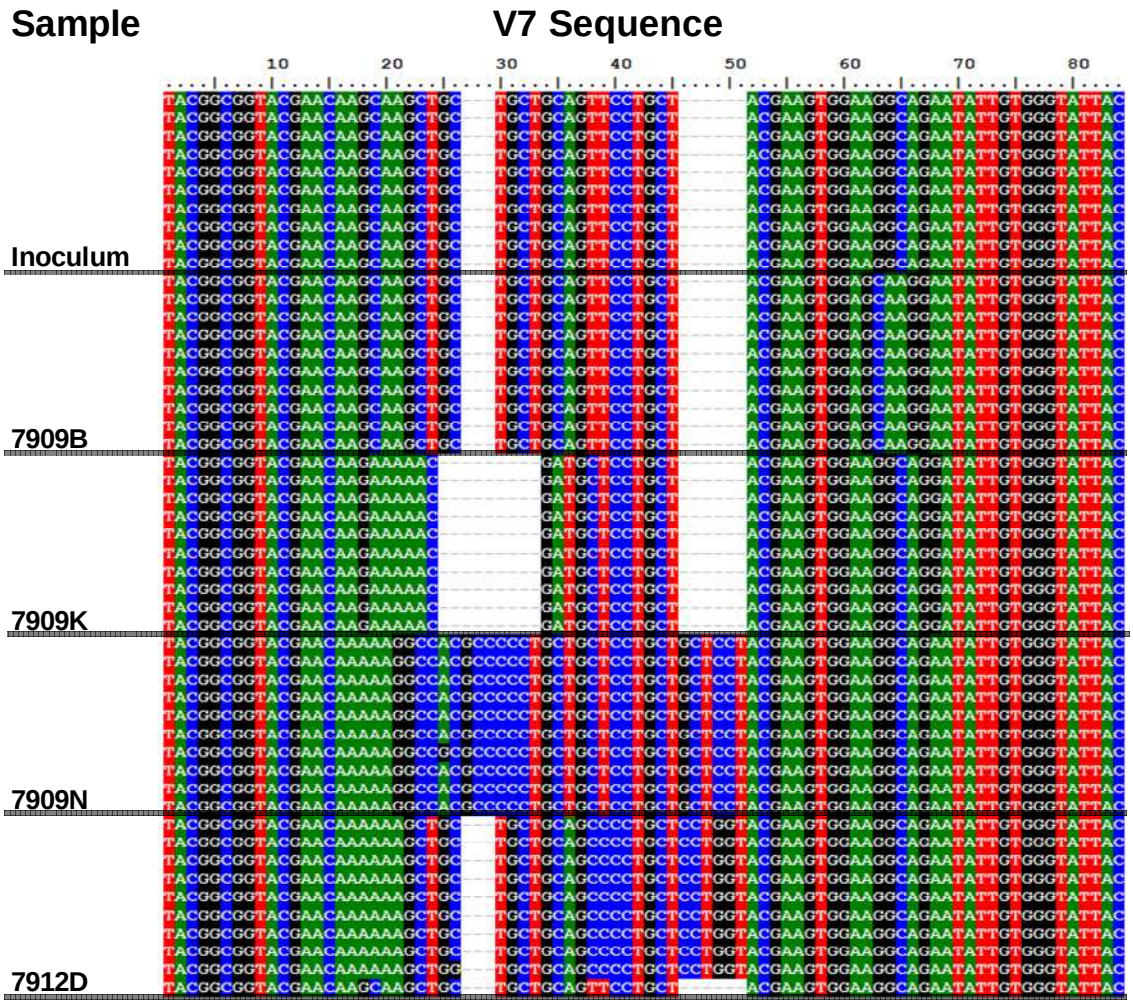

Figure II-1. Examples of TprK V7 sequences

Examples of TprK V7 sequences among secondary lesions, compared to the inoculum. For each lesion (identified by number on the left), DNA sequences were obtained from 10 *E. coli* clones containing the *tprK* ORF. Note that the sequence variation in V7 is seen as insertions/deletions and base changes. The identity, or near identity, of the 10 sequences within each lesion demonstrates the single-cell origin of the lesion.

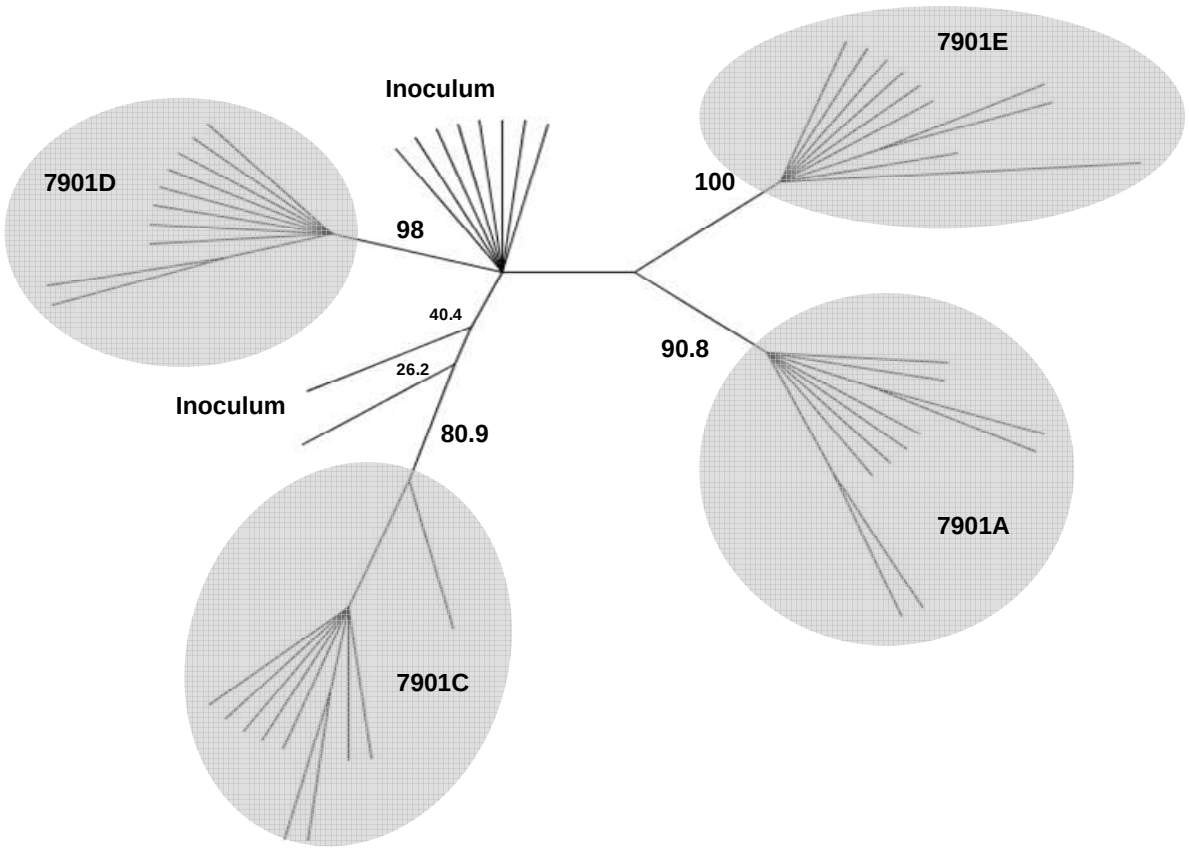

Figure II-2. Phylogenetic analysis of *tprK* DNA sequences from secondary lesions

Phylogenetic analysis of full ORF *tprK* DNA sequences from secondary lesions. A representative example of phylogenetic relationships among the inoculum and the individual secondary lesions (identified by rabbit number and lesion letter) that developed in rabbit 7901 is shown. Lesion 7901B did not contain amplifiable treponemes. Clusters enclosed by a shaded oval correspond to the sequences from 10 plasmid clones per lesion. Boot strap values of the major branches are shown, demonstrating separation of the lesion sequences from the inoculum sequence. Note that the sequences for each lesion are in tight clusters, consistent with a single cell origin for each lesion.

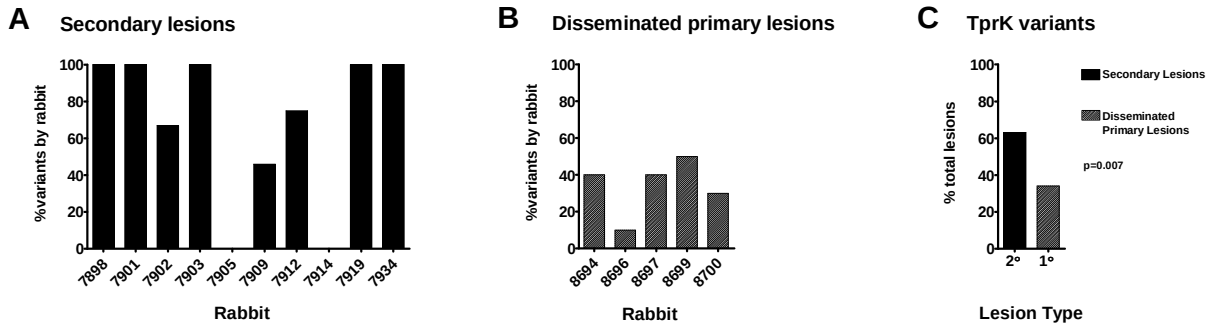

Figure II-3. Proportions of lesions arising from TprK variants

Proportions of lesions arising from TprK variants in secondary syphilis (A) vs. disseminated primary syphilis (B) for individual rabbits are shown. Combined data are shown in Panel C. Variants were identified by combination of FLA and sequencing of *tprK* amplicon from lesion biopsies. P-value determined by two-tailed Fisher exact test. Rabbits 7905 and 7914 did not develop secondary lesions.

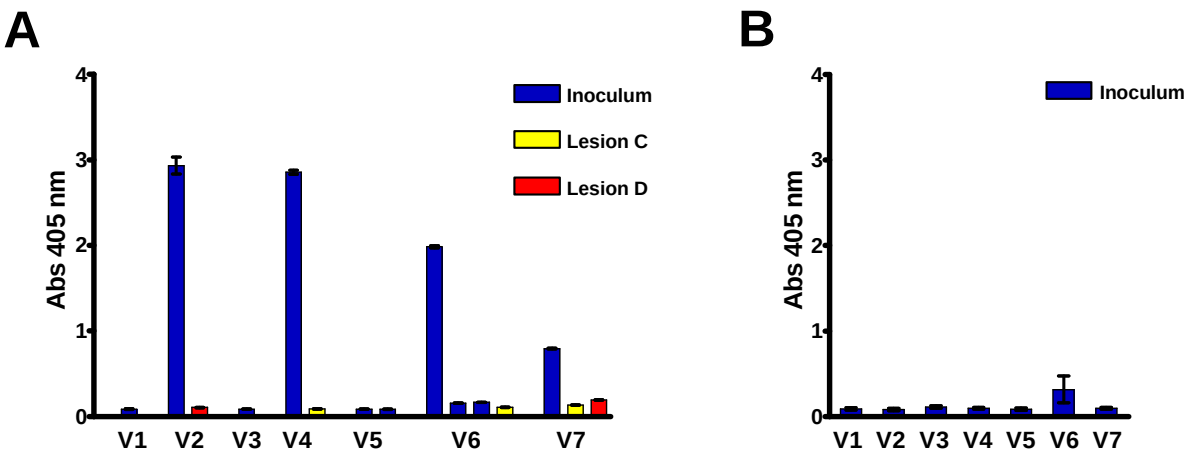

Figure II-4. Analysis of antibody reactivity in secondary syphilis

TprK variants found in secondary lesions escape antibody binding. Panel A: Antibody binding (ELISA) of antiserum (collected at the time of development of secondary lesions) from rabbit 7912 against synthetic peptides representing V region sequences identified in the inoculum (blue bars) and in two secondary lesions (red and yellow bars) that arose in this rabbit. Strong reactivity is seen to the predominant sequences of the inoculum V2, V4, V6, and V7 regions, but not to the V region sequences found in the secondary lesions. Note also that there is no antibody reactivity to the two minority V6 sequences that were present in the inoculum (also in blue). Sera from all rabbits with secondary syphilis were similarly tested, and showed the same pattern of reactivity to the majority inoculum sequences, but not to the minor inoculum sequences or the secondary lesions sequences. Panel B: Mean (+/- SE) ELISA results for antisera (collected at the time of lesion appearance) from rabbits with disseminated primary syphilis (n=5 rabbits), showing lack of antibody reactivity directed to the V region sequences from the inoculum.

III. Repeated Syphilis Infection is Facilitated by Antigenic Variation of TprK

Abstract

Despite development of opsonic anti-treponemal antibodies and specific cellular responses that can resolve early lesions, individuals can acquire repeated syphilis infections. We sought to identify molecular determinants that contribute to establishment of a new syphilis infection in an individual who has already developed immunity to an earlier infecting strain. Immunity to syphilis is expected to involve antibody mediated opsonophagocytosis, as this is the mechanism of treponeme clearance from lesions. Evidence shows that TprK, an outer membrane protein, undergoes antigenic variation in 7 discrete variable regions (V regions) that are targets of antibodies formed during infection. Variation is a result of recombination between the V regions and donor site sequences present elsewhere on the chromosome. The importance of TprK V region-specific immunity in reinfection is not known. We took advantage of naturally occurring mutants, isolated from the same parent strain, to test whether organisms with broader donor site repertoires are able to establish clinical superinfection in animals with immunity to a donor site-restricted mutant. We found that after an immunizing infection with a donor site-restricted isolate, challenge with isolates harboring full donor site repertoires produce active clinical infection. Interestingly, after an immunizing infection with an isolate with a full donor site repertoire, challenge with any isolate (no matter the donor site repertoire) expressing a very different TprK sequence was also able to superinfect. These data support our hypothesis that immunity to TprK V regions is a major factor in determining the specificity of immunity to infection and reinfection by *T. pallidum* strains.

Introduction

Kerani *et al.* found that 17.6% of new cases of syphilis in King county between 1992-2008 were reinfections (281). Reinfections were also found to account for 10% of syphilis cases in British Columbia in 2006 (282), 11% of cases in San Diego county, CA between 2004-2009 (283), and 19% in Sydney, Australia, in 2011 (284). Men who have sex with men (MSM) account for up to 91% of new syphilis diagnoses in the United States (52), and 6- 11% of those have a repeated infection within 2 years (283, 285). In Seattle and Paris, there was an introduction and gradual shift in prevalence of the 14d/g strain type from 0 to 100% between 2001 and 2012, replacing the previously prevalent 14d/f type (29, 101). This is noteworthy because the vast majority (>90%) of these cases were found among MSM, who are at increased risk of repeated infection (29, 101). The introduction of a heterologous strain into a population that may have developed partial immunity to a predominant previously circulating strain likely requires immune evasion mechanisms to establish symptomatic syphilis with infectious lesions.

The Sing Sing study of human inoculation in prison inmates showed that experimental reinfections are possible and that reinfection can occur in subjects with almost any stage of syphilis. Briefly, the investigators found that, among treated patients, 100% (11/11) of patients with early syphilis, 80% (4/5) of patients with a history of congenital syphilis, and only 50% (13/26) of patients in late latency were susceptible to symptomatic reinfection (i.e. developed cutaneous lesions at the challenge site) with the Nichols strain, while patients with untreated latent syphilis were unable to be symptomatically reinfected (0/5) (59). In sum, 66% of treated patients (various stages) were susceptible to reinfection with the Nichols strain. These data

suggest that immunity to reinfection develops slowly over the course of infection and into late latency.

In the rabbit model of syphilis infection, repeated infection (after treatment) was described in great detail by Magnuson and Rosenau. From this study, we know that there is a progressive increase in the ID_{50} needed to produce symptomatic homologous reinfection as the duration of the initial immunizing infection increases (104). These data recapitulate the observations of the human inoculation study: immunity increases over the duration of infection. As such, after prompt treatment of early syphilis, one would expect more symptomatic reinfections compared to persons treated later in their course of infection.

"Chancre immunity" is the term used to describe refractoriness to challenge, or lack of lesion development, in untreated persons. The term was first used to describe the failure to symptomatically reinfect patients using material from their own primary chancre to inject elsewhere on their body (11). These auto-inoculations presumably contained the homologous strain because the treponemes originated from the patients' own active lesions. In a review of studies by Finger and Landsteiner, Chesney reported heterologous challenge experiments in humans exhibiting active tertiary syphilis. Among the 15 attempted reinoculations with a human source of treponemes, 13 subjects developed lesions (286). Repeated infections can be defined in two ways: 1) "reinfection" after a patient is treated for their initial syphilis infection and 2) "superinfection" in which an untreated patient acquires infection by new treponemes.

The development of immunity during infection in the rabbit model of syphilis has been carefully studied: after a 3-4 month infection, rabbits develop immunity to the homologous strain, even after treatment with penicillin. This is manifested by the lack of lesion development after intradermal challenge and failure of treponemes to disseminate to lymph nodes (104).

However some, but not all, heterologous strains were able to superinfect, but there was no molecular information about the superinfecting "heterologous" strains (102). In our study, we sought to determine the unique characteristics that allow some strains to superinfect rabbits previously infected with another strain.

TprK is the only *T. pallidum* outer membrane protein (OMP) that is known to undergo sequence variation during infection (247) and is highly variable among strains (267). We have demonstrated that TprK antigenic variation results in immune evasion by the new variants (225, 242-244, 247-249). Sequence variation of the seven variable (V) regions of *tprK* occurs by segmental gene conversion between the *tprK* expression site and donor sites located in the 3' and 5' flanking regions of *tprD* (235, 245). Infection-induced antibodies are directed to V region sequences, which are predicted to be exposed on external loops (248). Even minor alteration of V region sequences by gene conversion abrogates antibody binding to new V regions and allows variants to escape clearance (243, 247). We believe that immune evasion is key to establishing superinfection by preventing the opsonization of new strains by antibodies generated against the original infecting strain (130, 131, 265). Recently, antigenic variation of TprK has been shown to facilitate the progression of syphilis from the primary chancre to development of the rash of secondary syphilis (Reid, submitted/Chapter2).

Antigenic variation of TprK is thought to function similarly to VlsE and MSP2 variation and immune evasion of *Borrelia burgdorferi* and *Anaplasma marginale*, respectively (259, 287). In these systems of antigenic variation, the repertoire of sequence variability (donor alleles) is critical for establishing superinfections (259), and variation of the expressed gene during infection is also essential (287). We hypothesize that TprK similarly plays an important role in facilitating *T. pallidum's* ability to cause repeated infections. We expect that isolates with full

donor site repertoires (50 donor sites at the *3' tprD* locus) will superinfect a rabbit previously infected with a donor site-restricted isolate (32 donor sites at the *3' tprD* locus), by utilizing its unique donor site sequences to produce novel TprK epitopes. Additionally, when challenge is conducted with *T. pallidum* isolates expressing very different TprK proteins compared to the initial infecting isolates, we expect that antibodies developed during the initial infection will fail to recognize the superinfecting isolate, thus allowing the treponemes to escape the established immune response and cause infectious lesions.

Because there is no system for creating specific mutations in *T. pallidum*, we exploited naturally-occurring *T. pallidum* mutants to identify the components of the TprK variation machinery involved in repeated infections. We demonstrate that a unique donor site repertoire or a unique TprK expression site is sufficient for superinfection.

Methods

Animals

Male New Zealand white rabbits were used for propagating strains and for the experiments described in this study. Animals were fed an antibiotic free diet and housed at 16-18℃. All protocols involving animals were approved in advance by the University of Washington Institutional Animal Care and Use Committee.

Strains used in this study

This study involved isogenic isolates derived from the Nichols strains obtained from University of Texas Health Science Center, Houston (a generous gift from Dr. Steven Norris), and a previously described isogenic isolate (Chicago C) of the Chicago strain (243). To

minimize any sequence variation in OMPs known to vary among strains, we chose to study strains of the same molecular strain type (14a/a); this typing scheme is based on the number of acidic repeat protein (ARP) repeats; *tprE, G, J* restriction fragment length polymorphism pattern; and *tp0548* sequence type (28, 230). Because the sequence of *tp0136* can also vary among strains (unpublished data), we also limited our examination to strains with identical *tp0136* sequences.

The Nichols Houston strain used in the first *T. pallidum* genome sequencing project (186, 245) was reported to contain 32 *tprK* donor sites in the *tprD* 3' flanking region. Subsequently, a subpopulation was identified within this strain in which a larger donor site region was seen, identical in size to that described by Centurion-Lara *et al.* in the Nichols Seattle and Chicago strains (245, 288). Utilizing the *in vivo* isolation protocol previously described (269), we obtained two isolates from the parent Nichols Houston strain: Nichols Houston E has a 1.2 kb deletion in the TprK donor sites, resulting in 32 donor sites (referred to as "donor site-restricted"); Nichols Houston J possesses a donor site repertoire of 50 donor sites (the full repertoire of donor sites) (Figure III-1). We maximized the likelihood that the two Nichols Houston isolates are isogenic outside of the 3' flanking *tprD* donor site deletion by isolating them from the same parent population. The sequence of the Nichols J donor site region is identical to that seen in Chicago C. Full genome sequence analysis is underway to determine the precise level of identity between the Nichols Houston E and J isolates and preliminary results indicate that, excluding the TprK donor and expression sites, these genomes are at least 99.993% identical (Giacani *et al.,* unpublished).

Previously, LaFond *et al.* reported that the Nichols strain undergoes TprK variation at a slower rate than other strains (242). We therefore examined the well-studied Chicago C isolate in our experiments, in addition to the Nichols Houston E and J isolates, because isolates from

the Chicago strain undergo *tprK* variation more quickly during infection. This may enable the Chicago C isolate to utilize its donor site repertoire more efficiently, thus potentially "out-varying" a Nichols isolate during infection, despite having identical donor site sequences and a 99.99% homology with the Nichols genome across excluding the *tprK* expression site (186, 270). Importantly, Chicago C has very different V region sequences at the *tprK* expression site compared to the Nichols isolates (Figure III-2).

All isogenic *T. pallidum* isolates were isolated as previously described (243, 269, 289). Briefly, naïve rabbits were infected intravenously with a parent strain, and treponemes were extracted from biopsies of the resultant lesions that developed on the rabbits' clipped backs. These were propagated by subsequent intratesticular infection to expand the population of "isolated" treponemes. Care was taken to minimize accumulation of *tprK* diversity during propagation steps before inoculation, although it is impossible to avoid all variation, particularly with the more rapidly-varying Chicago strain. A comparison of known *tprK*-related differences among the isolates is presented in Figures III-1 (donor sites) and III-2 (expression site).

Experimental infection

Propagation of treponemes

All isolates studied here were obtained from intradermal lesion biopsies. To obtain adequate numbers of treponemes, each isolate was propagated up to two times in rabbit testes to expand the population as previously described (269). Testes were removed at peak orchitis (~10 days post infection), scored with a sterile scalpel, and treponemes were liberated from the tissues in the presence of a filter sterilized 1:1 solution of 0.9% saline and normal rabbit serum (NRS). Normal rabbit serum was collected in-house and pooled from animals nonreactive in both VDRL and FTA-ABS tests for treponemal infections. Treponemes in the resulting

suspension were counted by dark field microscopy and diluted in sterile 50% NRS/saline for experimental infection.

Initial immunizing infection

Rabbits were initially infected intratesticularly (IT) with $5x10^7$ treponemes in each testis. Immunizing infection by this route was previously described (102) and has the benefit of avoiding interference with observation of lesions after subsequent intradermal challenge due to scarring. During the course of the immunizing infection, rabbits developed orchitis as treponemes multiplied. To reduce any discomfort caused by inflammation and swelling in the testes, buprenorphine was administered from development of orchitis until resolution. The immunizing infection was allowed to continue for 3 months (90 days), as previous studies had demonstrated development of homologous immunity by approximately 90 days (102, 104, 286, 290-292).

Intradermal challenge inoculation

Nichols strains are known to undergo *tprK* variation slowly (242) and did not accumulate detectable diversity during the 10 day IT infections required for propagation (data not shown). However, the Chicago C isolate did acquire considerable *tprK* variation during propagation, necessitating a subsequent re-cloning, as previously described (289), to obtain a more homogenous *tprK* population. Nichols Houston J was re-isolated as well, but because Nichols Houston E appeared clonal at *tprK,* it was not re-isolated for the challenge suspension. Challenge inoculations were performed intradermally (ID) 3 months after the primary intratesticular infection. ID challenge consisted of 0.1 ml of a 10^4 treponeme/ml suspension at marked sites along the rabbits' backs. Three groups of 5 rabbits initially infected with Nichols Houston E were challenged 8 sites with Nichols Houston E (homologous isolate), Nichols

Houston J, or Chicago C. Three groups of 4 rabbits initially infected with Nichols Houston J or Chicago C were challenged at 10 sites with Nichols Houston E, Nichols Houston J, or Chicago C. Following challenge, rabbits were monitored daily and their backs kept free of fur. At the time of challenge, 3 naïve rabbits were similarly infected with each challenge isolate. Intradermal challenge allowed for close monitoring of lesion development over time by measuring induration, color, ulceration, and healing, while allowing biopsies of individual lesions to be taken. The sites of challenge inoculation were biopsied (4mm diameter punch biopsies) at day 21, in at least one site per animal. Local lidocaine anesthesia was used for collection of biopsies.

DNA sequence analysis

DNA was extracted from skin biopsies as previously described (289). Tp47 (gene *tp0574*) was amplified by PCR and then blotted to nylon membranes for Southern hybridization as previously described (139), and used to determine the presence of treponemal DNA within skin biopsy samples. The *tprK* (*tp0897)* expression site was amplified from suspensions used for initial infection and challenge inocula and then cloned in *E. coli* as previously described for sequence analysis (30 clones from immunizing inocula and 10 from challenge inocula) (267). The *tprK* sequences aligned in Figure III-2 represent the majority sequence at each variable region; 74% (23/31) Chicago C, 94% (30/32) Nichols Houston E, and 83% (25/30) Nichols Houston J sequenced isolates. Primers used in this study are shown in Table III-1.

To examine the accumulation of TprK variants in lesions arising after challenge, we utilized a deep sequencing approach. DNA samples extracted from the biopsied lesions of Nichols Houston J challenge of Nichols Houston E-immunized rabbits (10 DNA samples) and Nichols Houston J infection of naive rabbits (6 DNA samples) were pooled in equal volumes.

The resulting pooled lesion samples as well as DNA from the initial and challenge inocula were used as template for variable region-specific PCR reactions that were subsequently run on an Illumina MiSeq sequencer for deep sequencing. PCR amplification of variable regions, library preparation, and paired-end sequencing were carried out by Covance Genomics Laboratory (now Labcorp, Seattle, WA). Because the maximum length for any TprK variable region studied thus far by Sanger sequencing was 197 base pairs (Chicago C V6), the 150-200 base pair read length of MiSeq is expected to provide full coverage of each variable region, utilizing sense and antisense primers. Prior to analysis, the primer sequences (located in conserved regions outside each variable region) were bioinformatically trimmed from MiSeq reads to decrease the effect of miscalls which occur most often at the 5' and 3' ends of each sequence. The total number of sequence occurrences within the pooled sample for each specific sequence was recorded, and used to determine the relative proportion of a specific variable region sequence among the 10 most prevalent sequences. Because each variable region is amplified individually in the preparation of the sequencing library, contiguous full-length *tprK* expression site sequences cannot be identified. Primers for variable region sequencing are found in Table III-1.

Antibody reactivity

After initial infection, but prior to challenge, blood was collected at day 90 from each rabbit, and sera stored at -80℃ until tested. Prior to use, serum samples were heated at 56℃ for 30 minutes. Synthetic peptides, designed to reflect individual V region sequences found in the initial infecting strains (Table III-2), were produced to >70% purity (GenScript Corporation, Piscataway, NJ) and used as antigens. Sera were tested at a 1/20 dilution for their ability to bind V region peptides by ELISA as previously described (243, 248) with the following modification: washing steps (4x30 second washes with 350 µl PBS 0.05% Tween 20) were carried out on a MultiWash Advantage plate washer (TriContinent, Grass Valley, CA). Each

sample was tested in triplicate. Serum reactivity (OD_{405}) to the tested peptides was normalized, by subtraction of NRS reactivity.

Statistical analyses

Two tailed Fisher's exact test was used to calculate the significance of differences in lesion development among groups of rabbits, and in comparing the proportions of TprK V7 variants in lesion samples.

Results

Patients are susceptible to repeated syphilis infections. Rabbit models of repeated infection were previously used to describe the development of homologous immunity to a single strain, and susceptibility to superinfection with select heterologous strains (102, 290). We sought to determine whether differences in the TprK antigenic variation system (donor site repertoire and *tprK* expression site) could account for the differences in ability of heterologous strains to superinfect rabbits that are immune to homologous challenge.

Homologous immunity to symptomatic superinfection

In previous studies, homologous immunity developed after 3 months of infection in the rabbit model (102, 290, 293). Although the Nichols strain has most often been used in these experiments, we needed to verify the ability of the individual Nichols isolates (Nichols Houston E and J) to induce homologous immunity after a 3 month immunizing infection. Furthermore, because homologous immunity and repeated infections have not been studied in the Chicago strain, we sought to determine whether our Chicago C isolate was able to induce homologous immunity.

After 3 month IT infections, Nichols Houston E and J rabbits failed to develop symptomatic superinfection after ID homologous challenge (Table III-3). No lesions were clinically discernible, and no treponemal DNA was detected at the challenge sites. After an immunizing infection of 3 months duration with the Chicago C isolate, homologous challenge resulted in near-complete homologous immunity: a single small papule developed very late (24 days post infection) at 1 of 40 challenge sites and was positive for treponemal DNA by T47 PCR and Southern blot. In sharp contrast, challenge isolates produced progressive indurated and ulcerating lesions at most inoculation sites (87-100%) in naïve control animals.

TprK donor site repertoire and ability to superinfect

With the confirmation of the development of homologous immunity in the rabbit model of repeated infection, results of heterologous challenges were examined. A unique MSP2 donor allele was shown to be critical for superinfection in *A. marginale* infections (259). Because MSP2 variation appears to be similar to TprK variation in *T. pallidum*, we hypothesized that *tprK* unique donor sites in the challenge isolate would enable symptomatic superinfection. We cannot genetically alter *T. pallidum*, presenting a formidable challenge when attempting to study specific differences between strains. Fortunately, we were able to isolate separate subpopulations of treponemes (naturally occurring mutants) with (Nichols Houston E) and without (Nichols Houston J) a large deletion corresponding to 18 donor sites, DS 28-45 in the *3' tprD* donor sites. The Nichols Houston E isolate is thus considered to be donor site-restricted (with only 32 donor sites) compared to Nichols Houston J (with a complete repertoire of 50 donor sites). We hypothesized that the additional 18 donor sites in the Nichols Houston J isolate would provide a repertoire of potential unique TprK variants that would enable evasion of existing anti-TprK immunity, resulting in symptomatic superinfection.

After an immunizing infection with Nichols Houston E (32 donor sites), challenge with Nichols Houston J (50 donor sites) resulted in symptomatic superinfection (21/40 lesions, Table III-4) despite the fact that the *tprK* expression site sequence in the Houston J challenge inoculum differed from Houston E at only V1 and V6. As expected, challenge with Chicago C, possessing the same repertoire of 50 donor sites as Nichols Houston J, also resulted in symptomatic superinfection (17/40 lesions). The presence of treponemal DNA in these lesions was confirmed with T47 Southern blot.

In the converse experiment, after an immunizing infection with Nichols Houston J (50 donor sites), challenge with the donor site-restricted Nichols Houston E failed to produce lesions and no treponemal DNA was detectable in biopsied challenge sites (Table III-4). Challenge of Nichols Houston J-immune rabbits with Chicago C resulted in clinical superinfection (25/40 lesions). This is notable because the two strains (Nichols Houston J and Chicago C) have identical donor site repertoires: there were no novel donor sites in Chicago C compared to Nichols Houston J. Challenge strains produced typical lesions at most sites in naïve rabbits (80-100%). These data suggest that unique donor sites may permit superinfection even by strains with very similar *tprK* expression site sequences (Nichols Houston E and J, derived from the same parent strain), but unique donor sites are not required for superinfection by isolates that do not originate from the same parent strain (despite having 99.99% genome sequence homology).

Isolates expressing *tprK* variants are able to superinfect

We next explored whether isolates with substantially different *tprK* expression site sequences could superinfect rabbits despite having the same donor site repertoires. Treponemes expressing different TprK V regions are expected to avoid recognition by

circulating antibody induced by the initial infecting isolate. Because no two isolates studied here have absolute identity in the *tprK* expression site V region sequences, they are all considered heterologous to each other. The *tprK* expression sites in Nichols Houston E and J differed only at V1 and V6, while Chicago C was different from Nichols Houston E at all 7 variable regions (Figure III-2).

In Table III-4, we show that Nichols Houston E-immune rabbits were able to be superinfected with each *tprK*-heterologous isolate (Nichols Houston J and Chicago C), although these18 unique donor sites might account for the development of unique TprK variants.

Nichols Houston J, however, has the same donor site repertoire as Chicago C, and superinfections of Nichols Houston J-immune rabbits by Chicago C, and vice versa, are achieved. It is possible that during the 3 month immunizing infection, the slow-to-vary Nichols Houston treponemes utilize a smaller portion of donor site repertoire compared to the rapidly-varying Chicago C. Chicago C also differs from the two Nichols isolates at all V region sequences in the *tprK* expression site (see Figure III-2). To our surprise, both Nichols isolates, including the donor site-restricted Nichols Houston E, were able to superinfect Chicago C-immune animals, though the treponeme-positive lesions that developed at the challenge sites were atypically flat and non-ulcerative (Table III-4). These attenuated lesions presented as flat macules that increased only in circumference. Erythema and early brief induration at the leading edge of these lesions was noted, with an area of central pallor. Photos comparing Nichols Houston E challenge of Nichols Houston J-immune rabbits (0/40 lesions), Chicago C-immune rabbits (40/40 atypical non-progressive lesions), and naive rabbits (24/30 lesions) and are shown in Figure III- 3.

Nichols Houston E was not expected to be able to “out vary” Chicago C to cause superinfection because it has a restricted repertoire of donor sites. The observation that Nichols Houston E can cause superinfection of rabbits previously infected with Chicago C demonstrates that unique donor sites are not required for symptomatic superinfection in previously infected rabbits. However, the finding that both Nichols Houston E and J were able to superinfect Chicago C-immune animals suggests that the expression of a different *tprK* sequence of the challenge isolate may be critical for superinfection.

TprK variants escape antibody recognition and cause symptomatic superinfection

If our hypothesis is that superinfecting isolates are immune escape variants, we expect that, in the challenge settings that did not result in symptomatic superinfection, there would be high titers of antibody to TprK V region epitopes expressed by the challenge treponemes. Conversely, we expect to see low or no antibody reactivity to challenge TprK V region epitopes in the setting of successful superinfection. We used synthetic peptides [6-25 amino in length (Table III-2) representing full-length TprK variable regions] in ELISA assays to identify antibody reactivity in sera collected 3 months after the initial immunizing infection, just prior to challenge.

Pre-challenge sera from Nichols Houston E-immune rabbits were tested against TprK variable region peptides from each challenge isolate (Figure III-4, panel A-C). As expected, these rabbits produced the greatest reactivity to homologous peptides, with high levels of antibody reactivity (>2 absorbance units) to V2 and V7 (Figure III-4, panel A). These rabbits also developed high levels of antibody to Nichols Houston J V2 and V7 peptides (Figure III-4, panel B), which were conserved between the isolates (Figure III-2). Nichols Houston E-immune rabbits failed to develop antibodies reactive with any Chicago C variable regions (Figure III-4,

panel C). This was consistent with the fact that the *tprK* expression sites of these two isolates differ at each variable region.

Nichols Houston J-immune animals developed antibodies that recognize V2 and V7 peptides from both Nichols Houston isolates (Figure III-4, panels D, E), again consistent with conserved sequences at these V regions. In Figure III-4, panel F, similar to panel C, very little antibody reactivity is seen to the Chicago C challenge isolate peptides. Again, this was expected because the Nichols Houston J and Chicago C *tprK* expression sites differ at each variable region. The mean antibody reactivity to Chicago C V4 was due to reactivity in only 1 of the 4 rabbits in that group.

As expected, Chicago C-infected rabbits produced robust antibodies against the homologous peptides (Figure III-4, panel I). These rabbits produced an intermediate (0.5-2 absorbance units) mean antibody response to the peptide antigens from Nichols Houston E and J (Figure III-4, panel G and H). This mean reactivity, however, was due to a single rabbit (rabbit 8345) who, interestingly, developed only a single erythematous, non-indurated lesion very late during the Nichols Houston J challenge infection (day 30 post-challenge).

<u>New TprK variants are selected by pre-existing antibody during superinfection</u>

The ability of Nichols Houston J to superinfect Nichols Houston E-immune rabbits suggests that Nichols Houston J treponemes have undergone TprK variation and escaped antibody binding. The Nichols Houston E-immune rabbits had high levels of antibody to V2 and V7 prior to the Nichols Houston J challenge. To examine the V region sequences of those treponemes found in the challenge lesions, we employed a high throughput sequencing approach using treponemal DNA extracted from lesion biopsies. Two variants were found at V7

in the challenge lesions, with frequencies of 24.5% and 6.0%. These frequencies are significantly higher than those found in pooled lesions that develop after Nichols Houston J challenge of naïve animals [0.39% and 3.48%, respectively ($p < 0.001$)], and are higher than were found in the Nichols Houston J challenge inoculum [0.19% and 2.22%, respectively ($p < 0.001$)]. The presence of high levels of antibody to the initial V7 sequence prior to challenge, and the high levels of V7 variants in the challenge lesions strongly suggests that pre-challenge antibody directed against V7 positively selected for V7 variant sequences, leading to successful superinfection. The contribution of variants in the other V regions is less clear: V1 and V4 were found to be invariant in the pooled DNA samples of lesions from Nichols Houston J challenge of Nichols Houston E-immune rabbits. V2, V3, V5, and V6 variants found in the challenge lesions ranged in prevalence (within the V region) from 0.55% to 7.22%.

Discussion

We had initially hypothesized that unique *tprK* donor sites are necessary for an isolate to establish superinfection during syphilis infection. To test this hypothesis, we utilized naturally occurring *T. pallidum* mutants to cross-challenge animals following a 3-month immunizing infection. Three isolates were isolated, each with a unique TprK sequence. Two isolates, Nichols Houston E and Nichols Houston J, arose from the same parent strain and, therefore, were expected to be isogenic outside of the *tprK* expression site and 3' *tprD* donor sites, where Nichols Houston E contains a 1.2 kb deletion, resulting in a restricted donor site repertoire of only 32 donor sites compared to 50 donor sites in Nichols Houston J. Preliminary genome sequencing analysis reveals that the Nichols Houston E and J isolates have over 99.993% identity outside of the TprK donor and expression sites (Lorenzo Giacani, personal communication). The third isolate, Chicago C, was heterologous to both Nichols Houston

isolates at the *tprK* expression site, but has the same donor site repertoire as Nichols Houston J (18 unique donor sites compared to Nichols Houston E).

We reasoned that, during the initial 3 month immunizing infection, the donor site repertoire of an isolate would be utilized to create new TprK variants necessary for persistent infection, and that any isolate with a higher number of donor sites would have an advantage in developing variants that could not be recognized by antibodies induce by another strain. The Nichols strains are known to vary *tprK* slowly, but an adaptive immune response does result in detectable accumulation of variants (242). While there is very little observed accumulation of diversity during routine 10-day IT propagation, LaFond *et al*. showed that, after 2 slow passages (60-70 days total) in rabbits, during which the immune response clears most of the treponemes from the primary site of infection, Nichols TprK variants increase in frequency due to immune selection (242). Because our initial infection progressed for 90 days, we expect that the Nichols isolates studied here were undergoing active TprK gene conversion, and using the donor sites to form new variants.

Our studies showed that the donor site-restricted isolate Nichols Houston E is unable to superinfect rabbits that are immune to the Nichols Houston J isolate with 50 donor sites at the *3' tprD* locus. Conversely, we showed that the two isolates (Nichols Houston J and Chicago C) with the 18 additional donor sites were able to superinfect rabbits immune to Nichols Houston E. While these results are consistent with our initial hypothesis that unique *tprK* donor sites are necessary for an isolate to establish superinfection, the converse studies in which Chicago C-immune rabbits were able to be superinfected with Nichols Houston E and J did not support this hypothesis.

Compared to the Nichols strains, Chicago C is known to more readily undergo TprK variation and active immunity further selects for variants (247). The inclusion of Chicago C in our experiments allowed for comparison with Nichols Houston J in which both isolates contain identical donor sites, but in which Chicago C has a higher likelihood of utilizing its donor site repertoire more fully during the 3 month initial infection. The comparison between Chicago C and Nichols Houston J allowed us to examine whether the broader utilization (and subsequent expression) of donors sites plays a role in cross-protection. We reasoned that the Chicago C-immune animals would have developed a broader V region antibody response directed against the new variants and would therefore be resistant to superinfection by Nichols Houston E and J treponemes.

Chicago C-immune rabbits had developed only modest antibody reactivity against the Nichols Houston E and J V regions and, while superinfection by Nichols Houston E and J did occur, the result was atypical attenuated lesions. Interestingly, the Chicago C-immune rabbit (rabbit 8345) with the highest level of antibodies to Nichols Houston J peptides developed only a single flat lesion after challenge at 10 sites, consistent with a role for antibody in protection. These results led us to consider whether challenge with an isolate hosting a different sequence in the *tprK* expression site, or development of such a variant early following challenge, may be sufficient for superinfection.

Anti-TprK antibodies have opsonic function (225) and it has been shown that antibodies raised against one V region sequence do not recognize variant V region sequences (243). Our experiments permitted us to ask whether a challenge inoculum expressing a variant TprK protein (compared to the initial inoculum) would allow those variant treponemes to evade antibody binding and establish infection. If a change in the expressed TprK is enough to abrogate antibody binding to important V regions, than we would expect each of our three

isolates to have superinfected the others. This occurred in Nichols-Chicago C pairings in which all of the V region sequences of the immunizing and challenge isolates were different: both Nichols Houston isolates could superinfect Chicago C-immune rabbits and vice versa.

However, this did not occur in the cross-infection studies between the two Nichols Houston isolates which differed in V1 and V6, but were identical in other V regions. Interestingly, in both sets of animals, high levels of antibodies developed only to the shared V2 and V7 sequences during the immunizing infection (Figure III-4 B and D). The only instance of superinfection between the two Nichols Houston isolates occurred with Nichols Houston J infection of Nichols Houston E-immune rabbits. In this case, ~~s~~equence analysis of the *tprK* expression site of treponemes in the lesions revealed a high-frequency V7 variant (24.5%). The significant increase in the frequency of this Nichols Houston J variant compared to its presence in the Nichols Houston J challenge inoculum (0.19%) suggests that this variant was positively selected by the V7 antibody that developed during the Nichols Houston E immunizing infection. Naïve animals, which did not have pre-existing V7 antibodies, had a very low frequency (0.39%), of this variant in their Nichols Houston J lesions.

Were the 18 additional donors sites in Nichols Houston J used to develop this new variant, consistent with our original hypothesis concerning a role for unique donor sites? Analysis of the sequences revealed that these 18 donor sites could have been used to create this variant; however, this would have required a large number of recombination steps. The most parsimonious route to creation of this variant can be found using donor sites that are shared between Nichols Houston E and J. Although we identified a V7 variant that appears to have been positively selected, we cannot definitively identify which donor sites were used to derive the variant sequence.

The effect of pre-existing immunity on repeated syphilis infection is complex and variable. Grassly *et al.* proposed that much of the periodic nature of syphilis outbreaks in the US since 1955 is due to the development of partial immunity from initial infection, with effects on subsequent exposures, and the waning of this immunity over time within populations (24). The idea that syphilis outbreaks are due to waning immunity may be over-simplistic in that it does not acknowledge relevant non-biological factors (e.g. changes in sexual practices, fluctuating levels of public health intervention among socially marginalized groups with high rates of partner change and poor access to healthcare, and shifting of outbreaks between disparate social groups) (25, 294). Nevertheless, population susceptibility to syphilis infection is almost certainly affected by prior infection. The ability of some pathogens to infect an individual who has prior immunologic experience reflects the higher fitness of that pathogen within a population. Variation of important antigens such as TprK provides an advantage *T. pallidum* to repeatedly infect individuals, effectively increasing the pool of people susceptible to infection.

In this study we focused on the development of symptomatic superinfection: that is, the formation of skin lesions after ID challenge among previously infected rabbits. Asymptomatic infections, in previously infected and antibiotic-treated rabbits have been described (292). This scenario is also seen in humans, as syphilis reinfection is commonly diagnosed in previously treated asymptomatic persons by increases in RPR titers. Rabbits that are asymptomatically reinfected still harbor treponemes in lymph nodes, and this is also likely to occur in asymptomatic human infection. While such asymptomatic infections are clinically important for an individual, they are less relevant from a public health standpoint because there are no lesions to transmit the infection to others. Symptomatic infections are of much greater concern for a population, as primary and secondary lesions contain many treponemes and present a superficial moist focus of transmissible treponemes. Because heterologous isolates are able to

cause symptomatic infections in individuals with intact homologous immunity, introduction of a new strain may result in a new round of clinical infection which may spread throughout an already-infected sexual network. Our studies demonstrate that waning immunity, as proposed by Grassly, does not appear to be necessary for the acquisition of new infections and subsequent transmission to new hosts.

In this report we isolated and used naturally-occurring *T. pallidum* mutants to study syphilis pathogenesis. Molecular changes that enable superinfection of an individual with syphilis immunity are advantageous to a bacterium only if it increases that cell's ability to cause productive transmissible infection. The basic reproductive rate (R_o) of infection is determined by β--the efficiency of transmissibility, c--the number of susceptible contacts), and D--the length of the infectiousness period (54, 55). The ability of a pathogen to superinfect hosts with previous exposure serves to increase the number of individuals who are susceptible to infection with the new strain. Thus, antigenic variation of TprK effectively increases the estimated R_o of syphilis infection. Pathogens with $R_o \approx 1$ are expected to persist in a population at constant endemic levels, while an $R_o > 1$ may be associated with outbreaks of infection (295). The transition from a $R_o \approx 1$ to $R_o > 1$ may represent the introduction of a new strain, with a unique TprK compared to circulating strains, into a population, thus causing an outbreak in an already endemic population. Recognizing the role of antigenic variation of TprK in the ability of new strains of *T. pallidum* to penetrate sexual networks with existing immunity is an important contribution to our understanding of outbreaks of disease in high-risk populations.

Tables and Figures

Table III-1. Oligonucleotides used in Chapter III

Target	Oligo	Sequence	Use
tp0897 (tprK)	Kll1-S	TCCCCCAGTTGCAGCACTAT	1,2
	Klr1-As	TCGCGGTAGTCAACAATACCA	1,2
	M13F-S	CAGGAAACAGCTATGAC	2
	M13R-As	GTAAAACGACGGCCAG	2
	9V-S	GATATTGAAGGCTATGCGGAGCTG	2
	9V-As	CCTCAAGGAAAGAAGTATCAGG	2
V1	V1-S	GTGGGCTCAGGTTTCGTTC	1, 3
	V1-As	CGCATAGACATTCCCCTCAC	1, 3
V2	V2-S	GGGGCTCACGTTTGATATTG	1, 3
	V2-As	CCGGTGAGCTCCACTTTAAT	1, 3
V3	V3-S	GAGCGTACGCGTGAAGATG	1, 3
	V3-As	TAGCAGCCAGAGCACACAGA	1, 3
V4	V4-S	CTTTGGGGTCTGTGTGCTCT	1, 3
	V4-As	AACGATACCCCAACGTCAAC	1, 3
V5	V5-S	TTGGGGTATCGTTGGTTCTC	1, 3
	V5-As	CCCAAATCAAGACCCTCAAG	1, 3
V6	V6-S	AAACCAAGGGGTCTGATCCT	1, 3
	V6-As	TAGACGATACGAACCCCAGA	1, 3
V7	V7-S	TGGGTGAGTATGGTTGGGTTA	1, 3
	V7-As	GCCGAATCTCCACCTTCTCT	1, 3
3’ tprK	3’K fam-S	CGCTTGATTTGGTAGGGATG	2
	3’K fam-AS	CTACCTCCGCTGCGTGTC	2
	V7-S2	GTCTGGGGTTCGTATCGTCAT	1,2
	Klr1-As	TCGCGGTAGTCAACAATACCA	1,2
tp0574 (T47)	T47-S	CGTGTGGTATCAACTATGG	1
	T47-AS	CAACCGTGTACTCAGTGC	1
	5’ biotin	GTTGACGTGTTTGCCGATGGACAGCCTA	4
tp0136	TP0136Ins-S	ACACAACGGCTGCGAATACT	1,2
	TP0136Ins-As	ACCGACCGTGCCCCATACT	1,2
	Tp0136-S	GAAGAGGGCGTTTTGTGTGT	1
	Tp0136-AS	AGGGCTTGCTCAGATGAAGA	1
3’tprD DS	3DF-S	ATTGCCGAGAGCATCTGGT	1,2
	3’DF-2-AS-6	GCACGGCGCTGTTCAGTAA	1,2
	3DF-As	CCGCTCTCCTTCCCATAAGA	2
	3DF-S1	GCCTCATACCGACTGGGAG	2

	3DF-S2	CCTGCTCCTGGTACGAAGT	2
	3DF-S3	CAGCAGGAACTCCCAGCACGT	2
	3DF-As1	GGGCGGACGTCCCACACC	2
	3’DF-1-2-AS	ATTGCGTCTTTTGCGCGCG	2
	3’DF-1-3-AS	AAACGCGGCGCTGTGGTTT	2
	3’DF-1-4-AS	GGGAGTTGGGCTCGGGC	2
	3’DF-1-5-AS	CTCGGGCTTGGGTGAAGG	2
	3’DF-1-6-AS	ACACCCTGGGTGAAAAGACT	2
	3’DF-1-7-AS	GTACGTGCTGGGAGTTCCT	2
	3’DF-1-8-AS	TGTGCGGTCATGGCCATGT	2
	3’DF-1-9-S	CGCAGGCCAGCAATGTATTT	2
	3’DF-1-10-AS	TTTGGTTTGCCCTCCCAG	2
	3’DF-1-11-S	CAAGCAGGGAGGACGCGT	2
	3’DF-1-12-AS	CTTCGGGAATCGAGTGTAGT	2
	3’DF-1-13-S	TCGTACTGCGCGTCATCGA	2
	3’DF-1-14-S	TGGACTATCCATTCCCCATC	2
	3’DF-1-15-AS	GTTGGGCTGGGGGAAATAG	2
	3’DF-1-16-S	TATCGCCCCAACGCACGC	2

1, PCR Amplification; 2, Sanger Sequencing; 3, Illumina Sequencing; 4, Probe

Table III-2. Oligopeptides used in Chapter III

Peptide	Sequence	Length
V1C1	GIAYEDGSAGNLKH	14
V1C2	GIASDGSAGNLKH	13
V1E1	GIASEDGSAGNLKH	14
V1E2	GIASKDGSAGNLKH	14
V1J1	GIAYENGGAQPLKH	14
V1J2	GIAYENGGAGALKH	14
V2C1	PAGVTPSKY	9
V2C2	WEGKPNGNVPAGVTPSKY	18
V2C3	REGKLNGNVPAGVTPSKY	18
V2C4	WEGKPNGKAPAGVTPSKY	18
V2CJ	WEGKDSQGNVPAGVTPSKY	19
V2EJ	WEGKDSKGNVPAGVTPSKY	19
V2J1	WEAKDSKGNVPAGVTPSKY	19
V2J2	WKGKDSKGNVPAGVTPSKY	19
V3C1	ILWDVGAK	8
V3C2	GYATARAGADILWDVGAK	18
V3C3	GYATAQPPANILWDVGAK	18
V3C4	GYAQAAAADILWDVGAK	17
V3C5	GYATARAQLPAVAPANDILWDVGAK	25
V3C6	TARAQLPAVAPANDILWDVGAK	22
V3EJ	GYAQARALAAGAK	13
V3J1	GYAQAARALAAGAK	14
V3J2	GYAQARALAAGAE	13
V4C1	TDVGHKKENAANVNGTVGAD	20
V4C2	TDVGHKKNAANVNGTVGAD	19
V4C3	TDVGHKKNGANVNGTVGAD	19
V4EJ	KKNGAQGTVADALLTLGYR	19
V5C1	SQASNVFQGVFLTT	14
V5C2	SQASNVFQGVFLTTPMQKD	19
V5C3	SQASNVFQGVFLTNAMQKD	19
V5C4	SQASNVFKDVFLTNAMDMQTH	21
V5C5	SQASNVSQGVFLNMAMTAH	19
V5C6	SKASNVFQGVFLTTPMQKD	19
V5EJ1	ASNVFGGVFLN	11
V5EJ2	SKASNVFGGVFLNMAMREH	19
V5J1	SKASNVFGGVFLNMAMTAH	19
V5J2	SKASNVFKDVFLNNAMDMQTH	22

V5J3	SKASNVFGRIFLNMAMREH	19
V5J4	SNASNVFGGVFLTNNMLQH	19
V6C1A	YKVLKAR	7
V6C1B	GAAVPAAADDI	11
V6C2A	PVHYKVLKARARA	13
V6C2B	ARAGAAVPAAADDIYFP	17
V6C3A	YKVLKAPALPGAAVPAA	20
V6C3B	KAPALPGAAVPAAADDI	20
V6C4A	YKVLKARARAAAAAGAG	20
V6C4B	LKARARAAAAAGAGVDIYFP	20
V6C5	PVHWKARARAGAAVPAAADDI	21
V6C6A	PVHYTVLTGPQARAGAAVPA	20
V6C6B	GPQARAGAAVPAAADDIYFP	20
V6C7	PVHYKVLKARALPGAPVPAI	21
V6C8A	YKVLKARARAGAAAGVP	20
V6C8B	GAAAGVPALPGAAADDIYFP	20
V6C9	YKVLKARARAVPPARVDI	21
V6C10A	PVHYKVLKAQARAVAAGVPP	20
V6C10B	AQARAVAAGVPPAADDIYFP	20
V7C1	YKVLKALPGAD	11
V7C2	PVHYKVLKALPGADIHFP	18
V7C3	PVHYKVLKAPAAADIHFP	18
V7C4	PVHWKALPGADIHFP	15
V7EJ1	TPPAAP	6
V7EJ2	YGGTNKKATPPAAPATKWSKE	21
V7J1	YGGTNKKATPPAAPTKWKAG	20
V7J2	YGGTNKKATPPAAPATKWKAE	21

Table III-3. Development of homologous immunity after 90-day initial infection

	Homologous challenge		Challenge of naïve rabbits		
Immunizing isolate	Lesions	DNA[1]	Lesions	DNA[1]	p-Value
Nichols Houston E (32 DS)	0/40	0/5	24/24	3/3	<0.001
Nichols Houston J (50 DS)	0/40	0/8	28/30	6/6	<0.001
Chicago C (50 DS)	1/40	1/9	26/30	6/6	<0.001

P values for the difference in the number of lesions that develop after challenge of naïve vs. previously infected rabbits were calculated using Fisher's two-tailed exact test

[1] Lesions were biopsied at day 21

Table III-4. Isolates with a full repertoire of 50 donor sites (DS) are able to superinfect

	Challenge isolate					
	Nichols Houston E (32 DS)		**Nichols Houston J (50 DS)**		**Chicago C (50 DS)**	
Immunizing isolate	Lesions	DNA[1]	Lesions	DNA[1]	Lesions	DNA[1]
Nichols Houston E (32 DS)	0/40	0/5	21/40[2]	10/10	17/40[2]	5/5
Nichols Houston J (50 DS)	0/40[3]	0/8	0/40	0/8	25/40[2]	6/8
Chicago C 50 (DS)	40/40*[2]	7/8	22/40*[2]	7/7	1/40	1/9
Naïve control	24/30	5/6	28/30	6/6	26/30	6/6

Results of homologous challenge, shaded boxes, are also shown in Table III-3

[1] Lesions were biopsied at day 21 and assayed for the presence of treponemal DNA by T47 Southern blot

[2] $p<0.001$ P values for the difference in the number of lesions that develop after homologous vs. heterologous challenge were calculated using Fisher's two-tailed exact test

[3] no significant difference compared to the homologous challenge

*Attenuated lesions

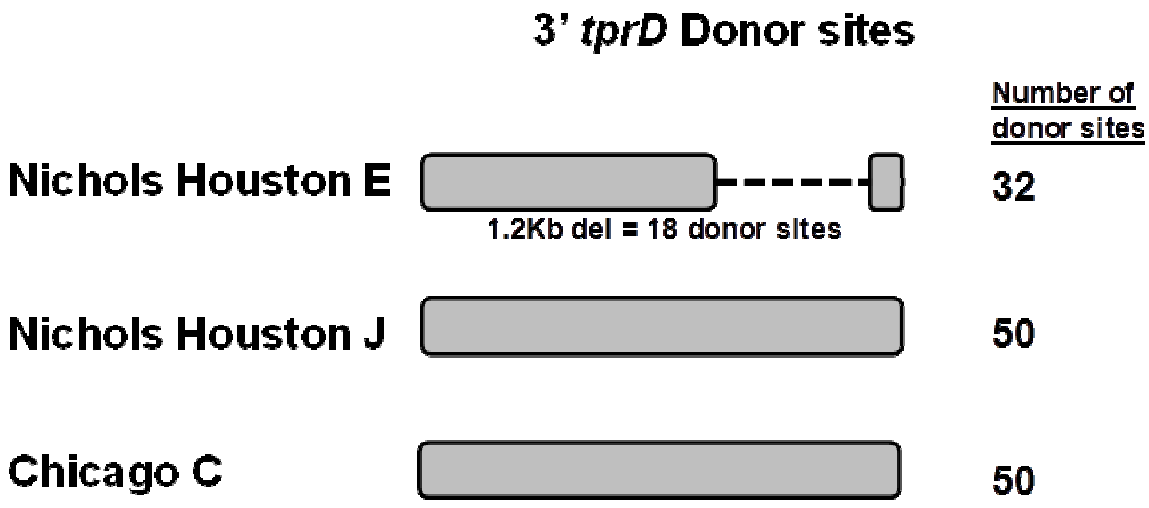

Figure III-1. Comparison of strains studied

Nichols Houston E and J were isolated from the same parent strain. Nichols Houston E has a 1.2 kb deletion in the 3' flanking region of *tprD*, resulting in 18 fewer TprK donor sites than Nichols Houston J. Chicago C was similarly isolated from its parent strain, and is identical to Nichols Houston J in both number and sequence of donor sites. Both parent strains studied are molecular strain type 14a/a, with additional identity at *tp0136*. Chicago C is reported to have 99.99% genome sequence identity with Nichols (32, 35).

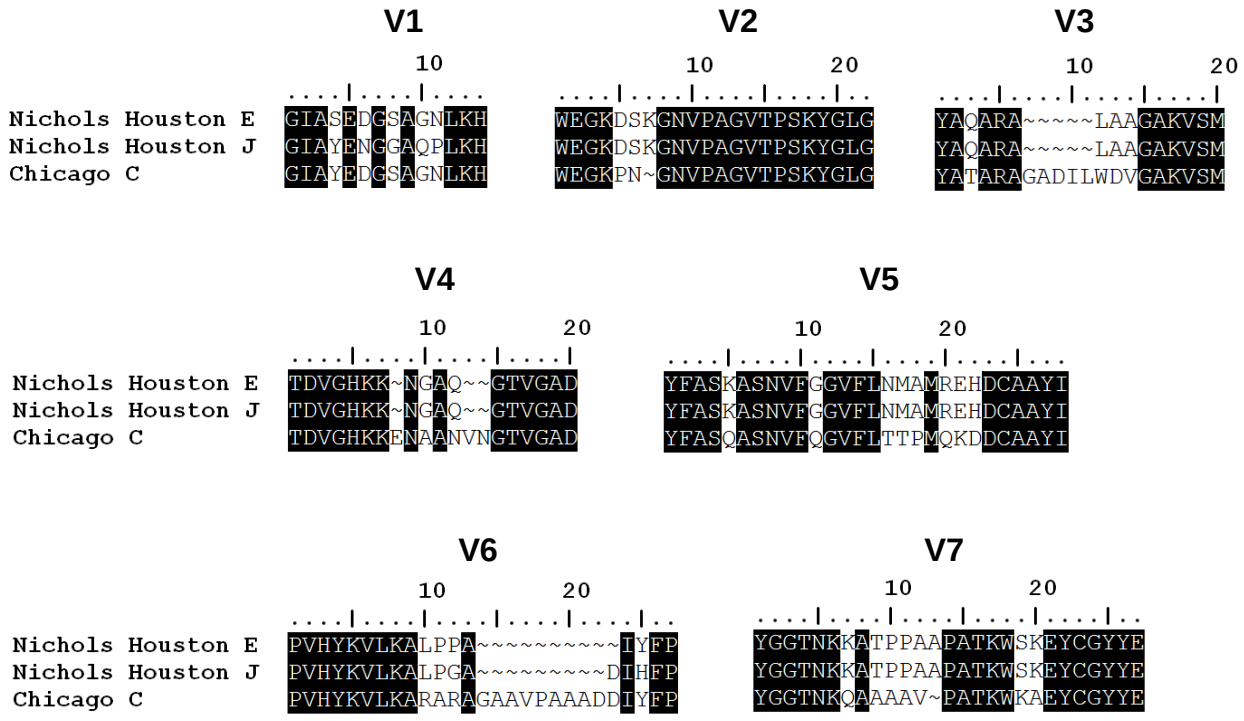

Figure III-2. Alignment of majority TprK sequences

Amino acid alignments of the variable regions are shown. Sequences represent the majority sequence in both the immunizing and challenge inocula.

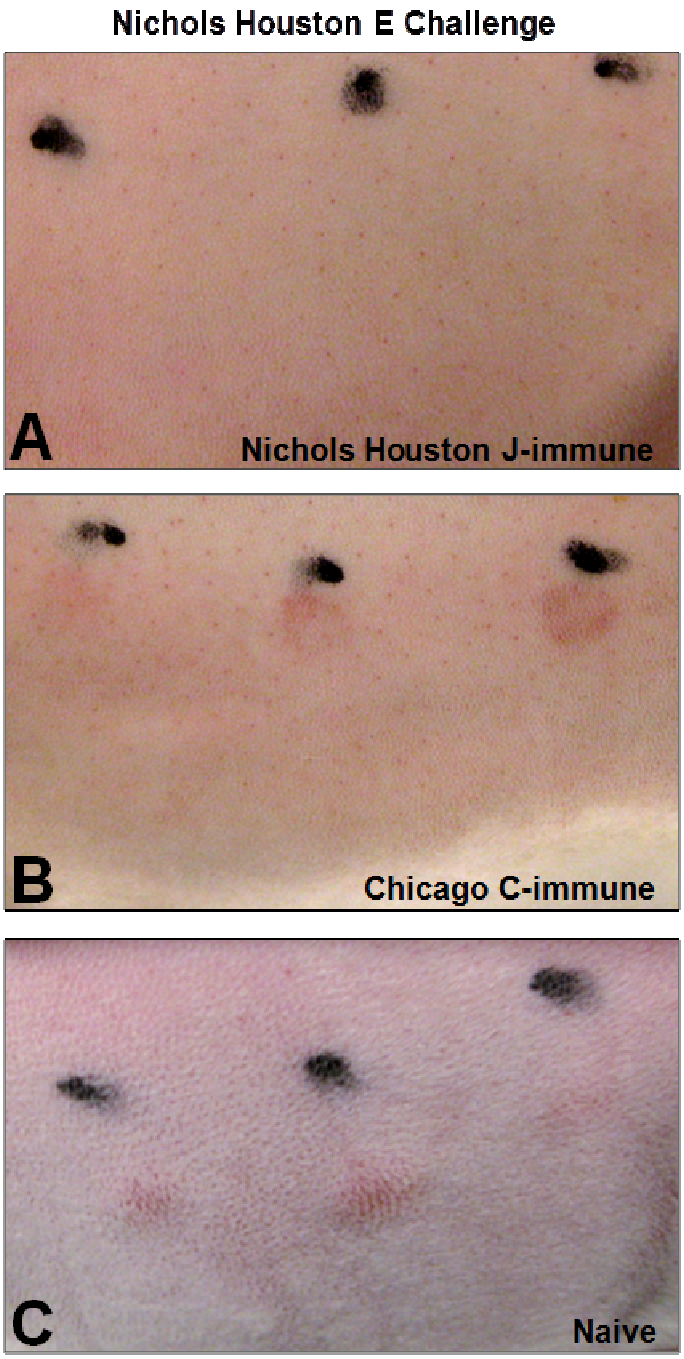

Figure III-3. Nichols Houston E challenge sites

Intradermal injection sites 3 weeks post-challenge with Nichols Houston E. Panel A) Nichols Houston J (50 donor sites)-immune rabbit with no lesion development. Panel B) Chicago C (50 donor sites)-immune rabbit with flat, erythematous lesions fading induration. Panel C) Naïve rabbit with typical lesion development with erythematous, indurated and raised lesions.

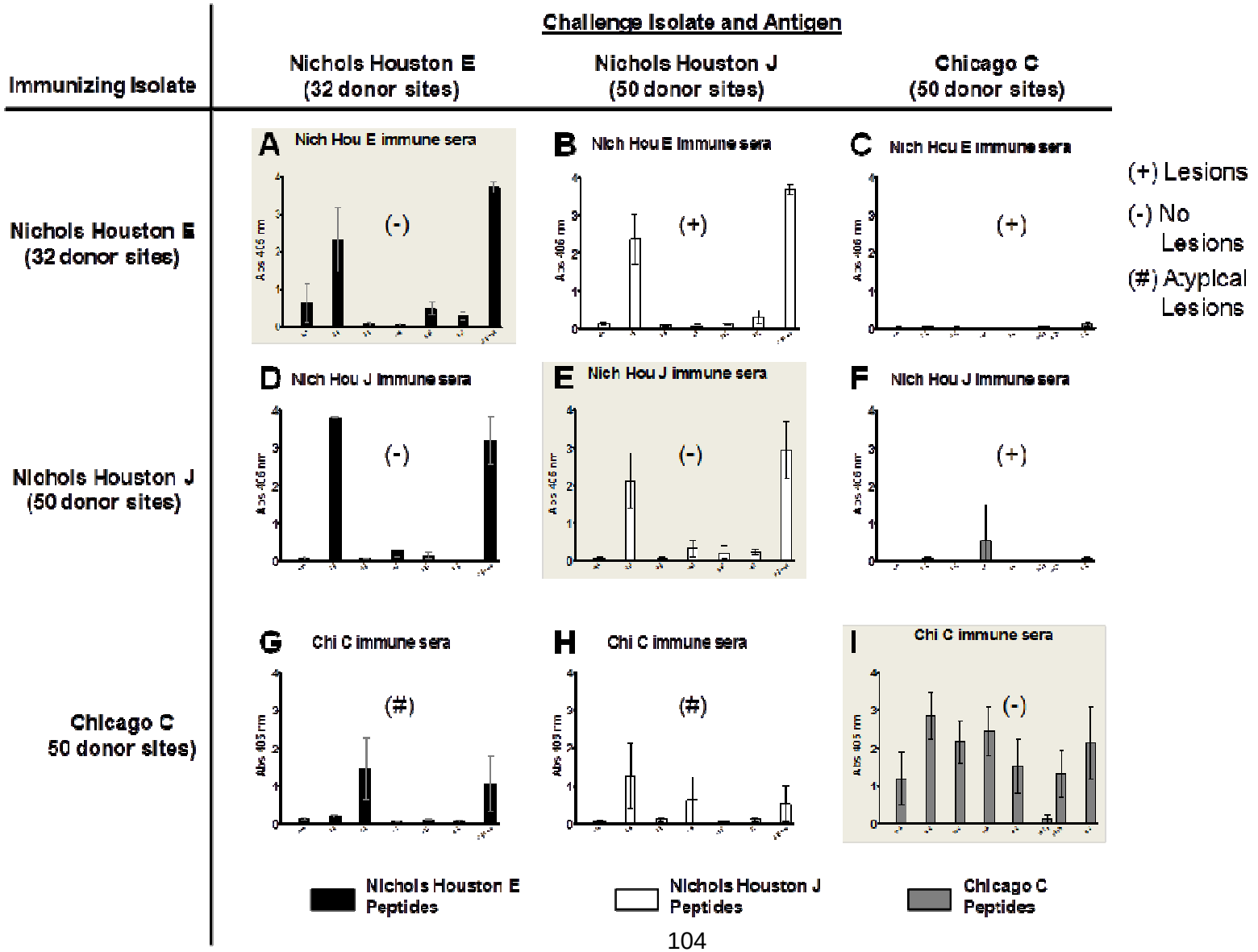
Challenge Isolate and Antigen
Immunizing Isolate
Nichols Houston E (32 donor sites)
Nichols Houston J (50 donor sites)
Chicago C (50 donor sites)
A Nich Hou E immune sera
B Nich Hou E immune sera
C Nich Hou E immune sera
D Nich Hou J immune sera
E Nich Hou J immune sera
F Nich Hou J immune sera
G Chi C immune sera
H Chi C immune sera
I Chi C immune sera
(-)
(+)
(#)
Abs 405 nm
(+) Lesions
(-) No Lesions
(#) Atypical Lesions
Nichols Houston E Peptides
Nichols Houston J Peptides
Chicago C Peptides

Figure III-4. Pre-challenge antibody reactivity

Sera collected 3 months after initial infection were tested by ELISA for antibody reactivity to synthetic peptides representing challenge inocula V regions. White bars represent reactivity to Nichols Houston J peptides, grey bars represent reactivity to Chicago C peptides, and black bars represent reactivity to Nichols Houston E peptides. Antibody responses prior to homologous challenges are highlighted by a grey background. Immunizing isolates are arranged by rows while challenge isolates are arranged by column. (-) indicates lack of lesion development, (+) indicated presence of lesions and (#) indicates presence of attenuated lesions at 21 days post challenge.

IV. Conclusions and Future Directions

The typical natural history of untreated syphilis infection begins with the development and ulceration of the chancre. These hard, indurated ulcers with necrotic centers then heal spontaneously. Experimental evidence implicates clearance of treponemes by opsonic antibodies and IFNγ-activated macrophages mediating opsonophagocytosis. After or concurrent with the resolution of the primary chancre, disseminated secondary lesions develop all over the body. The rash of secondary syphilis may appear as macular, then papular lesions of the skin (including the palms of the hands and soles of the feet) and mucous patches on mucosal membranes. These lesions also heal spontaneously by the same mechanism of opsonophagocytosis, but the rash may reappear in successive waves. After healing of the lesions of primary and secondary syphilis, an individual enters a period of latency where there are no signs or symptoms of pathology from infection. An individual can remain latently infected for a lifetime, with few if any consequences and a low risk of infecting another person. In a subgroup of untreated persons, tertiary disease manifests many years after initial infection as granulomatous inflammation, resulting in gummatous destruction of tissues.

Much of our knowledge of the natural history of syphilis has come from observations of untreated people. Such experiments are not ethical, especially after safe and effective antibiotic treatment was described (168). The study of syphilis pathogenesis in the rabbit model of infection has greatly advanced the field over the years. Rabbits exhibit almost identical skin pathology after intradermal infection as that seen in the human primary chancre. Experimental syphilis in rabbits also develops in stages, with some reports even describing central nervous system involvement (201) and tertiary disease (204). From the Sing Sing study of reinfection, we know that previously infected (both treated and untreated) people are able to be reinfected

or superinfected (59). Interestingly, there was a reduced risk of reinfection the longer the duration of the initial infection. From studies of reinfection in rabbits, we know that, after a 3 month initial infection with the Nichols strain, rabbits develop "chancre" immunity, defined by their inability to be symptomatically reinfected with the homologous strain, Nichols (102). Only certain heterologous strains are able to re infect these chancre-immune animals, as seen by the development of skin lesions upon intradermal challenge. The observations and subsequent laws of Abraham Colles and Giuseppe Profeta provide very early examples of chancre immunity in humans, in which an infected infant is unable to re infect its mother and vice versa.

The progression from primary to secondary syphilis has been puzzling because there is an established immune response involving circulating anti-treponemal antibodies. How are treponemes able to escape immune clearance during healing of the primary chancre to persist and seed secondary lesions? Similarly, how are treponemes able to establish new infections in individuals who have already mounted an immune response during previous episodes of syphilis infection? Based on the work described in this dissertation, antigenic variation is the likely means of immune evasion. And aside from phase variation of a select group of predicted OMPs (234), TprK is the only treponemal gene known to undergo antigenic variation via sequence variation during infection.

Briefly, multiple lines of evidence support TprK's role in syphilis pathogenesis by antigenic variation. The sequence changes are confined to seven variable regions (245, 267), and it is these variable regions that contain B cell epitopes, while the conserved regions contain T cell epitopes (248). Sequence changes abrogate antibody binding to the variable regions (243), and *in silico* predictions support a beta barrel structure of TprK with each variable region exposed to the host environment where antibody can select for new variants (Figure I-4). Antibodies, raised to portions of recombinant TprK, are opsonic and partially protective upon challenge. Despite the studies supporting TprK's outer membrane location and ability to

undergo antigenic variation in response to immune pressure, a single group continues to refute TprK's characterization as a variable outer membrane protein (217, 237, 239).

Obtaining a crystal structure of a membrane protein is no easy task, but would help to clarify TprK's structure and cellular topology. Specifically, determining which regions (amino vs. carboxy termini and variable vs. conserved regions) are exposed may aid in rational vaccine design. We know that T cells are important in immune clearance, and that the conserved regions contain T cell epitopes. Additionally, it would be useful to be able to identify TprK (in addition to all other predicted OMPs) on intact treponemes. Although indirect immunofluorescence using agarose microdroplets is a promising approach, the technique is still unreliable and has not been readily transferred to other laboratories. Another method employed by other researchers is heterologous expression of the gene in a cultivatable treponeme that has a similar cell wall structure, as previously described by Cameron *et al.* (118) using *T. phagedenis.* Lastly, developing a method to genetically manipulate *Treponema pallidum* would allow for mutational analysis of the TprK antigenic variation system. By completely deleting the repertoire of donor sites for *tprK* gene conversion, one could assess the mutant's ability to persist during experimental infection.

This work contributes to further understanding of syphilis epidemiology by using the basic reproductive rate (R_o) as a conceptual framework. R_o is directly proportional to three parameters specific for an infectious disease: β (the transmission efficiency), c (the number of susceptible contacts an infected individual makes); and D (duration of the infectious period). The ability of infected individuals to develop some immunity to reinfection after recovery (entering latency) greatly impacts the estimated R_o value as described by Grassly *et al.* (24). The efficiency of transmission is intrinsic to the specific etiologic agent and its route of transmission. *Treponema pallidum* has a low ID50 of about 50 organisms and a doubling time of 30-33 hours. Although there are generally more lesions present on the body during

secondary syphilis, the transmissibility of infection is similar to that found after contact with primary chancres.

The third parameter of R_o is the duration of infectiousness. Although many different pathologic mechanisms may aid in a treponeme's ability to persist in an infected person for decades, we propose that antigenic variation of TprK is the most important. Persistence permitting development of the primary to secondary stages of syphilis can more than double the length of the infectious period of the skin lesions in early syphilis. As described by Kassowitz law, transmissibility of infection declines after the early (primary and secondary) stages of syphilis. The changing of TprK exposed epitopes (on the variable regions), facilitates treponemal immune escape from opsonization by antibodies directed against the major TprK sequences found in the inoculum. As demonstrated in Chapter 2 in the rabbit model of experimental syphilis, we were able to show that individual secondary lesions are seeded from single treponemes, and that these individual treponemes are TprK variants. These TprK variants may originate as minority (less than 10% of the inoculum) subpopulations or they may develop by gene conversion during active infection. Like life rafts in an ocean of inoculum-specific anti-TprK antibody, TprK variants escape the immune response induced by the TprK expressed by the majority population in the inoculum. Because TprK is the only known treponemal gene to antigenically vary during the course of infection, we believe that the TprK antigenic variation mechanism is vital in prolonging infectiousness.

The experiments outlined in Chapter 2 stimulate many questions regarding TprK and secondary syphilis. The first is whether TprK is constitutively expressed in multiple tissues and at different times during infection. Giacani *et al.* showed that in the rabbit model of infection, *tprK* is preferentially expressed, compared to the other Tpr proteins, by multiple strains in primary intratesticular lesions (226). Expression of *tprK* is also maintained during primary intradermal lesions for at least 5 weeks post infection (247), but the possibility of a mechanism

of phase variation (as seen in other Tpr's) remains. We do not have information regarding the expressed levels of *tprK* in secondary lesions. Although we measure antibody to portions of TprK throughout infection, it is possible that there is a drastic decrease in surface expression of TprK epitopes, enabling treponemes to escape opsonization. By repeating this study and collecting lesion biopsies for RNA extraction, one could directly measure *tprK* message during the course of primary and secondary syphilis by synthesizing cDNA and targeting a conserved region with a Taqman probe for real time RT-PCR quantification.

During natural syphilis infection, patients may suffer from recurrent episodes of secondary syphilis. In the aforementioned study of secondary syphilis in rabbits, animals were followed (serologically and by keeping their backs shaved) for only 100 days. With a larger data set and a longer observation period, we may be able to detect examples of recurrent secondary lesions in the rabbit model. Should such an example develop, I hypothesize that recurrent lesions would demonstrate the characteristics described for rabbit secondary lesions: they will be seeded by new single TprK variant treponemes and they will develop in the absence of variant-specific antibody. Data from Chapter 2 suggest that variation in a single V region is sufficient for immune escape. The presence of conformational epitopes that span multiple V regions would support this claim, and utilizing native folded TprK variants as antigens in ELISA assays would allow us to compare the reactivity of linear peptides against more complex epitopes. Alternatively, the use of cyclic synthetic peptides (simulating the external V region loops predicted in Figure I-4) may allow detection of conformational epitopes by ELISA.

From our analysis of the data from secondary lesions in 10 rabbits, it became clear that a single rabbit was an outlier in that he developed far more secondary lesions compared to the other animals. Although this animal produced comparable serologic responses to lipoproteins, lipids (cardiolipin-lecithin), and TprK antigens and followed a similar course of infection (development of indurated, then ulcerating, and healing lesions), he developed a large

proportion of lesions containing inoculum TprK sequences. We hypothesize that, if lesions had been followed over time, the secondary lesions seeded by inoculum treponemes would have been cleared more quickly than those seeded by TprK variants. Because these secondary lesions were small (less than 0.5 cm in diameter), it was technically difficult to obtain a lesion biopsy for sequencing leaving enough lesion to monitor its progression or resolution. A potential method to obtain a sample for TprK sequencing while leaving the lesion intact for further observation is to take a lesion aspirate. The insertion of a fine needle into the indurated epidermis of the lesion may increase inflammation in the area, but can be controlled. The other drawback to this technique is the potential failure to collect enough treponemes to amplify TprK by PCR. Indeed, even after taking full epidermis thickness biopsies that fully encompassed the entire lesion, DNA prepared from several secondary lesions contained very little treponemal DNA, and full length TprK amplicons were unattainable.

An unexpected finding from the study of secondary syphilis in rabbits was that, among the animals followed serologically after ID infection, two (7905, 7914) developed significantly higher antibody titers to a TprC peptide compared to the other 8 rabbits. Interestingly, these two rabbits were the only ones that failed to develop any secondary lesions. Although this is meager evidence in a small sample set, it may suggest that those animals that develop higher antibody titers to TprC (a recently characterized OMP) are better able to control their treponemal load such that they do not develop secondary lesions. In their characterization of TprC, Anand *et al.* show that during experiment infection, rabbits develop antibody to the amino and carboxy termini of TprC (236). However, the sera of patients with confirmed secondary syphilis do not react to either portion of TprC (112). Additionally, sera from the same set of secondary syphilis patients failed to react to portions of another recently characterized OMP, Tp0326; the treponemal BamA orthologue (227).

These observations of the lack of reactivity to TprC in human sera suggest that patients and rabbits that develop secondary syphilis do so because they are not producing sufficient opsonic antibody to important OMPs. Further experiments should be conducted to better describe this phenomenon. Anand *et al.* proposed that only the carboxy terminus of TprC is surface exposed, although we found that the amino terminus contains targets of opsonic antibody (238). In addition to the 20aa peptide from the carboxy terminus of TprC (Figure IV-Figure *IV-1*~~1~~) that was tested here, a library of peptides spanning the entire TprC open reading frame should be tested. This may aid in clarifying the topology of TprC in the outer membrane. Furthermore, sera from rabbits who fail to produce secondary lesions but instead enter early latency should be tested by ELISA using other candidate OMPs, along with sera from patients presenting with all stages of disease (particularly early and late latency). I hypothesize that the individuals who clear their primary lesions and enter latency, will have higher titers of antibody to candidate OMPs compared to individuals with active secondary disease. It was previously reported that OMPs exist at low concentrations on the *T. pallidum* surface and that TprC is poorly expressed (280). The overall poorly antigenic nature of the *T. pallidum* outer membrane in addition to potential epitope shielding by other molecules, may account for the variability in which infected individuals mount effective antibody responses.

The third and last component of R_o is the number of susceptible individuals with whom an infected person has contact. From the Sing Sing study of reinfection, we know that, during the course of infection, individuals may develop partial immunity to repeated infection. The phenomenon of chancre immunity is described in humans, particularly where infected mothers are “immune” to reinfection by their congenitally infected infants (Colles law), while infants with congenital syphilis have immunity to reinfection as well (Profeta’s law). From the human studies of reinfection, it is also evident that early treatment results in an increased likelihood of susceptibility to repeated infection. Additionally, this finding was recapitulated in the rabbit

model of infection in which, after 3 months of infection, animals develop immunity to symptomatic challenge by the homologous strain. An important study by Turner and McLeod revealed that, after establishment of chancre immunity, only heterologous strains are able to reinfect (or superinfect) rabbits (102). Because these experiments were carried out before molecular techniques were developed and the strains are no longer available, we sought to determine the molecular determinants of treponemal repeated infection (either superinfections or reinfections after treatment).

Contemporarily, we see repeated infections in current outbreaks of syphilis among MSM. There are few documented examples in the literature of repeated infections after adequate treatment, but the use of molecular typing proves that a new strain was acquired (28, 73, 101). In *Anaplasma marginale*, Futse *et al.* demonstrated that a single unique donor cassette for the antigenically variable protein MSP2 was sufficient to allow superinfection of the host by a second strain (296, 297). In this system, the donor cassettes are analogous to the TprK donor sites that are located at the 3' and 5' flanking regions of *tprD*. Similarly, we sought to determine whether unique donor sites were sufficient to allow *T. pallidum* superinfection in the rabbit model of syphilis.

Chapter 3 describes how naturally occurring Nichols strain mutants were exploited to examine the effect that a large reduction in donor sites has on superinfection. The results showed that the strain with a reduced TprK donor site repertoire was unable to cause symptomatic superinfection in rabbits previously infected with a strain containing the complete donor site repertoire, while the strain with the full donor site repertoire was able to cause lesions in rabbits previously infected with the strain containing the diminished donor site repertoire. Additionally, from this study, we observed that it is possible for strains with identical donor site repertoires to super-infect if the challenge inoculum TprK sequence is sufficiently different from the initial infecting strain. So despite developing anti-TprK antibody and immunity to challenge

by the homologous strain (possessing a comparable OMP profile), some strains expressing a variant TprK sequence are able to establish new infectious lesions in previously infected animals. These data lead to a correlation between TprK variation and the ability of *T. pallidum* to overcome pre-existing immunity in an individual to establish a new infection. By permitting repeated infection, TprK variation increases the value of the second parameter of R_o, c (the pool of susceptible contacts which an infected person may come in contact), further increasing the reproductive rate of syphilis. This is the first study into syphilis pathogenesis utilizing *T. pallidum* mutants derived from a single parent strain (Nichols Houston E and Nichols Houston J) (288). These naturally occurring mutants were isolated using the "cloning" method developed by our group, and provide an example of how identifying and harvesting mutants can facilitate experimentation even though we cannot yet genetically manipulate *T. pallidum*.

By sequencing *tprK* expression sites from the lesions developing after superinfection, we hoped to identify which donor sites were incorporated into each variable region. We hoped that comparing the donor site usage profile back to the donor site repertoire would tell us whether utilization of unique donor sites contained in the deleted region had facilitated superinfection. However, it became clear that determining, in a non-biased way, which donor site was used is extremely difficult. This is due to the previously described mosaic pattern of gene conversion, in which theoretically small segments (few nucleotides) of donor sites or even whole donor sites can be incorporated into a *tprK* variable region (Figure III-3). Often one of several donor sites might have contributed the sequence found in the new variant, and it was impossible to know which was responsible. Utilization of a sequence searching program (SeqSearch, a gift from Dr. John Mittler) did little to address this problem, as user manipulation of the program output is still necessary, thus introducing possible bias. A more robust program that is able to provide the most parsimonious predicted donor site usage pattern would greatly advance the ability to study TprK gene conversion.

In the comparison of Nichols Houston E and Nichols Houston J, it was recognized that the parent strain contained two subpopulations, one of which possessed a large deletion in the donor sites. Giacani *et al.* provided a detailed comparison of the *tprK* gene conversion mechanism (including donor site repertoires), for different strains, subspecies and species. Aside from the large 1.2kb deletion described in the Nichols Houston parent strain, a 51 base pair insertion has been found in two strains recently isolated from patients, UW 104 and UW 126 (235). Although these few examples of changes in the donor sites are noted, there is still a very high degree of donor site conservation among syphilis strains, prompting us to wonder about the prevalence of unique donor site repertoires circulating naturally. Is *tprK* donor site usage a factor in current outbreaks of syphilis in discrete geographical regions? We propose conducting a targeted sequencing screen of multiple *T. pallidum* isolates from ongoing outbreaks. Grimes *et al.* described the introduction of a new strain type into an ongoing syphilis outbreak in MSM in Seattle [(29) and Figure I-2]. Sample sets such that described in Seattle are potentially a rich source of contemporary strains that can be examined for changes in donor site repertoire that might be responsible for replacement of one strain type with another in a small population of highly exposed individuals.

Syphilis has a complicated history in society. There are multiple examples of ethical wrong-doing centered on human experimentation, and stigmatization of this disease and other STI's persists today. In contrast to other bacterial STI's, syphilis causes a complex natural history and can persist in an individual for life, sometimes progressing to cause serious disease in the elderly person or the next generation. Furthermore, the etiologic agent itself is extraordinarily difficult to study. This is due in part to the architecture of the cell wall, which makes the Treponema genus unique among gram negative bacteria, and causes the bacteria to be extremely fragile with an unstable outer membrane during experimental manipulations. *T. pallidum*'s small genome accounts for the its limited metabolic capacity and likely contributes to

the uncultivable nature of the bacteria *in vitro*; making the use of animal models a necessity for routine propagation. Additionally there is only modest agreement on surface localization of predicted OMP's among research groups. This is partially due to the technical intricacies involved in developing and repeating assays while circumventing damage to the outer membrane. In this work, we have used our knowledge of TprK to examine, in a more clinical context, the role of antigenic variation in the complicated natural history of syphilis infection and in *T. pallidum's* success as a pathogen over the past 600 years.

Figures

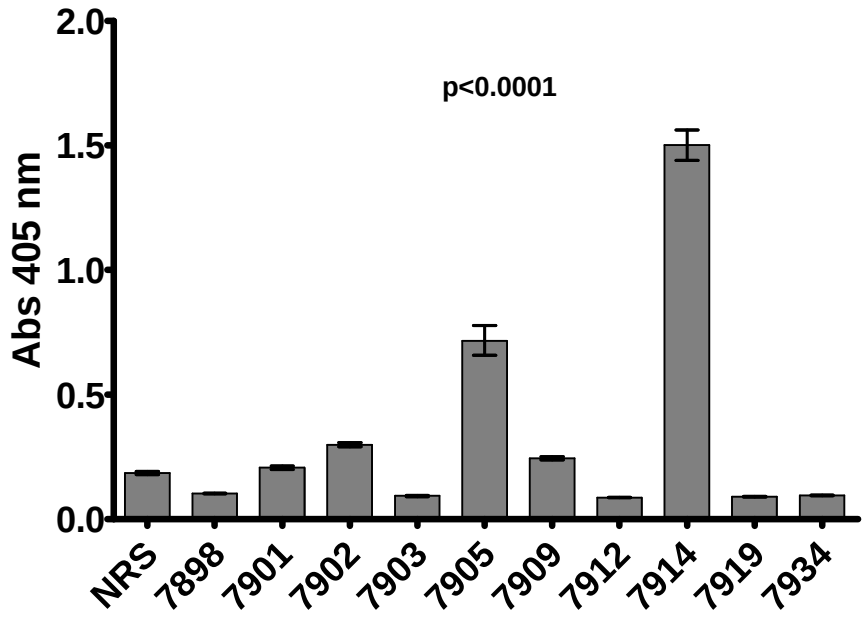

Figure IV-1. Serologic reactivity to TprC

ELISA detection of antibody reactivity to a synthetic peptide representing a portion of the carboxy terminus of TprC (sequence: QWEQGVLADAPYMGIAESIW) in serum from intradermally infected rabbits 100 days post infection. P-value determined by one way analysis of variance.

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architecture and exists as periplasmic and outer membrane-spanning conformers. J Bacteriol **195:**2060-2071.

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the *tprK* gene of *Treponema pallidum* during infection. Molecular Microbiology **52:**1579-1596.

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297. **Rodriguez JL, Palmer GH, Knowles DP, Jr., Brayton KA.** 2005. Distinctly different *msp2* pseudogene repertoires in *Anaplasma marginale* strains that are capable of superinfection. Gene **361:**127-132.

CURRICULUM VITAE

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PERSONAL DATA:
Maiden name: Tara Harriet Brinck
Place of birth: Anchorage, Alaska
Citizenship: U.S.A.

EDUCATION:

2005	B.S., Biology, University of Maryland, Baltimore County
2005-present	M.D., School of Medicine, University of Washington, Seattle
2014	Ph.D., Pathobiology, Department of Global Health, University of Washington, Seattle

POST-GRADUATE TRAINING:

2009-2012	Postgraduate Research Assistant (Graduate Student), Pathobiology Program, Department of Global Health, University of Washington, Seattle (Treponemal Research Laboratory, Sheila A. Lukehart, Ph.D.)
2011	"Bridging the Career Gap for Underrepresented Minority Scientists" workshop participant
2012-2014	Predoctoral Fellow, STD/AIDS Research Fellowship Program, University of Washington, Seattle

SPECIAL AWARDS AND HONORS:
Fellowships

2002	Juvenile Diabetes Research Foundation Scholar
2003	Project IMHOTEP Fellowship
2004	Fogarty Minority International Research Training Scholar
2001-2005	Meyerhoff Scholar
2005-present	Medical Scientist Training Program Fellow
2012-2014	Predoctoral STD/AID Research Training Fellowship

Awards

2004-2006	FASEB MARC travel award
2010-2012	NIAID Research Supplement to Promote Diversity in Health-Related Research
2010	Carl Strom travel award, Gordon Research Conference on the Biology of Spirochetes

PROFESSIONAL ORGANIZATIONS:
Endocrine Society
American Society for Microbiology
Student National Medical Association

TEACHING/TRAINING RESPONSIBILITIES:
Teaching Responsibilities

2002-2005 Math and Science tutor at Catonsville High School, Baltimore, MD
2005 Athlete tutoring in Chemistry and Biology at University of Maryland, Baltimore County Baltimore, MD
2012 UW Teaching Assistant, PABIO 536: Bioinformatics

Laboratory training of undergraduates
Eric Kim, Undergraduate Recipient of Mary Gates Award for Undergraduate Research, currently Emergency Department Technician
Stephanie Brandt, Undergraduate, currently Graduate Student, Indiana University
Mark Fernandez, Undergraduate Recipient of Mary Gates Award for Undergraduate Research

SPECIAL LOCAL RESPONSIBILITIES:
2006-2007 Treasurer, UW chapter of the Student National Medical Association (SNMA)
2007 Co-Chair, UW chapter of the Student National Medical Association (SNMA)
2006-2008 Volunteer Recruiter, NW Kidney Health Fest for African American Families

PROFESSIONAL SERVICE
Meeting organization
2012 Program Committee: selected speakers for oral presentations for 2012 Gordon Research Conference on the Biology of Spirochetes. Ventura, CA.
2007 Assisted in organizing the Region 1 Student National Medical Association Regional Conference. Seattle, WA.

Community service as a scientist
2004 SARS and Monkey Pox response team. Distributed SARS-related educational material to international travelers disembarking in Anchorage, AK from Asia.
2003-2006 Minority Access to Research Careers (MARC) Peer-Advisor, Experimental Biology conference
2006-2008 Young, Gifted and Black event panel member; discussed career opportunities in science and medicine with high school students
2007 Essence of Success; discussed career opportunities in science and medicine with high school students
2005-2008 Minority Association of Pre-Health Students (MAPS UW); discussed lab opportunities and pre-health education with UW undergraduates

RESEARCH INTERESTS
Antigenic Variation
STD pathogenesis
STI molecular epidemiology

MEDICAL INTERESTS
Women's health
Tropical medicine
Public health
Ethnic and racial health disparities

RESEARCH SUPPORT
2005-2008 USPHS/NIH T32 GM 007266. "University of Washington Medical Scientist Training Program: MD/PhD." PI: Lawrence A. Loeb.
2008-2014 USPHS/NIH R01 AI 42143. "*Treponema pallidum*: Pathogenesis-Associated Molecules." PI: Sheila A. Lukehart.
2009-2012 USPHS/NIH R01 AI63940. "Antigenic Variation of TprK." PI: Sheila A. Lukehart. Diversity Supplement
2012-2014 USPHS/NIH T32 AI 007140. "University of Washington STD & AIDS Research Training Grant." PI: Sheila A. Lukehart.

2014-present USPHS/NIH T32 GM 007266. "University of Washington Medical Scientist Training Program: MD/PhD." PI: Marshall S. Horwitz.

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Original Refereed Journal Publications

Marra CM, Sahi SK, Tantalo LC, Godornes C, Reid T, Behets F, Rompalo R, Klausner JD, Yin Y-P, Mulcahy F, Golden MR, Centurion-Lara A, Lukehart SA. 2010. Enhanced Molecular Typing of *Treponema pallidum*: Geographical Distribution of Strain Types and Association with Neurosyphilis. J Infect Dis. 202:1380-8. PMC3114648.

Giacani L, Brandt SL, Puray-Chavez M, Brinck Reid T, Godornes C, Molini BJ, Benzler M, Hartig JS,Lukehart SA, Centurion-Lara A. 2012. Comparative Investigation of the Genomic Regions Involved in Antigenic Variation of the TprK Antigen among Treponemal Species, Subspecies, and Strains. J Bacteriol.194: 4208-25. PMC3416249.

Centurion-Lara A, Giacani L, Godornes C, Molini BJ, Brinck Reid T, Lukehart SA. 2013. Fine Analysis of Genetic Diversity of the *tpr* Gene Family among Treponemal Species, Subspecies and Strains. PLoS Negl Trop Dis 7(5): e2222. PMC3656149

Reid TB, Molini BJ, Fernandez MC, Lukehart SA. 2014. Antigenic Variation of TprK Facilitates Development of Secondary Syphilis. Infect. Imm. In revision.

Reid TB, Molini BJ, Giacani L, Iverson-Cabral S, Fernandez MC, Centurion-Lara A, Lukehart SA. 2014 Repeated Infection by the Syphilis Spirochete is Facilitated by Antigenic Variation of TprK. In preparation.

Abstracts

Poster presentations

2004 "Mutations in the *aire* gene leading to APECED disease" presented at Annual Biomedical Conference for Minority Students. Dallas, Texas.

2010 "Does Local Immune Environment Affect the Rate of *tprK* Sequence Change in *Treponema pallidum*?" presented at Gordon Research conference on the Biology of Spirochetes. Ventura, CA.

2012 "Molecular determinants of *Treponema pallidum* Superinfection" presented at Gordon Research conference on the Biology of Spirochetes. Ventura, CA.

2013 "Molecular determinants of *Treponema pallidum* Superinfection" presented at Infectious Disease Society of America, IDWEEK 2013. San Diego, CA.

2014 "TprK escape variants seed lesions in secondary syphilis" presented at Gordon Research conference on the Biology of Spirochetes. Ventura, CA.

Oral Presentations

2003 "MRSA in the Alaska Native Medical Center". Arctic Investigations Program, CDC and Morehouse School of Medicine. Anchorage, AK.

2010 "Does Local Immune Environment Affect the Rate of *tprK* Sequence Change in *Treponema pallidum*?" presented at Gordon Research conference on the Biology of Spirochetes. Ventura, CA.

2014 "TprK escape variants seed lesions in secondary syphilis" invited to present at Gordon Research conference on the Biology of Spirochetes. Ventura, CA.