Monica E. Embers *Editor*

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"He who knew syphilis knew medicine"
-Sir William Osler

There was a young man from Back Bay
Who thought syphilis just went away
He believed that a chancre
Was only a canker
That healed in a week and a day.

But now he has 'acne vulgaris'
Or whatever they call it in Paris;
On his skin it has spread
From his feet to his head,
And his friends want to know where his hair is.

There's more to his terrible plight:
His pupils won't close in the light
His heart is cavorting,
His wife is aborting,
And he squints through his gun-barrel sight.

Arthralgia cuts into his slumber;
His aorta is in need of a plumber;
But now he has tabes,
And saber-shinned babies,
While of gummas he has quite a number.

He's been treated in every known way,
But his spirochetes grow day by day;
He's developed paresis,
Has long talks with Jesus,
And thinks he's the Queen of the May.

Anonymous

Introduction

Long-term survival by an infectious microbe within the host requires the ability to fend off the immune response or become undetectable. Pathogens may utilize varied strategies for this purpose, but the spirochetes are uniquely adapted to combat host immunity on several fronts to ensure persistence for long periods of time. The symptoms and disease course of these systemic pathogens reflect their ability to spread through the host without generating acute responses from immune system recognition. The pathogenic spirochetes are indeed masters of immune evasion and persistence.

The Order Spirochaetales contains three families, encompassing nine Genuses, three of which are associated with disease in humans: Borrelia, Leptospira, and Treponema. Most research has been focused on the species from which the more common diseases result: Lyme disease, Syphilis, Leptospirosis, relapsing fever, and periodontal disease. In general, these organisms are genetically complex and utilize a unique variety of virulence factors, many known and likely many yet to be discovered. Each, however, has distinctly become adapted to different environments for sustenance and transmission.

For the tick-borne *Borreliae*, immune evasion essentially begins as the spirochetes traverse the salivary glands of the tick into the mammalian host. We know that factors in tick saliva inhibit the immune response both generally, and in specific assistance to the *Borrelia* spirochetes. From that point, dissemination, tissue invasion, and seclusion, both immunological and physical, ensues. The spirochete bacteria are well adapted for tissue invasion, with their small-diameter, long-width, and periplasmic flagella. Other commonalities amongst the spirochetes are their fastidious growth in microaerophilic environments and the systemic nature of disease caused by them.

In this book, we explore the many mechanisms by which the most prevalent Spirochetal pathogens persist in a healthy immune-competent host. Among them are the direct and indirect suppression of host immune signals, phase and antigenic variation, escaping recognition by host complement proteins, and seclusion into immune-privileged sites. We also explore antibiotic therapy for control of infection. A baffling topic which warrants further research.

Much of the emphasis for this compendium on the ability of the spirochetal pathogens to evade immune responses in the host and persist falls on the Lyme disease

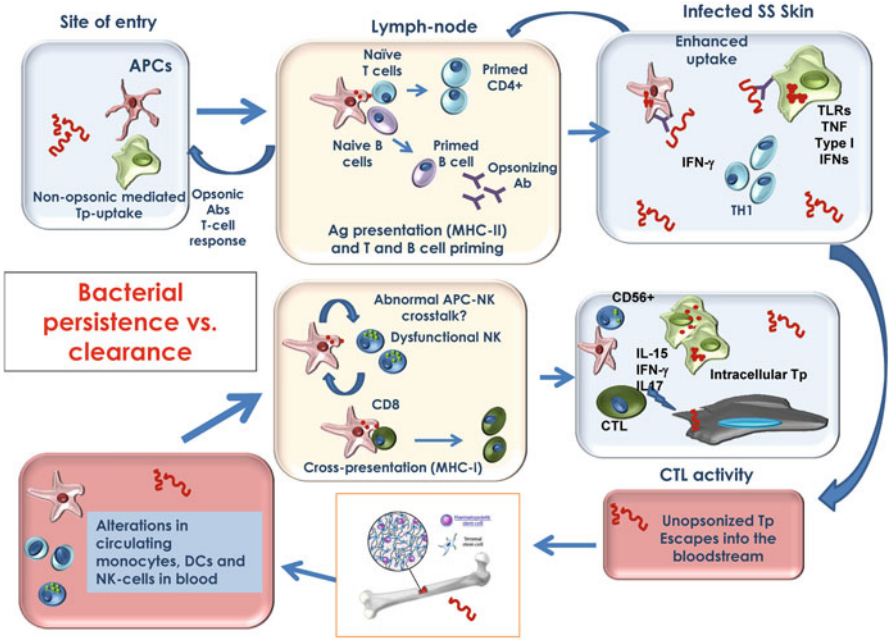

Fig. 1 Model of syphilis pathogenesis that integrates innate and adaptive immune responses to the bacterium (kindly provided by J. Salazar)

spirochete, *Borrelia burgdorferi*. This is largely because it is the most studied of the spirochetes and in part due to impact of disease caused by this organism in the USA and Europe.

The Treponemes

Spirochetes of the Treponema genus cause diseases of current and historically high prevalence worldwide, such as syphilis (*T. pallidum* subspecies pallidum) and periodontitis (*T. denticola*). Less prevalent are T. pallidum ssp. responsible for Bejel and Yaws, and *T. carateum*, responsible for the disease named Pinta in Central and South America. Research over the years on *T. pallidum* has revealed multiple mechanisms of immune evasion, affecting the host response at many levels.

The progression of syphilis follows several different phases (primary, secondary, and tertiary) with variation amongst individuals. At each level of infection, a shift in the balance of immune response versus immune evasion by *T. pallidum* can lead to either clearance or persistence. Using past and current research findings, Dr. Juan C. Salazar has proposed a model of immunologic events in the early stages of syphilis. As shown in Fig. 1, phagocytosis by antigen-presenting cells (APCs) is central to the immune response against *T. pallidum.* After primary infection, spirochetes

replicate at the site, elude the innate response, and rapidly disseminate to the skin and other tissues. Initial uptake by phagocytic cells is inefficient due to absence of opsonic antibodies, but the small number that have phagocytosed spirochetes migrate to the draining lymph nodes where they present antigen to naïve T and B cells. Newly sensitized T helper cells return to the primary lesion (chancre), where they recognize *T. pallidum* antigens on APCs and secrete interferon-γ (IFN-γ), which activates tissue macrophages. Concomitant increases in opsonic antibodies facilitate phagocytosis and clearance, resulting in chancre resolution. Meanwhile, treponemal spirochete densities at other sites in the skin begin to increase and trigger local inflammatory responses, resulting in the skin lesions characteristic of secondary syphilis. While primed $CD4^+$ and $CD8^+$ T cells, along with opsonic antibodies may be present, the outer membrane of the spirochete provides few antigenic targets and some treponemal subpopulations may become resistant to opsonophagocytosis. The predominance of $CD8^+$ T cells in secondary syphilis lesions may indicate a misdirected cellular immune response resulting from inefficient $CD4^+$ T cell priming in the lymph nodes. Lack of effective opsonization will, thus, prevent uptake by tissue-based macrophages, leading to further dissemination through the bloodstream to other end organs and tissues, including the bone marrow. In this compartment, *T. pallidum* may affect development of monocytes, dendritic cells, and natural killer (NK) cells. Over time, the generation of more antigen-specific T cells, IFN-γ-producing $CD56^+$ NK cells, and treponeme-specific opsonic antibodies may then lead to control of spirochete infection.

Four chapters of this book relate to the Treponema species. Two chapters are dedicated to research findings on the mechanisms of invasion and establishment of infection by the agents of Syphilis (S. Houston and C. Cameron) and periodontal disease (J. McDowell and R. Marconi). The subject of molecular genetic variation of a specific antigen, TprK, by the *T. pallidum* spirochete is included in a subsequent chapter by L. Giacani and A. Centurion-Lara. Finally, the potential for syphilis spirochetes to resist antibiotic treatment is explored in a chapter authored by L. Stamm.

The Leptospires

Leptospirosis is the most common zoonosis worldwide, but is relatively rare in humans, with 100–200 cases in the USA (>50% in Hawaii); however, this is probably an underestimate. The disease is caused by the spirochete *Leptospira interrogans* (multiple serotypes), which infects via invasion of intact mucous membranes or abraded skin. Leptospirosis was first described by Adolf Weil in 1886 as an "acute infectious disease with enlargement of spleen, jaundice and nephritis." Interestingly, leptospirosis was thought to be a primary cause of death in Native Americans in 1620 (present-day Massachusetts) before the arrival of Pilgrims. A comprehensive and elegantly written chapter by S. Faisal, S. McDonough, and Yung-Fu Chang describes the disease and pathogenic mechanisms utilized by *L. interrogans*.

The Borreliae

The Lyme disease spirochete. While pathogenic spirochetes are found worldwide, those associated with Lyme disease are prevalent in Europe and the USA, with emergence in the Asian continent. Even within the USA, the disease appears to be slowly expanding from northern States to the middle and southern USA. New species have also been isolated and identified, such as *Borrelia lonestari* (Fig. 2) and *Borrelia bissettii* from the southern USA. However, their actual pathogenicity and prevalence remain to be elucidated.

Since the discovery of the cause–effect relationship between *B. burgdorferi* and Lyme disease, only a few studies have probed into the acquired immune response to this pathogen specifically. Very recent studies have uncovered not only multiple mechanisms utilized by the pathogen to escape immune recognition but also a disconcerted response to infection of mice, specifically with respect to the B cell response.

It has become clear that protection afforded by experimental animal models against *B. burgdorferi* relies on the humoral (antibody) response (Barthold and Bockenstedt 1993; Barthold 1999; Fikrig et al. 1994; McKisic and Barthold 2000; Belperron et al. 2007). In several of these studies, passive transfer of immune serum either prevented infection or controlled immune-mediated disease pathology. However, while *B. burgdorferi* antigens generate antibodies that can be protective, the adaptation and selection of spirochetes throughout the course of infection preclude clearance by the host antibody response. This is due in large part to molecular adaptation by these spirochetes. A gene array analysis of the mRNA expression of 137 putative lipoprotein genes revealed two phases of expression (Liang et al. 2002). At primary infection, prior to the generation of significant antibody titers, most (116 of 137) lipoprotein genes were transcribed. In the second phase, at the advent of the humoral response, between 17 and 30 days post-infection, transcription was downregulated for most of these genes. This result suggests that immune selection or signaling may drive the molecular adaptation. More recent results from the Liang lab indicate that *Borrelia burgdorferi* lipoproteins may serve to protect the spirochetes from innate responses. In fact, the exact identity of the lipoprotein constitution may not be as important as the simple presence of the lipoprotein cloak itself (Xu et al. 2008).

As the story of immune evasion by *B. burgdorferi* unfolds, it appears that this spirochete has somehow developed the means to delay a proper immune response, allowing its molecular adaptation and dissemination to occur, unrecognized. The absence of immunity to reinfection by *B. burgdorferi* in immune-competent hosts (Piesman et al. 1997; Nowakowski et al. 2003) prompted inquiry into the apparent inability of these hosts to generate an effective memory response. Early infection with *B. burgdorferi* was shown to elicit hypercellularity in the draining lymph nodes of infected mice (Fig. 3), seen as lymphadenopathy (Tunev et al. 2011). This was the result of abundant B cell proliferation, which occurred without concomitant T cell proliferation. A testament to the uncanny immune evasive tactics of this pathogen was their accumulation within the lymph node during this intense response. In terms of the specificity of antigen and isotype, abnormally high proportions of antibodies were IgM and they targeted select few dominant *B. burgdorferi* antigens.

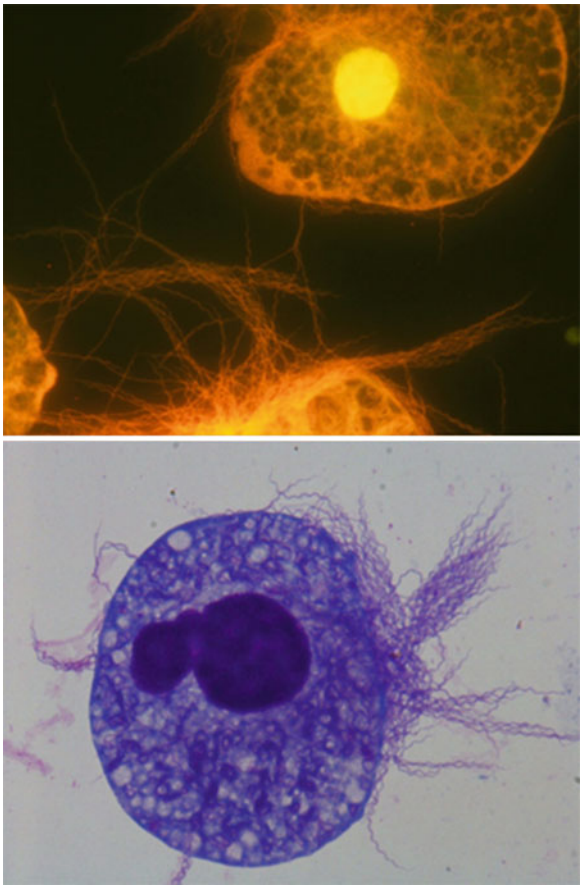

Fig. 2 Bundles of Borrelia lonestari spirochetes grown with AAE2 (Amblyomma americanum embryonic) tick cells. Preparations were stained with acridine orange (top) or Diff-Quick (bottom). Photos courtesy of Dr. Andrea Varela-Stokes, Mississippi State University

The authors of this work have probed further into the phenomenological ineffective B cell response to *B. burgdorferi*. Recent studies by the N. Baumgarth lab, in collaboration with S. Barthold have again found ample B cell accumulation in mice in response to infection with *B. burgdorferi,* much of which occurs without a strong induction of germinal centers. Looking at the response over time, the authors found that T-cell dependent B cell responses in germinal centers do occur, but that they are slow to develop and decline rapidly in comparison to what is seen with other infections, despite the continued presence of *B. burgdorferi* antigens (Hastey et al. 2012). So, not only is there a predilection towards the generation of low-affinity IgM responses, but the T-dependent response may be delayed to a point that host adaptation by the spirochete likely supersedes the generation of antibodies that would be effective at clearing the pathogen.

Relapsing Fever (RF) spirochetes. The relapsing fever spirochetes can be transmitted by either the body louse (epidemic RF) or by soft ticks (endemic RF). For

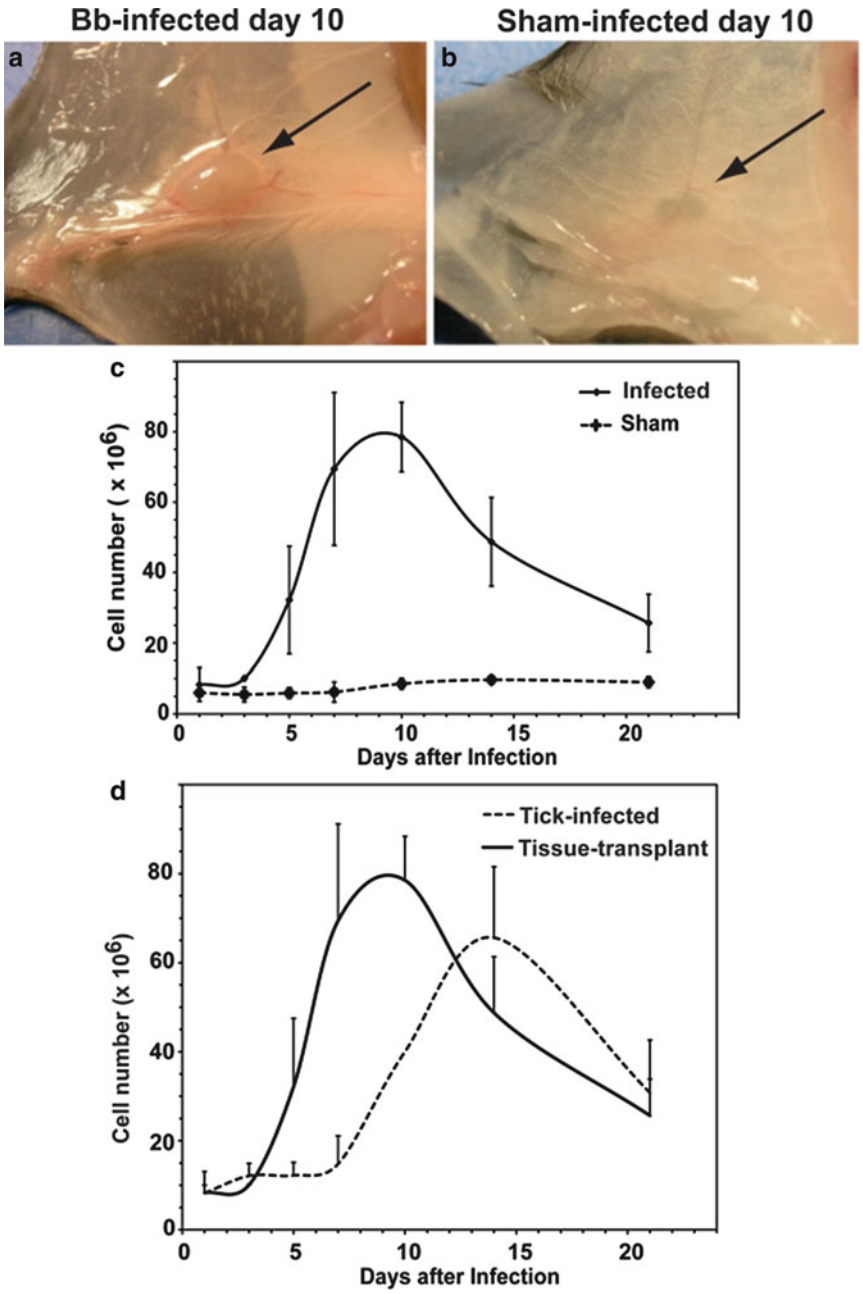

Fig. 3 Infection with host-adapted *B. burgdorferi* induces lymphadenopathy near the site of infection. Lymphadenopathy of the right inguinal lymph node of C57BL/6 mice 10 days after infection via subcutaneous transplantation of small pieces of ear from *B. burgdorferi*-infected (**a**) or noninfected (**b**) congenic SCID mice into their right dorsal tarsal region. (**c**) Shown are mean cell numbers ± SD of right inguinal lymph nodes from groups of four mice per time point. (**d**) Comparison of lymph node cellularity (mean ± SD) obtained from draining lymph nodes in (**c**) and from the axillary lymph nodes of tick-infected mice. Reproduced from (Tunev et al. 2011) with permission from PLoS Pathogens

one of the more common agents of RF in the USA, *B. hermsii,* a New World relapsing fever species, approximately 30 serotypes have been derived from a single bacterial cell. The serotype of a Borrelia cell depends on its major surface antigen. The 30 or so antigens are divided approximately equally between two families: Variable Large Proteins (Vlp) of approximately 36 kDa and Variable Small Proteins (Vsp) of approximately 20 kDa. In Chapter 9, we (Embers and Lopez) discuss RF prevalence and disease, along with host immune responses and mechanisms for antigenic variation by the spirochetes, including gene conversion/full or partial, rearrangement, and hypermutation.

Seven chapters of this book are dedicated primarily to the Borreliae. Covering seclusion by *B. burgdorferi* into immune-privileged sites and the establishment of chronic (untreated) infection is a chapter by Robert Gilmore Jr. With regard to active evasion of host immunity, the role of complement-binding proteins (Peter Kraiczy and Reinhard Wallich), antigenic variation (Troy Bankhead) and modulation of immune mediators (Dennis, Gautam and Dixit) are discussed. Finally, we (Embers and Barthold) examine the topic of persistence by the Lyme disease spirochete following antibiotic therapy.

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Contents

Part I
Invasion and Dissemination

Chapter 1
Treponema pallidum Dissemination; Facilitating Immune Evasion and Bacterial Persistence

Simon Houston and Caroline E. Cameron

1.1 Dissemination Potential of *Treponema pallidum*

1.1.1 T. pallidum, the Causative Agent of Syphilis

Syphilis is a chronic multistage disease, usually transmitted sexually or in utero, which affects more than 12 million people globally each year and is caused by the bacterium *T. pallidum* subsp. *pallidum*. Although developing nations account for the majority of new syphilis infections (Gerbase et al. 1998), rapidly increasing syphilis infection rates have also been observed in Europe (Righarts et al. 2004), Australia (Jin et al. 2005), and North America (Kent and Romanelli 2008) over the course of the past decade. Two additional ways in which syphilis infections impact health are congenital syphilis, which remains a major health concern in developing nations and results in spontaneous abortion, stillbirth, postpartum death or significant newborn malformations (Walker and Walker 2007), and the well-documented capacity of *T. pallidum* infection to significantly increase the risk of HIV transmission and acquisition (Nusbaum et al. 2004).

T. pallidum is a spirochete bacterium with a small genome (~1.14 Mbp) encoding approximately 1,040 proteins (Fraser et al. 1998; Matejkova et al. 2008). The bacterium is inherently fragile due to its unusual envelope arrangement in which the peptidoglycan layer is found within a cytoplasmic-membrane-proximal location rather than occupying the typical outer-membrane-proximal location as in conventional Gram-negative bacteria (Izard et al. 2009; Liu et al. 2010). This unusual ultrastructure and associated extreme fragility make laboratory manipulation of this bacterium exceedingly difficult. Further, the bacterium is an obligate human pathogen which

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to date has not been successfully cultured continuously in vitro. The combination of experimental limitations associated with research on this pathogen and lack of conserved virulence factors that can be easily identified via bioinformatic analyses of the *T. pallidum* genome has hindered the identification of virulence factors that contribute to the infection process of this pathogen.

1.1.2 The Highly Invasive Nature of T. pallidum

The highly invasive nature of *T. pallidum* has been well established through numerous in vitro and in vivo laboratory studies (Cumberland and Turner 1949; Raiziss and Severac 1937; Riviere et al. 1989; Thomas et al. 1988, 1989). The widespread clinical manifestations associated with syphilis infection further emphasize the highly invasive nature of *T. pallidum*. Establishment of the initial localized infection is usually made evident by the appearance of a painless lesion, known as a chancre. It has also been demonstrated in vivo that the bacteria are capable of invading the tissue barrier and undergoing rapid and widespread dissemination via the circulatory system within hours of exposure (Cumberland and Turner 1949; Raiziss and Severac 1937). The highly invasive nature of *T. pallidum* is further underpinned by the fact that the pathogen can be detected in several sites distal to the primary infection site, including the kidneys, liver and central nervous system (CNS), during the early stages of syphilis. *T. pallidum* is also one of only a few highly invasive pathogens capable of traversing the vascular basement membrane of the brain (the blood–brain barrier; BBB), which is composed primarily of collagen type IV and laminin, but also contains collagen type I, entactin, fibronectin, perlecan, and vitronectin (Carbonell et al. 2009).

1.1.3 Spread of T. pallidum During Each of the Stages of Infection

There are four stages of syphilis: primary, secondary, latent, and tertiary. During primary syphilis, *T. pallidum* replicates at the site of infection and forms a single chancre which spontaneously heals within 3–8 weeks (Baughn and Musher 2005; LaFond and Lukehart 2006). Despite this formation of a characteristic, localized lesion, in vivo studies have demonstrated that *T. pallidum* does not remain localized at the site of infection but instead is able to enter the bloodstream minutes after experimental inoculation (Cumberland and Turner 1949; Raiziss and Severac 1937) followed by widespread dissemination within hours (Collart et al. 1971). Consistent with these observations, by the secondary stage of infection *T. pallidum* can be found to affect multiple organs and tissue sites, including the skin, oral cavity and tongue (Mindel et al. 1989), stomach (Greenstein et al. 1994), kidney (Bansal et al. 1978; Tourville et al. 1976), liver (Hira et al. 1987; Lee et al. 1971; Mindel et al. 1989), spleen (Hira et al. 1987; Mindel et al. 1989), and CNS (Chapel 1980; Lukehart et al.

1988; Rolfs et al. 1997). Latent syphilis is divided into two phases: (1) early latent syphilis, which comprises the year following secondary syphilis infection and which may involve a relapse of secondary symptoms, and (2) late latent syphilis, an asymptomatic period of infection lasting for at least 1 year (Kent and Romanelli 2008; LaFond and Lukehart 2006). The tertiary stage of syphilis, which occurs in approximately 30 % of untreated cases, further emphasizes the highly invasive nature of the pathogen, as the disease produces widespread clinical manifestations, including cardiovascular syphilis, neurosyphilis, and gumma formation which most commonly affects the skin, bone, and liver (Singh and Romanowski 1999). Furthermore, several studies have successfully demonstrated the presence of *T. pallidum* (or *T. pallidum* DNA) in cerebrospinal fluid (CSF) of patients with neurosyphilis (Lukehart et al. 1988; Marra et al. 2004), tertiary stage gummas (Handsfield et al. 1983; Zoechling et al. 1997), and tertiary stage syphilitic aortitis (O'Regan et al. 2002).

1.2 Spread of *T. pallidum* to Immunologically Privileged Sites

One mechanism by which *T. pallidum* may evade the immune system and promote chronic infection is through dissemination to, and invasion of, immunologically privileged sites. These are body sites in which there is a reduced or absent innate immune response, and include the brain, eyes, placenta, fetus, ovaries and testicles. It is believed that immune privilege is critical for the protection of specific vital organs where an inflammatory immune response would result in organ destruction and failure (Ferguson et al. 2002). In vivo animal studies have demonstrated that *T. pallidum* is capable of infecting known immunologically privileged sites, including the brain (Collart et al. 1971; Rosahn et al. 1948), testes (Sell et al. 1980), and eyes (Marra et al. 1991). PCR analyses have also confirmed the presence of *T. pallidum* in the eye (Muller et al. 2007; Rajan et al. 2006) and CSF (Burstain et al. 1991; Hay et al. 1990; Marra et al. 1996; Noordhoek et al. 1991) of syphilis patients. This capability may allow the pathogen to take advantage of the reduced immune surveillance and subsequently aid bacterial survival for extended periods of time in widely distributed anatomical sites. Bacteria residing in these immunologically privileged sites may explain, at least in part, the capacity of *T. pallidum* to persist during latency despite the induction of a vigorous immune response within non-immunologically privileged host sites, and would provide a reservoir of replicating bacteria that could contribute to the recurrences of spirochetemia that are observed during early latency.

1.3 Mechanisms of Dissemination

Numerous studies have demonstrated that *T. pallidum* is capable of invading the tissue barrier and undergoing rapid widespread dissemination via the circulatory system (Cumberland and Turner 1949; Mahoney and Bryant 1934; Raiziss and Severac 1937; Riviere et al. 1989; Sell et al. 1980; Thomas et al. 1988, 1989).

Although the virulence factors involved in *T. pallidum* dissemination remain to be definitively identified, several mechanisms have been proposed to be responsible for this striking aspect of treponemal pathogenesis.

1.3.1 Attachment Mechanisms

Bacterial attachment to host components is an essential early step in dissemination and invasion of many pathogenic bacteria, including *T. pallidum*. One common mechanism by which pathogenic bacteria interact with their host is through the expression of surface-exposed adherence factors or adhesins. Adhesins that have been implicated or proven to be involved in host component interactions include fimbriae, surface polysaccharides (including bacterial capsules) and, as may be the case with *T. pallidum*, surface exposed outer-membrane proteins.

1.3.1.1 Adhesins and Host Attachment

T. pallidum has been shown to bind to several mammalian cell types (Baseman and Hayes 1980; Fitzgerald et al. 1975, 1977; Hayes et al. 1977; Thomas et al. 1988, 1989) as well as isolated components of the extracellular matrix, including collagen, laminin, and fibronectin (Cameron 2003; Fitzgerald and Repesh 1985; Fitzgerald et al. 1984; Thomas et al. 1985). Data regarding the molecular identity and contribution of *T. pallidum* adhesins to host–pathogen interactions and dissemination have only recently begun to emerge. Palzkill and colleagues (Brinkman et al. 2008) identified the treponemal protein Tp0136 as a fibronectin-binding adhesin that elicits a strong immune response during human and experimental animal infection and exhibits extensive sequence polymorphism among *T. pallidum* subspecies and strains. An additional study used in silico analysis of the sequenced *T. pallidum* genome (Fraser et al. 1998) to identify 27 potential outer membrane proteins (Cameron 2003), 10 of which were investigated further based on their predicted high likelihood of surface exposure and possible role as adhesins (Cameron 2003; Cameron et al. 2004). Two of the proteins, Tp0155 and Tp0483, when purified in their recombinant forms, were found to bind in a dose-dependent manner to matrix fibronectin and matrix and soluble fibronectin, respectively. Furthermore, the two proteins inhibited attachment of *T. pallidum* to fibronectin-coated slides (Cameron et al. 2004). An additional *T. pallidum* adhesin, Tp0751, was identified and shown to bind specifically to a variety of laminin isoforms (Cameron 2003), abundant glycoprotein components of the basement membrane that underlies endothelial cells. Heterologous expression of Tp0751 on the surface of the non-pathogenic spirochete *Treponema phagedenis* was also shown to confer laminin binding on this non-adherent spirochete (Cameron et al. 2008). More recently it has been shown that Tp0751 is capable of binding human fibrinogen (Houston et al. 2010), a key structural protein that is integral to the process of blood coagulation. Under normal

physiological conditions within the host, fibrin clot formation is induced upon conversion of soluble fibrinogen to insoluble fibrin via activation of thrombin by the coagulation cascade (Levi et al. 2004). It has been reported that this process is likely to play an important role in bacterial containment and inhibition of bacterial dissemination (Rivera et al. 2007). Similar to other invasive bacteria (Chang et al. 2005; Liu et al. 2007), the interaction of *T. pallidum* with coagulation proteins such as fibrinogen may contribute to the rapid widespread dissemination of this pathogen by interfering with this host defence mechanism to limit the spread of infectious agents. Collectively these findings suggest that *T. pallidum* is capable of attaching to a broad range of host components that have a widespread anatomical distribution, a necessity for such a highly invasive bacterium.

1.3.2 Motility

For many flagellated pathogenic bacteria, motility is an important virulence factor which aids in bacterial colonization, nutrient acquisition, and dissemination of infection (Ottemann and Miller 1997). Similar to other spirochetes, *T. pallidum* exhibits a corkscrew-like motility through rotation around its longitudinal axis. Unlike the surface-exposed flagella found in other motile bacterial species, *T. pallidum* cellular propulsion is accomplished through a motility system that is unique to spirochetes whereby the flagellar filaments are located in the periplasmic space between the inner and outer membranes (LaFond and Lukehart 2006). The *T. pallidum* flagellar filaments are composed of several proteins, including the sheath protein, FlaA, which overlies the core proteins FlaB1, FlaB2, and FlaB3 (Cockayne et al. 1987; Norris et al. 1988; Radolf et al. 1986). This unusual structural arrangement facilitates treponemal immune evasion through sequestering of these highly immunogenic motility proteins within a subsurface cellular locale. In addition, the corkscrew-like motility promotes movement through more viscous material compared to externally flagellated bacteria (Charon and Goldstein 2002). In turn, this virulence attribute contributes to enhanced tissue penetration, dissemination of infection, and invasion of a wide variety of distant host sites (Berg and Turner 1979; Liu et al. 2010; Norris 1993).

1.3.3 Ultrastructure

T. pallidum is characterized by several unusual ultrastructural features which may aid in dissemination of infection, evasion of the immune response and bacterial persistence.

1.3.3.1 Low Protein Content in Outer Membrane

It has been demonstrated that the outer membrane of *T. pallidum* is relatively immunologically inert compared to other pathogenic bacteria, a characteristic

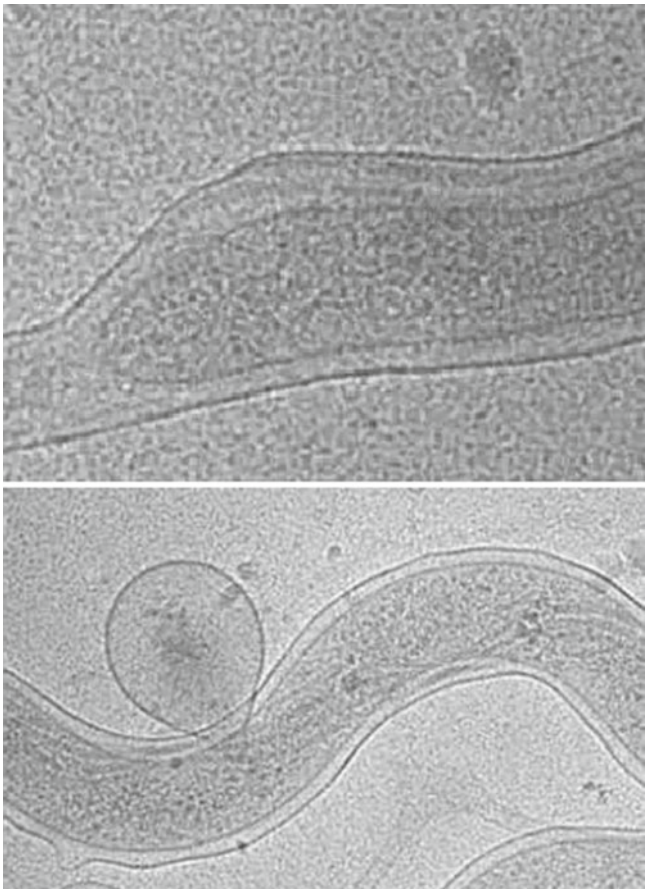

Fig. 1.1 Cryo-electron micrographs of *T. pallidum* show outer membrane (OM) blebs (*bottom*), and the bulge in the OM over a basal body (*top*). Provided by Justin Radolf

which is thought to be responsible for the ability of this "stealth" pathogen to establish long-term latency (Cameron 2005; Radolf 1995). The underlying mechanism responsible for the lack of immunological reactivity observed with the *T. pallidum* outer membrane (Fig. 1.1) has been investigated using several elegant microscopy techniques, including freeze-fracture electron microscopy and cryo-electron tomography (Fig. 1.2) (Cox et al. 1992, 2010; Izard et al. 2009; Liu et al. 2010; Radolf et al. 1989; Walker et al. 1989). These studies have confirmed that there is a paucity of integral outer membrane proteins on the surface of this bacterium, with freeze-fracture investigations providing evidence for approximately 100-fold fewer integral membrane proteins present on the surface of *T. pallidum* compared to *Escherichia coli* (Walker et al. 1989). The use of in vitro *T. pallidum* immobilization assays combined with freeze-fracture investigations has shown that aggregation of outer membrane proteins in the presence of immune rabbit

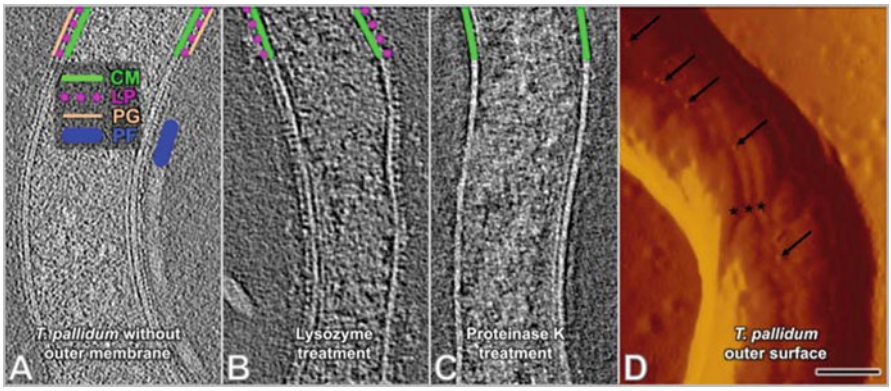

Fig. 1.2 Confirmation of *T. pallidum* peptidoglycan layer, cytoplasmic membrane surface proteins, and rare outer surface proteins. Cells in all *panels* were previously treated with distilled water to remove the outer membrane and expose subsurface structures. (**a**) Cells without subsequent enzyme treatment. Locations of the cytoplasmic membrane (*green line*), surface proteins (*purple circles*), peptidoglycan (*orange line*), and a periplasmic flagellum (*blue line*) are shown. (**b**) With lysozyme treatment. The peptidoglycan layer was removed, whereas the putative lipoproteins (*purple circles*) are still visible on the outer surface of the cytoplasmic membrane. (**c**) With proteinase K treatment, the layer of putative lipoproteins was removed. (**d**) Presence of intramembranous particles on the outer surface of *T. pallidum*, as revealed by scanning probe microscopy (SPM). Freshly prepared, Percoll-purified *T. pallidum* were applied to a mica surface, air-dried, and visualized by SPM. A portion of one *T. pallidum* cell is shown using the amplitude mode. Small protrusions are visible on the outer surface (*arrows*) and often are located on the bulge in the outer membrane created by the underlying periplasmic flagella (*asterisks*). Scale bar is 100 nm. Reproduced from (Liu et al. 2010) with permission from the Journal of Molecular Biology

serum requires an extended period of 16 h, which is a considerably longer timeframe than observed for similar aggregation within other bacteria. (Blanco et al. 1990). This emphasizes the rare nature of outer membrane proteins within *T. pallidum*. Given that surface-exposed proteins represent the first antigens to be targeted by the immune system following infection, these findings help explain the immunologically inert nature of the *T. pallidum* outer membrane. The presentation of this unusual bacterial surface to the host would directly aid evasion of the immune response and, accordingly, would facilitate bacterial dissemination and establishment of persistent infection.

1.3.3.2 Lipoproteins, Lipopolysaccharide and the Proinflammatory Response

One way in which proteins are often found attached to the bacterial surface, including within the related spirochetes *Leptospira* and *Borrelia*, is through outer membrane anchorage via an N-terminal lipid-modified cysteine residue (Haake 2000). Whole genome sequencing of *T. pallidum* originally indicated the presence of only

22 putative lipoproteins compared to 105 in *Borrelia burgdorferi* (Fraser et al. 1998). More recently, computational analyses and in silico studies indicate that there are 46 potential lipoproteins in the *T. pallidum* genome, whereas the genome of *B. burgdorferi* was shown to contain 127 putative lipoprotein genes (Setubal et al. 2006). Recent studies using high resolution cryo-electron tomography suggest that, unlike the situation within the related spirochetes *Leptospira* (Haake and Matsunaga 2010) and *Borrelia* (Liu et al. 2009), the majority of lipoproteins found within *T. pallidum* are localized to the outer leaflet of the cytoplasmic membrane (Izard et al. 2009; Liu et al. 2010). To date only two predicted lipoproteins have been suggested to be surface exposed within *T. pallidum*. The first, Tp0136, is a fibronectin-binding protein that was suggested to be surface-exposed through a combination of immunofluorescence and immunoelectron microscopy (Brinkman et al. 2008), although an independent investigation reported discordant results for surface exposure of this protein within *T. pallidum* (Cox et al. 2010). The second predicted lipoprotein, Tp0751, is suggested to be surface-exposed through the functional capacity of this protein to bind to host components laminin (Cameron 2003) and fibrinogen (Houston et al. 2010), and through the observation that heterologous expression of Tp0751 on the surface of the related spirochete *T. phagedenis* induces lipidation of this protein (Houston et al. 2010). Although the definitive answer of whether these predicted lipoproteins are surface-exposed within *T. pallidum* awaits further investigation, it is apparent that lipoproteins, if present on the treponemal surface, mirror the rare occurrence observed for integral membrane proteins. Since it has been shown that *T. pallidum* lipoproteins function as proinflammatory agonists (Akins et al. 1993; Radolf et al. 1995; Salazar et al. 2002) and elicit strong antibody responses (McGill et al. 2010), suggesting a propensity for these molecules to elicit a vigorous immune response during infection, the predominant location of *T. pallidum* lipoproteins within a subsurface locale undoubtedly contributes to the immune evasion capacity of this pathogen and its ability to successfully disseminate and establish chronic infection. In addition, *T. pallidum* does not possess lipopolysaccharide (LPS) (Belisle et al. 1994; Fraser et al. 1998; Hardy and Levin 1983), a major inflammatory-inducing endotoxin which activates the innate immune system via Toll-like receptor 4 (TLR4) and which is associated with the outer membrane of Gram-negative bacteria (Ulevitch and Tobias 1999). The unusual absence of this bacterial surface component would cause a reduction in the proinflammatory response induced by *T. pallidum* during infection, thereby directly enhancing its chance of survival within the host environment.

1.3.4 Chemotaxis

Evidence indicates that *T. pallidum* may utilize chemotaxis to further promote survival and dissemination during infection. Thirteen chemotaxis genes, including four methyl-accepting chemotaxis protein (MCP)-encoding genes, which potentially

encode for proteins with specificity for amino acids and carbohydrates, were identified from the whole genome sequence (Fraser et al. 1998). A putative *T. pallidum* MCP gene, *mcp1*, has also been cloned and shown to encode a 64 kDa protein which is highly homologous to at least 69 other chemotaxis proteins. Furthermore, the authors were able to demonstrate the existence of a 64 kDa methylated *T. pallidum* protein using intrinsic radiolabelling with L-[methyl-3H] methionine (Hagman et al. 1997). Two operons encoding putative chemotaxis (Che) response regulators were also identified in the *T. pallidum* genome (Fraser et al. 1998) and four of these chemotaxis proteins (CheA, CheW, CheX, and CheY) were expressed in vitro (Greene et al. 1997). Each of the *che* genes was found to be co-transcribed using reverse transcriptase-PCR and shown to exhibit significant homology to known chemotaxis genes (Greene et al. 1997). The presence of so many potential chemotaxis genes in the relatively small *T. pallidum* genome further suggests that the pathogen uses chemotaxis in response to nutrient gradients and hostile host environments, processes which likely contribute to *T. pallidum* dissemination.

1.3.5 Antigenic Variation of the Tpr Protein Family

Evidence suggests that *T. pallidum* also utilizes antigenic variation as a mechanism to facilitate immune evasion. The *T. pallidum* repeat (*tpr*) gene family is comprised of 12 paralogous genes (*tprA-L*) divided into three families (subfamily I–III) (Centurion-Lara et al. 1999a). Several of these proteins are predicted to be located in the outer membrane and most have been shown to elicit an immune response during experimental syphilis infection (Leader et al. 2003; Morgan et al. 2002; Sun et al. 2004). Recently, it has been shown that members of this protein family undergo both antigenic (Centurion-Lara et al. 1999b, 2004; LaFond et al. 2003) and phase variation (Giacani et al. 2007, 2009). In particular, *tprK* has been shown to undergo strain-specific antigenic variation at seven discrete variable regions, whereas *tprC* and *tprD* exhibit strain-independent variation (Centurion-Lara et al. 2004; LaFond et al. 2006; LaFond and Lukehart 2006). Together with antigenic variation, it has been proposed that "off/on" gene regulation, or phase variation, of Tpr proteins for which an immune response has, or has not, been generated, respectively, may aid *T. pallidum* in escaping detection by the host immune response (LaFond and Lukehart 2006). Antigenic variation by *T. pallidum* is discussed in more detail in Chap. 2.

1.3.6 Proteolytic Degradation of Host Components

Pathogenic bacteria often express surface-exposed and extracellular proteases as a means to facilitate host component and tissue destruction, an important mechanism that can contribute to dissemination of infection and tissue invasion. Potential host-interacting proteases were not specifically identified following *T. pallidum* complete

genome sequencing (Fraser et al. 1998). However, the highly invasive nature of the pathogen supports their existence. Five potential haemolysins with sequence similarity to *B. burgdorferi* proteins were identified (Fraser et al. 1998), although direct haemolytic activity was not demonstrated. One study did report that *T. pallidum* is capable of inducing interstitial collagenase (matrix metalloprotease-1, MMP-1) expression when added to human dermal fibroblast cultures (Chung et al. 2002). Its target, collagen-1, is the major component of connective tissues and bones and is also found in all organs. However, no further studies have been published regarding this mechanism, so it is difficult to ascertain the exact contribution of host protease up-regulation by *T. pallidum* in bacterial dissemination.

By using heterologous protein expression and in vitro protease assays, it has been demonstrated that soluble recombinant forms of the Tp0751 adhesin produced in both *E. coli* and insect expression systems were capable of degrading human fibrinogen and laminin. The authors also demonstrated that the observed protease activity was abolished by metalloprotease inhibitors, including the zinc-chelator 1,10 phenanthroline, but not by serine or cysteine protease inhibitors. Inductively coupled plasma-mass spectrometry revealed that the protease binds zinc and calcium (Houston et al. 2010). These results indicate that Tp0751, which has been designated pallilysin, is a zinc-dependent host component-degrading protease. The ability of pathogenic bacteria to degrade fibrinogen is recognized as an important virulence factor by which the pathogen prevents or inhibits coagulation-based containment. This mechanism may be of particular importance at the initial site of infection and during dissemination via the circulatory system. Likewise, laminin is a major component of basement membranes, obstacles which *T. pallidum* must traverse during the course of infection. This study provides the first description of a *T. pallidum* protein that is capable of degrading human proteins and thus provides novel insight into a potentially important mechanism of *T. pallidum* dissemination.

1.4 Effective Vaccine Design

The limited effectiveness of current worldwide public health programs in controlling syphilis highlights the need for the development of an effective vaccine. In light of increasing global *T. pallidum* infection rates, the extreme morbidity associated with the tertiary stage of syphilis, and the fact that syphilis infection significantly increases the risk of HIV acquisition and transmission, development of an effective syphilis vaccine would have a significant impact on global public health by preventing sexually and congenitally transmitted syphilis infections and by reducing transmission and acquisition of HIV. In order for a vaccine to be effective, it would have to not only prevent dissemination of infection, but also prevent establishment of later stages of the disease. It is probable that the identification of rare surface-exposed outer membrane proteins will be essential in syphilis vaccine development, as these antigens are the first targets of the immune system (LaFond and Lukehart 2006) and are also most likely to induce protective immunity since clearance of *T. pallidum* is dependent upon opsonophagocytosis and subsequent killing by