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Angiogenesis in secondary syphilis: role of the bacterioferritin TpF1, antigen of *Treponema pallidum*

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Summary

Syphilis is a chronic inflammatory disease transmitted by the spirochete *Treponema pallidum.* The bacterium is usually acquired by direct contact, generally sexual; the disease is worldwide spread and it represents an important global health problem. Syphilis is indeed a major sexually transmitted disease, particularly in developing countries, but an increasing number of new cases have also been recently noted in USA and Europe.
Syphilis is a progressive and multistage disease with diverse and wide-ranging manifestations.
Syphilis is an evolving disease and can be divided in three main stages. In the first stage, a red papule appears at the site of inoculation, usually genitals. Within the first few days, the papule ulcerates, producing the typical chancre of primary syphilis, which is painless and filled with treponemes. The chancre is accompanied by regional lymphadenopathy. If untreated, bacteria can also proliferate in the chancre and can be transported via lymphatics to the bloodstream, from which they disseminate throughout the body, giving rise to secondary syphilis: this represents the second stage and is a systemic disease. The most common manifestation of secondary syphilis is a disseminated mucocutaneous rash, which usually resolves spontaneously. In untreated patients, the symptoms are absent for a variable period of time, which is called latent stage. Afterwards, one-third of untreated patients can develop symptomatic late syphilis: the third stage of the disease, which is subdivided into gummatous syphilis, neurosyphilis and cardiovascular syphilis.
This disease could be cured with an antibiotic treatment, but, if not adequately treated, syphilis can lead patients to death.
Nowadays there are no available vaccines to prevent syphilis in particular due to the difficulties in cultivating *T. pallidum in vitro* make more difficult to study this pathogen as well as the identification of its virulence factors.

Little is known about the mechanism by which the bacterium causes the clinical manifestations that accompany the different stages of the disease, however it is established that *T. pallidum* is able to invade and survive in a wide variety of tissues and organs and that it promotes the formation of new blood vessels. Angiogenesis could have a crucial role in syphilis pathogenesis, at least for two reasons: i) the bacterium has limited metabolic capabilitie, thus probably it requires support from the host and it may derive most essential macromolecules from the blood. ii) Increased vascular permeability is one of the first stages in angiogenesis and the organism could take advantage of the vascular leakage to access to and egress from the bloodstream, resulting in systemic spread.

Another distinctive feature of syphilis consists in the fact that it becomes chronic in untreated patients and this probably reflects the ability of the bacterium in eliciting a T regulatory response, which could be associated with the fading of the host effector immune responses against the pathogen. We have recently demonstrated that the bacterioferritin TpF1, a major antigen of *T. pallidum*, plays a pivotal role in driving this suppressive immune response, by modulating the release of specific cytokines by monocytes.

TpF1 is a protein homolog to another immunomodulant antigen produced by the bacterium *Helicobacter pylori* and called HP-NAP. Both these proteins belong to the Dps-like family, a versatile group of bacterial stress miniferritins with a nearly spherical dodecameric structure.

HP-NAP, besides interacting with neutrophils, monocytes and dendritic cells and modulating their activity (as TpF1 does), also binds to endothelial cells, in which it is internalized by transcytosis.

Considering the homology between HP-NAP and TpF1 and the fact that angiogenesis is a characteristic of the secondary syphilis, we wondered whether TpF1 could interact with endothelial cells and promote their proliferation.

We have demonstrated that TpF1 induces proliferation and migration of human endothelial cells (HUVECs); moreover, the protein activates endothelial cells to form microcapillary-like structures *in vitro* inducing

marked changes in the cell pattern with the formation of tubules assembled by elongation and joining of the cells; a similar pattern was observed with VEGF. With the aim of understanding the mechanism underlying the activity of TpF1, we have addressed the possibility of an indirect effect mediated by VEGF or IL-8, both proangiogenic factors. We have excluded any contribution by VEGF: indeed, the latter is not released by the endothelial cells upon TpF1 stimulation. On the contrary, we have found that TpF1 triggers the release of IL-8 and, notably, the blockage of the cytokine strongly prevents the angiogenic activity of the bacterial protein inhibiting endothelial cell proliferation, migration and micro capillary-like structures formation. We also verified that the secretion of IL-8 relies on the induction of intracellular cAMP, produced by cellular adenylate cyclases, which is able to induce gene transcription through the activation of the two transcription factors NF-κB and CREB (cAMP response element-binding protein).

Finally we have observed that TpF1 induces new blood vessels formation *in vivo* in a zebrafish model. Moreover TpF1 promotes, in zebrafish, IL-8 gene expression suggesting a role for this cytokine in the TpF1-induced angiogenesis process also *in vivo*.

Sommario

La sifilide è una malattia infiammatoria cronica trasmessa dalla spirocheta *Treponema pallidum*. Il batterio è spesso trasmesso attraverso contatto diretto, generalmente sessuale; la malattia è diffusa a livello mondiale e rappresenta ancora oggi un importante problema sanitario globale. La sifilide è, infatti, la principale malattia a trasmissione sessuale, specialmente nei paesi in via di sviluppo, un crescente numero di nuovi casi è comunque stato riscontrato anche in USA e in Europa. La sifilide è una malattia progressiva e multistadio con manifestazioni cliniche diverse e ad ampio raggio.

La sifilide è una malattia in continua evoluzione ed è caratterizzata da tre stadi principali. Nel primo stadio, una papula rossastra appare nel sito d' infezione, generalmente i genitali. Entro i primi giorni, la papula tende ad ulcerare, dando origine alla chancre tipica della sifilide primaria, una lesione indolore contenente un alto numero di treponemi. La chancre è inoltre accompagnata da linfoadenopatia regionale. Se la malattia non è trattata, i batteri possono proliferare all'interno della chancre e, attraverso la via linfatica, raggiungere il flusso sanguigno dal quale disseminano in tutto l'organismo dando così origine alla sifilide secondaria: questa rappresenta il secondo stadio della malattia ed ha carattere sistemico. Nei pazienti non trattati, i sintomi possono rimanere assenti per un periodo variabile di tempo denominato sifilide latente. Successivamente, un terzo dei pazienti non sottoposti a trattamento può sviluppare i sintomi dell'ultimo stadio della sifilide; la sifilide terziaria. La sifilide terziaria può essere a sua volta suddivisa in gomma sifilitica, neurosifilide e sifilide cardiovascolare.

Questa malattia può essere curata attraverso una semplice terapia antibiotica ma, se non adeguatamente trattata, può portare alla morte del paziente. Ad oggi non sono ancora disponibili vaccini volti a prevenire la sifilide, in particolare la difficoltà di coltivare T. palliudm *in vitro* rende

complicato lo studio di questo patogeno e, quindi, l'identificazione dei suoi fattori di virulenza.

Il meccanismo attraverso cui il batterio provoca le tipiche manifestazioni cliniche che accompagnano i differenti stadi della malattia risulta, ancora, perlopiù sconosciuto, ad ogni modo è stato dimostrato che *T. pallidum* è in grado di invadere e sopravvivere in un'ampia varietà di tessuti e organi e che promuove la formazione di nuovi vasi sanguigni. L'angiogenesi potrebbe avere un ruolo cruciale nella patogenesi della sifilide almeno per due ragioni: i) il batterio ha limitate capacità metaboliche, dunque è probabile che esso richieda supporto dall'ospite e che derivi la maggior parte delle macromolecole essenziali dal sangue. ii) l'aumento della permeabilità vascolare è una delle prime fasi dell'angiogenesi e l'organismo potrebbe trarne vantaggio per accedere e uscire dal circolo sanguigno, portando ad una diffusione sistemica della malattia.

Un'ulteriore caratteristica distintiva della sifilide risiede nella capacità di diventare cronica nei pazienti non trattati, una condizione che probabilmente riflette l'abilità del batterio nell'indurre una risposta T regolatoria la quale, a sua volta, può indurre l'indebolimento degli effettori della risposta immunitaria dell'ospite contro il patogeno. A tal proposito abbiamo recentemente dimostrato che la batterioferritina TpF1, uno dei principali antigeni di *T. pallidum*, gioca un ruolo fondamentale nel dirigere questa risposta immunitaria soppressiva, modulando il rilascio di specifiche citochine da parte dei monociti.

TpF1 è una proteina omologa ad un altro antigene immunomodulante prodotto dal batterio *Helicobacter pylori* e chiamata HP-NAP. Entrambe queste proteine appartengono alla famiglia *Dps-like*, un gruppo variegato di mininferritine batteriche caratterizzate da una struttura dodecamerica approssimativamente sferica.

HP-NAP, oltre ad interagire con neutrofili, monociti, cellule dendritiche e a modulare la loro attività (analogamente a TpF1), sono in grado di legare le cellule endoteliali nelle quali sono poi internalizzate per transcitosi.

Considerando dunque l'omologia esistente tra HP-NAP e TpF1, e considerando che l'angiogenesi è una caratteristica peculiare della sifilide

secondaria, ci siamo chiesti se TpF1 potesse interagire con le cellule endoteliali e promuovere la loro proliferazione.

Abbiamo dimostrato in primo luogo come TpF1 induca la proliferazione e la migrazione di cellule endoteliali umane (HUVEC); inoltre, la proteina è in grado di attivare le cellule endoteliali promuovendo la formazione di strutture simil-capillari in vitro attraverso l'induzione di marcate modifiche nella distribuzione cellulare e nella formazione di tubuli assemblati mediante l'elongazione e la giunzione delle cellule; un simile comportamento è stato osservato anche in presenze del VEGF.

Al fine di comprendere il meccanismo alla base dell'attività di TpF1, abbiamo considerato la possibilità di un effetto indiretto mediato dal VEGF o da IL-8, entrambi fattori pro-angiogenici. Abbiamo escluso qualsiasi contributo del VEGF; quest'ultimo non viene rilasciato dalle cellule endoteliali in seguito al trattamento con TpF1. Al contrario, abbiamo osservato che la stimolazione con TpF1 promuove il rilascio di IL-8, in particolare, l'inibizione di tale citochina previene fortemente l'azione angiogenica indotta dalla proteina batterica inibendo la proliferazione cellulare, la migrazione e la formazione di strutture tubulari. Abbiamo inoltre osservato che la secrezione di IL-8 risiede nella produzione di cAMP, prodotto dall'adenilato ciclasi, il quale è in grado di indurre l'espressione genica attraverso l'attivazione dei due fattori di trascrizione NF-κB e CREB (*cAMP response element-binding protein*).

Infine abbiamo verificato che TpF1 induce la formazione di nuovi vasi sanguigni in vivo, in un modello di *zebrafish*. Inoltre, TpF1 promuove anche in *zebrafish* l'espressione genica di IL-8 suggerendo dunque, anche in vivo, un ruolo per questa chemochina nel processo angiogenico indotto da TpF1.

Introduction

1.1 Syphilis and *Treponema pallidum*

1.1.1 Syphilis: an historical overview

Syphilis is a chronic, inflammatory and usually sexually-transmitted disease, caused by the spirochaete *Treponema pallidum* subs. *pallidum*. Since it's recognition as a new disease, in Europe in the 15th century, syphilis has been subject of great mystery and legend [1] (Figure 1). The name syphilis comes from a Latin poem, "*Syphilis sive de morbo gallico*", describing an Italian shepherd boy, who contracted the "French disease" in the early 16th century [2].

Figure 1. Angelo Bronzino. An Allegory, 1545 (formerly called, Venus, Folly, Cupid and Time) Courtesy Trustees of the National Gallery, London [3].

The first recorded epidemic of syphilis occurred in 1945 in Italy, when the French king Charles the VIII invaded Naples. When the invading army, including chiefly mercenaries, was disbanded and the soldiers returned home, syphilis was disseminate across Europe [4]. A number of prominent historical figures are reported, or assumed, to have contracted syphilis in the course of their lives. Francesco I de' Medici (1541–1587), second Grand Duke of Tuscany, had suffered from malaria and syphilis [5].

It is generally accepted that also Gerard de Lairesse (1641–1711), a draughtsman, theatrical set designer, lecturer, writer, theoretician, and one of the most celebrated Dutch renaissance painter suffered from congenital syphilis [6]. Sir William Osler's, a canadian physician, uses Rembrandt's Portrait (Figure 2) of de Lairesse to describe the stigmata of late congenital syphilis:

"Growth is slow, development tardy, and there are facial and cranial characteristics which often render the disease recognizable at a glance. A young man of nineteen or twenty may neither look older or be more developed than a boy of ten or twelve [..] The forehead is prominent, the frontal eminences are marked, and the skull may be asymmetrical. The bridge of the nose is depressed, the tip retrousse´.The lips are often prominent, and there are striated lines running from the corners of the mouth."

Figure 2. Rembrandt's Portrait of de Lairesse (The Metropolitan Museum of Art, Robert Lehman Collection, 1975) [6]

Although syphilis has been widely studied, the exact origin of the disease is still controversial. In this regard two main theories have been proposed: the Columbian theory (or New World theory) and the pre-Columbian theory (or Old World theory). The New World theory asserts that syphilis was endemic to the Caribbean and brought to Europe by Columbus's crew after his first travel in 1942. On the contrary, the Old World theory

hypothesizes that the disease was endemic in central Africa and arrived in Europe long before Columbus's voyage [7]. Recently, it has been developed a new hypothesis, which probably integrates the other two: the so-called Unitarian theory. This theory assess that treponematosis had a worldwide distribution and each social group was exposed to a kind of treponematosis characteristic to its geographic location, climatic condition and stage of cultural development. In other words, this theory supports the idea that different treponematosis such as yaws, bejel, pinta as well as syphilis, represent an adaptive responses of *Treponema pallidum* to peculiarities of the environment, culture, and contacts with other population [8].

1.1.2 Syphilis: epidemiology

Syphilis, which is only a human disease, since there is no animal reservoir, is usually transmitted by sexual contact or from mother to infant. *T. pallidum* may also occasionally be transmitted as a blood-borne infection. If left untreated, syphilis typically follows a prolonged course consisting of several stages. In a small proportion of cases, the infection can lead patients to death as consequence of neurological or cardiovascular complication.

The disease represents an important global health problem counting the onset of approximately 12 million of new cases per year, with particular incidence in developing countries (Figure 3), due to lack of prenatal tests, antibiotics therapies or preventive measures; however, an increasing number of new cases have also been recently noted in the United States and Europe.

Most cases occurs in sub-Saharan Africa, southeast Asia and south America, where congenital syphilis represents the main cause of perinatal and neonatal death [2]. Since the 1950s, periodic epidemics have been

observed in the United States, with peaks around 1990 followed by a progressive decline of prevalence from 1993 until 2000.

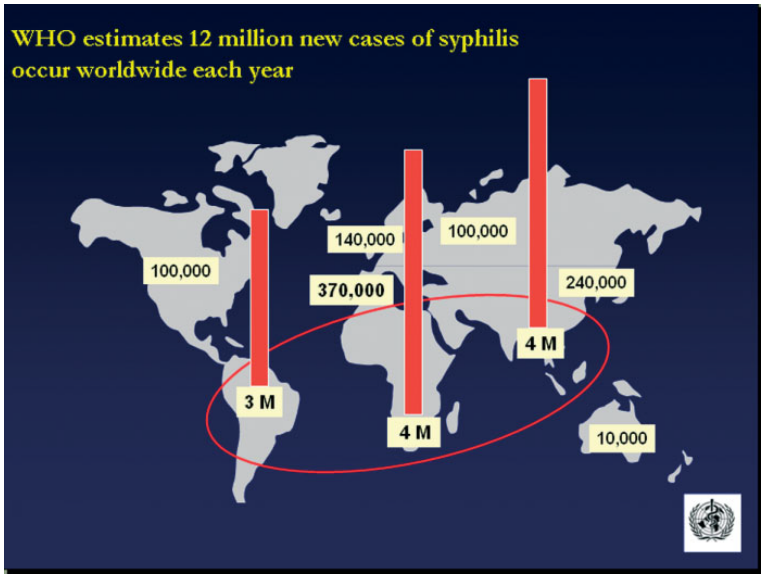

Figure 3. The global distribution of syphilis cases. [2]

During the last decade, however, a significant increase in syphilis incidence has been reported in several countries, including the United States, Canada, and some areas of Central and Eastern Europe. The main causes of the increased prevalence in syphilis epidemiology probably relies on sexual promiscuity, prostitution and a decreasing use of barrier protection due to a false sense of security that sexually transmitted diseases are currently curable and to a lack of pertinent knowledge. Nowadays, about 50–60% of new cases of syphilis occur in men who have sex with other men and are strongly associated with HIV and high-risk sexual behavior. Indeed, several studies report that patients who have been infected with HIV are susceptible to a co-infection with syphilis and vice versa, with 30-60% cases of co-infection with HIV in some urban centers such as Los Angeles, Chicago and Huston [7].

Over the past two centuries, syphilis symptoms have become less severe, thanks to the availability of effective treatments and to a concomitant decrease of the bacterium virulence if compared with epidemics of the XV

century. Syphilis pathogenesis has indeed changed over the time turning from an acute, debilitating and high morbidity disease, able to kill within a few years, into a chronic and milder form with infected patient who can survive for decades [9]. This evolution of the diseases towards a less aggressive form could result either from a progressive loss of virulence factors by the bacteria or from the fact that syphilis killed off the most susceptible population right at the beginning of its devastating existence [8].

1.1.3 Syphilis: pathogenesis and clinical manifestation

Syphilis is a progressive and multistage disease with diverse and wide-ranging manifestation, each characterized by a peculiar symptomatology. Once acquired, syphilis passes throughout a series of four different, but overlapping stages, commonly referred to as primary syphilis, secondary syphilis, latent syphilis and tertiary syphilis [7]. The stages of the disease were described for the first time by Philippe Record in the mid-1800s [1].

1.1.3.1 Primary syphilis

The infection initiate when *Treponema pallidum* penetrates dermal micro abrasions or passes across intact mucosa, probably moving through the junctions between cells. The primary cutaneous lesion begins as a small red macule appearing at the site of inoculation, usually genitals. Within the first few days, the macule enlarges to a papule, with a range in diameter from 0.5 to 1.0 cm, and then ulcerates producing the typical chancre of primary syphilis (Figure 4). This sore is painless and filled with bacteria, is solitary round or oval; it has sharp indurated margins and a firm button-like cartilaginous base with a convex eroded surface and a raw-ham color; it shows a thin serous discharge and a hemorrhagic border without pus. The chancre is also accompanied by regional lymphadenopathy [10].

Since the chancre is painless and usually located in an inconspicuous anatomical site, diagnosis of syphilis is sometimes delayed until later disease manifestation become apparent. Clinical evaluation can also be complicated by the fact that the appearance of primary chancres in some individuals does not fit the classic description; indeed, only about half of affected individuals has the typical chancre, while the other half shows a non-indurated ulcer with irregular border or chancroid-like lesion.

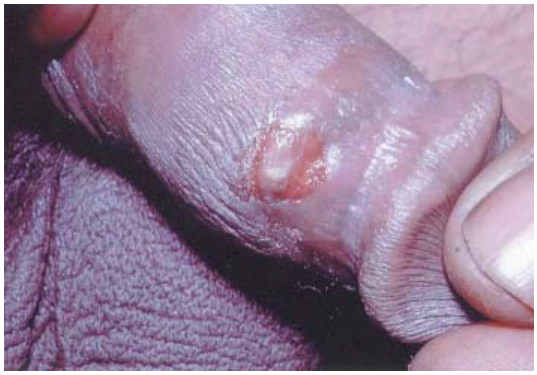

Figure 4. Primary chancre [11]

Usually, primary lesion develops 3 weeks after the infection and it resolves spontaneously within 4 to 6 weeks, but may still be discernible in about 15% of patient at the onset of secondary syphilis.

1.1.3.2 Secondary syphilis

Although secondary syphilis generally appears after the healing of the chancre of primary syphilis, there is no a sharp demarcation between primary and secondary cutaneous syphilis [10].

Within hours from the infection, and during the evolution of primary stage, *T. pallidum* proliferates in the chancre and can be transported via lymphatic to the bloodstream, from which bacteria disseminate throughout the body, giving rise to the secondary syphilis: this represents the second stage of the disease that becomes systemic. The most common manifestation of secondary syphilis is a disseminated muco-cutaneous

rash (Figure 5). Pale and discrete macular lesions appear initially on the trunk and proximal extremities, on the palms of the hands and soles of the feet and, in 4% to 11% of patients, *T. pallidum* infection of hair follicles results in alopecia of the scalp.

In rare individuals, lesion may become necrotic, a condition called *lues maligna* but, generally, the lesions are inconspicuous and may remain undetected [1].

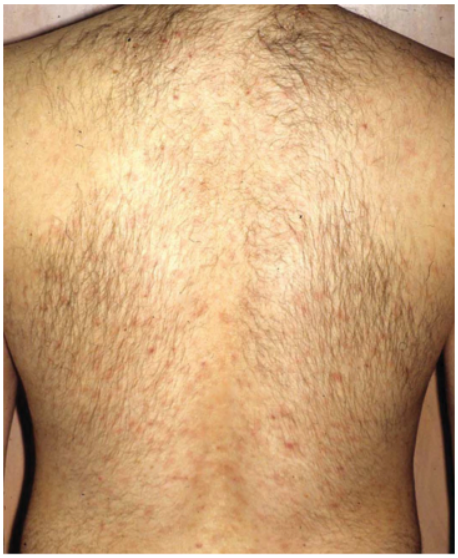

Figure 5. Mucocutaneous rash in secondary syphilis [10]

Although many patients with secondary syphilis do not experience any clinical evidence, the remaining patients display a variety of lesions including: sore throat, malaise, headache, fever, weight loss, nausea, arthritis, periostitis, myalgia [10]. Secondary syphilis can also be accompanied by gastric and renal involvement and hepatitis. Approximately 5% of individuals with secondary syphilis experiences early manifestation of neurosyphilis, including meningitidis and ocular disease [1].

1.1.3.3 Latent Syphilis

If untreated in the primary or secondary stage, syphilis progresses to the latent stage during which the bacterium is probably localized in

immunologically privileged sites, that is usually characterized by absence of symptoms. The latent stage includes early latency and late latency. Patients with syphilis in the early latency stage remain infectious, whereas those with syphilis in the late latency stage are thought to be non-infectious [12]. During latent syphilis, organisms may reach the bloodstream intermittently and can infect the developing fetus during pregnancy. Latent syphilis, if not cured by an antibiotic therapy evolves into the tertiary syphilis [1].

1.1.3.4 Tertiary syphilis

About one-third of individuals with latent syphilis develops the typical clinical manifestation of tertiary syphilis [1].

Tertiary syphilis is mainly characterized by a persistent low-level burden of pathogens, against which a potent and detrimental immune response is mounted [12]. In this regard, it is believed that many of the protean symptoms of secondary and tertiary human syphilis, are manifestation of immune reactions that fail to clear the organism. The inability of the host in eliminating the microorganism results in a chronic inflammatory response that, in turn, can culminate in the tissue destruction [10] (see below).

Tertiary syphilis is characterized my three main clinical manifestations: the gumma (15% of untreated patients), cardiovascular syphilis (10% of untreated patients) and late neurosyphilis (6.5% of untreated patients) [1].

- The gumma: it is a granulomatous, nodular lesion with variable central necrosis (Figure 6) (Lanfond & Lukehart, 2006). The formation of a granuloma reflects the inability of the host in removing the cause of an inflammation (foreign body or infectious agent), despite a strong recruitment and activation of macrophages at the site affected. Macrophages are often arranged around a central necrotic zone, essentially walling off the necrotic tissue, and exhibit an epithelioid appearance. Surrounding the epithelioid cells

are varying numbers of lymphocytes, plasma cells, macrophages, along with fibroblasts and connective tissue scaring [10].

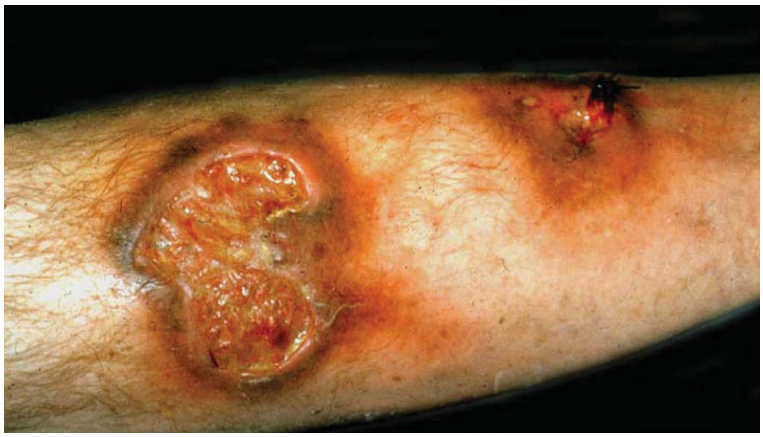

Figure 6. Tertiary cutaneous syphilis - the gumma [10]

These destructive lesions most commonly affect the skin and bones, but they may also occur in the liver, heart, brain, stomach and upper respiratory tract.
The gumma is the most characteristic lesion of tertiary syphilis but, unless it affects a vital organ, does not cause serious complication: for this reason it is also defined as “late benign syphilis” [1].

- Cardiovascular syphilis: prior to the advent of penicillin therapy, most of the deaths caused by syphilis were associated with cardiovascular impairment. Typically, one of the most frequent alterations of the cardiovascular system during syphilis, is represented by aortitis, an inflammation mainly concerning the ascending aorta. In most of cases aortitis is uncomplicated and asymptomatic.
  If a complication occurs, in 10% of individuals with untreated syphilis, it may be aortic regurgitation, coronary ostial stenosis or saccular aneurysm [1].

- Neurosyphilis: in untreated or undertreated patients, *T. pallidum* can reach the cerebrospinal fluid. In this scenario four possible events may occur: spontaneous resolution, asymptomatic neurosyphilis, syphilitic meningitidis, or progression to late neurosyphilis [7].

  The most common symptoms of neurosyphilis include vertigo, insomnia, personality changes, loss of consciousness and epilepsy [1].

1.1.3.5 Congenital Syphilis

Despite the fact that *T. pallidum*, in most cases, is sexually acquired, it can be also transmitted from mother to foetus at any time during pregnancy, resulting, if untreated, in congenital syphilis.

Antibiotic treatment of the mother during the first six months of pregnancy is, in many cases, sufficient to prevent negative outcomes, but later treatment or lack of treatment may result in foetal death, foetal damage, or birth of an infected infant. Like the acquired syphilis, congenital syphilis includes different stages: early congenital syphilis (manifestations appears within 2 years of life; usually after 2 or 10 weeks after the delivery), late congenital syphilis (manifestation appears after 2 years of life), and residual stigmata. The first symptom of early syphilis (up to 50% of new-borns) is a persistent rhinitis, named “snuffles”, caused by *T. pallidum* invasion of the nasal mucosa. The bacterium can further invade the bones and cartilage of the nose and palate, leading to gummatous destruction later in life. Early congenital syphilis is also characterized by skin lesions resembling those of the secondary disease of adults, usually accompanied by desquamation of the skin of palms and soles, condylomata lata, and mucous patches. Manifestations of late congenital syphilis usually appear two years after birth; between 5 to 25 years after birth, interstitial keratitis may cause damage to the cornea and iris, and eighth-nerve deafness may be apparent. Other signs include neurosyphilis, arthropathy, bilateral effusion of the knees and elbows, gummatous periostitis of the palmate

and nasal septum. Many of these manifestations progress despite an adequate treatment [1].

1.1.4 Diagnosis and treatment

For most of the 20th century, the gold standard method for the diagnosis of syphilis was the direct identification of *T. pallidum* by dark field microscopy or by direct fluorescent antibody test (DFA). Both methods were applied on the exudate of the lesions or on the infected tissue and they represented the main diagnostic methods for primary syphilis. The accuracy of dark field microscopy strongly relies on the operator's experience and does not permit to discriminate between *T. pallidum* and non-pathogenic commensal treponemal species [4].

A Tp-specific polymerase chain reaction (Tp-PCR) has been recently considered from The US Centers for Disease Control and Prevention (CDC) as a valid diagnostic method along with the dark-field microscopy (DFM), which is still considered the reference test, despite its limits [13].

Another group of tests useful for syphilis diagnosis is represented by serological tests which are in turn divided into "treponemal" tests, and non-treponemal tests.

Treponemal tests are based on the use of lyophilized *T. pallidu*m or lysate of pathogenic *T. pallidum* to detect antibodies that are present in the sera of syphilitic patients and are specific for the bacterium. These tests comprise serum fluorescent treponemal antibody absorption test (FTA-ABS) and microhemagglutination test for *T. pallidum* (MHA-TP).

Non-treponemal tests detect antibodies that are not specifically directed against the *T. pallidum* bacteria. These antibodies are produced by the body when an individual has syphilis but may also be produced in several other conditions. Examples of non-treponemal test are the Venereal Disease Research Laboratory (VDRL) and the Rapid Plasma Reagin (RPR) card test [14]. The latter test, for example, measures IgM and IgG antibodies to lipoidal material released from damaged host cells as well as

to lipoprotein-like material, and possibly cardiolipin released from the treponemes.

The traditional approach for syphilis serodiagnosis utilizes a two step analysis: a first screening with a non-treponemal test followed by a confirmatory test based on a more specific determination.

Serological diagnosis is widely available but it's utility is affected by false-positive results and by the need of equipped laboratories, [2].

After 60 years of use, penicillin remains the best pharmaceutical approach for the treatment of syphilis, though doxocycline and tetracycline can be also employed for efficient *T. pallidum* eradication (except for pregnant woman), in case of penicillin allergy.

In the 1990s, azithromycin emerged as a particularly attractive alternative to penicillin therapy for syphilis. However, a study performed on *T. pallidum* isolated from patients in different USA countries and in Ireland, identified macrolide-resistant strains among these population.

Several efforts have been devoted to the development of an effective potential vaccine. It has been demonstrated that the immunization of rabbits with some treponemal molecules (i.e. TpN15, TpN47, endoflagella) leads to the production of reactive antibodies, some having a great opsonic activity. Nevertheless, the protection against *T. pallidum* was only partial and resulted in an attenuation of the clinical manifestation of the disease and in a more rapid healing of lesions.

Despite the fact that immunization induces a strong humoral immune response, the incomplete protection of the host suggests that a cell-mediated immune response is critical for counteracting the infection and for clearing the bacterium. Moreover, the first targets of the immune response, represented by outer membrane proteins, are very rare in *T. pallidum*. For this reason, is clear that the identification of molecules on the surface of *T. pallidum* is essential for the development of an effective syphilis vaccine; their discovery and use in a multivalent vaccine will lead to the production of an effective vaccine for syphilis [1].

1.2 Biology of *Treponema pallidum*

Since it's recognition in the 15th century as a new disease, syphilis has been subject of great mystery and legend. In the middle of 18th century John Hunter, an eminent Scottish physician and venereologist, in order to verify whether syphilis and gonorrhea were the same disease, inoculated himself with pus from an individual infected with a sexually transmitted disease. Unfortunately for Hunter, and also for the medical community, the patient was infected with the etiological agents of both the diseases: this caused decades of medical and scientific confusion. In 1838 Philippe Ricord, a French physician, published the results of some studies demonstrating that syphilis and gonorrhea were distinct infections [1](Lanfond & Lukehart, 2006). The etiology of syphilis remained unclear until 1905, when Schaudinn, a German parasitologist, examining a fresh preparation of a material provided by the dermatologist Erich Hoffmann from an eroded papula in the vulva of a woman with secondary syphilis [15], was able to discern a transparent, delicate spiral organism. The bacterium was named by Schaudinn and Hoffman *Spirochaeta pallidum* because of the extraordinary difficulty in staining the organism; short time later Schaudinn proposed the name *Treponema* [16], [10].

Nowadays is well established that the causative agent of syphilis is *Treponema pallidum* subsp*. pallidum, a* bacterium belonging to spiral-shaped bacteria, the *spirochaetaceae* (the spirochetes), and related to other pathogenic treponemes that cause non-venereal diseases such as bejel, yaws, and pinta (Figure 7). A peculiar feature of *T. pallidum*, as well as of other spirochetes such as *Borrelia burgdorferi* (etiological agent of Lyme disease) and *Leptospira interrogans* (etiological agent of leptospirosis), is its ability to efficiently swim in a high viscous gel-like environment, such as connective tissue, where most of the flagellated bacteria are slowed or stopped. Motility is thought to play a pivotal role in widespread dissemination of spirochaetal infections and in establishing the chronic infection [14]. *T. pallidum* varies from 6 to 15 μm in length and is 0.2 μm in diameter; the spiral-shaped body of *T. pallidum* is surrounded by

a cytoplasmic membrane, which is enclosed by a loosely associated outer membrane. A thin layer of peptidoglycan between the membranes provides structural stability. Endoflagella, organelles that allow for the characteristic corkscrew motility of *T. pallidum*, are located in the periplasmic space.

T. pallidum has a small genome constituted by a single, circular chromosome of about 1.4 Mb containing approximately 1041 open reading frame (ORFs): a genome smaller than that of the majority of other bacteria.

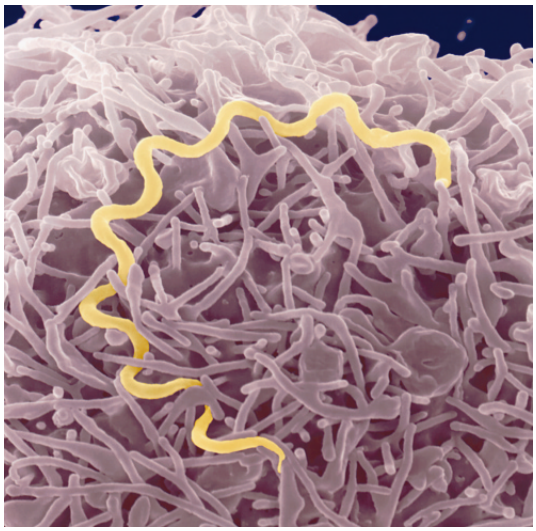

Figure 7. *T. pallidum* on the surface of a human lymphocyte [17]

Probably due to the size of the genome, *T. pallidum* lacks genes that encode many metabolic functions. The bacterium is indeed able to carry out glycolysis but, on the same time, it lacks tricarboxylic acid cycle enzymes and an electron transport chain; pathways for the use of alternative carbon source for energy, for *de novo* synthesis of enzymes, cofactors and nucleotides are also absent. Despite the bacterium lacks pathways involved in amino acid and fatty acid synthesis, it possess enzymes for the interconversion of fatty acids and amino acids. The severe defect of biosynthetic pathways suggests that the bacterium acquires most essential macromolecules directly from the host, exploiting

interconversion pathways to generate others.

An interesting aspect of the metabolism of *T. pallidum* is the absence of porin-like proteins, making unclear how nutrients can cross the outer membrane to gain the periplasmic space. Recent studies suggest that the presence of putative outer membrane proteins may alter the permeability of the outer membrane facilitating TM flux of hydrophilic solutes by the insertion of amphipathic α-helices into the external bilayer allowing non selective diffusion of nutrients into the periplasm [18].

T. pallidum, human-obliged pathogen, does not survive outside the mammalian host, and the infectious capability is lost within few hours after its isolation. To obtain sufficient organisms for experimental manipulation, *T. pallidum* must be propagated in rabbit [19] and this represents the biggest obstacle to the syphilis research. The generation time of *T. pallidum* is unusually slow; it doubles in 30-33 h *in vivo*, and in 30-50 h *in vitro*. Several biological factors may contribute to the slow replication rate of *T. pallidum.*

The organism, besides a weak ATP production, lacks enzymes such as catalase and oxidase that detoxify reactive oxygen species; for this reason the *in vitro* survival of *T. pallidum* is prolonged by low oxygen concentrations. In addition to its sensitivity to oxygen, *T. pallidum* also lacks the typical heat shock response regulated by σ 32. The resulting heat lability may contribute to the slow growth of the organism [1].

1.3 *Treponema pallidum* proteome

Despite a severe lack of metabolic capabilities, sensitivity to oxygen, and decreased viability in an environment warmer than the body temperature, *T. pallidum* is able to invade and survive in a wide range of tissues and organs, causing chronic infection and a broad spectrum of clinical manifestation in the host. Moreover, the bacterium displays highly invasive capabilities: in rabbit infection, *T. pallidum* enters the bloodstream within

minutes after intratesticular or intradermal inoculation; similarly, organisms applied to rabbit genital mucosa are found in deeper tissues within hours [1]. These *T. pallidum* infectious capabilities relies on the presence of a wide range of bacterial proteins involved in many aspects of syphilis pathogenesis such as attachment to the extracellular matrix, motility and inflammation.

The endoflagella of *T. pallidum*, responsible for its great motility in the host, are composed by multiple polypeptides arranged into an outer sheath and a central core [20]. Three proteins, FlaB1 (34,5 kDa), FlaB2 (33 kDa) and FlaB3 (31 kDa) form the flagellar core which is covered with a sheath made up of subunits of the 37 kDa FlaA protein. FlaA subunits form chains, on the outer surface of the flagellum, which are attached to the flagellum central core [21]. Cell-mediated and humoral immune responses to endoflagella are induced during syphilis infection and most of the flagellar proteins are recognized by antibodies from both infected humans and rabbits. A peculiar feature of *T. pallidum* is that it lacks lipopolysaccharide (LPS), the typical endotoxin present on the surface of many gram-negative bacteria that causes inflammation and fever. On the other hand, mostly localized in the periplasmic space and probably released after *Treponema* autolysis [22], there are several lipoproteins endowed with pro-inflammatory activity that induce the expression of inflammatory mediators via toll-like receptor (TLR2) triggering. The 47-kDa lipoprotein TpN47 induces cultured endothelial cells expression of adhesion molecules such as ICAM-1, VCAM-1 and E-selectin, In accordance,, dermal injection of synthetic analogs of the TpN17 and TpN47 lipoproteins has shown to induce transient infiltration by PMNs, at the site of injection. Moreover TpN47 activates macrophages to express some pro-inflammatory cytokines such as TNF-α, IL-1β, IL-6, IL-8. TNF-α is also induced by TpN15, TpN17 and TpN38, whereas stimulation of macrophages with TpN17 activates IL-1β production [1].

The outer membrane of *T. pallidum* contains a very few number of proteins, if compared with other pathogens, and for this reason they are named Rare Outer Membrane Proteins (TROMPs) [20].

The observation that only those treponemes that had been physically disrupted reacted with anti-*T. pallidum* antiserum, suggests that the surface of *T. pallidum* is non-antigenic and this permits the organism to evade the immune response [23].

1.4 TpF1

TpF1, also known as TpN19, 4D antigen or TP1038, was one of the first *T. pallidum* proteins cloned in *E. coli* and is one of the best characterized. This protein has been identified and purified for the first time in the 1984 [24]. It is a glycoprotein of 190 kDa, belonging to the Dps (DNA binding proteins from starved cells)-like proteins family and localized in the periplasmic space of the bacterium. The crystal structure of the protein was resolved for the first time in the 2006 by Thuminger and colleagues (Figure 8) [22].

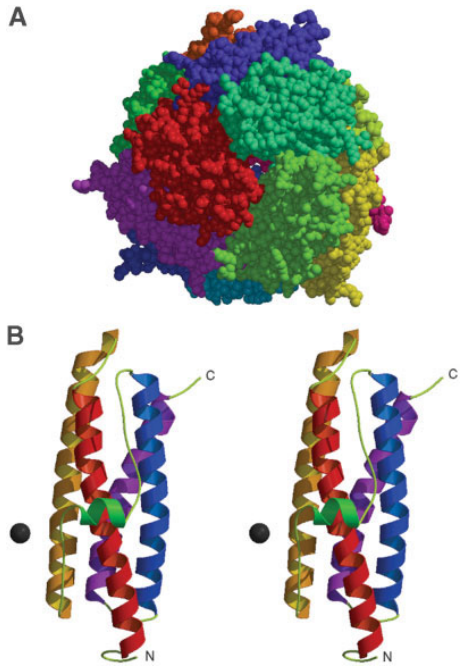

Figure 8. Crystal structure of TpF1.(A) Van der Waals representation of the dodecamer of TpF1. Each subunit is colored differently. (B) Stereoview of the ribbon representation of TpF1 monomer [22].

TpF1 is a dodecamer, about 90 Å in diameter, displaying 32 symmetry. Each subunit of the protein (19 kDa) folds in a way very similar to other mininferritins: a four-helix bundle, with helices B and C connected through a long stretch that includes a short helix. The deduced amino acid sequence contains two cysteine residues, which are presumably involved in interchain disulfide bonds whose reduction is required for the dissociation of the oligomeric structure [25]. Considering that disulfide-bonded proteins have been demonstrated in the outer membranes of several bacteria pathogens, it has been suggested that TpF1 could fulfill a structural role in the outer membrane of *T. pallidum* [26]. However, in the 1988 Cunningham and colleagues demonstrated that the protein derived from the periplasmic space [27].

The arrangement of the 12 monomers of TpF1 results in a nearly spherical shell typical of mininferritins, with an internal cavity where the iron is stored. TpF1 is highly immunogenic and specific antibodies were detected in about 95% of syphilitic sera by radioimmunoassay [28]; notably no antibodies against the 19 kDa monomer were found [24]. Immunogenic properties of the protein are difficult to rationalize on the basis of the crystal, and they may depend on the flexible N-terminal domain which protrudes from the surface of the spherical shell [22]. Recently, Babolin and colleagues have demonstrated that TpF1 activates the inflammasome, thus leading to the production of pro-inflammatory cytokines such as TNFα, IL-6 and IL-1β in human monocytes. Moreover, it has been demonstrated that TpF1 drives the development of T regulatory response that may, instead of being beneficial to the host, allow long-term persistence of the bacteria and contribute to the genesis and maintenance of the chronic disease [29].

1.3 The host immune response to *T. pallidum*

The outcome of the *T. pallidum* infection depend on the duration and the site of the infection and on the immune status of the infected individual. Since the growth of *T. pallidum* is strictly dependent on the temperature (the internal temperature of the body is too high for optimal growth), the major combat zones consist in the external surfaces of the body, in which immune effector mechanisms try to annihilate a huge amount of proliferating bacteria. On the same time, lymphadenopathy or splenomegaly represent the evidence that an immune response develop also in the internal organs; clinical manifestations of the tertiary stage, such as granuloma, due to a chronic frustrated inflammation, reflects an inadequate immune response to a persistent infection [10].

Altought *T. pallidum* is a temperature sensitive bacterium, it has been reported that it quickly gains access to deeper tissues and the bloodstream [30]. Moreover the bacterium induces dermal cells to produce matrix metalloproteainase 1 [31], that, by degrading the matrix, may help the bacterium to penetrate tissues.

Usually, polymorphonuclear cells (PMNs) are the first cells that infiltrate the site of infection. Accordingly, PMNs are seen in both experimentally-induced and naturally-acquired early syphilis lesions, although infiltration is transient and the number of PMNs is low if compared with other acute bacterial infections; the inability of PMNs to adequately control *T. pallidum* is demonstrated by the progression of infection [1]. Another cell type present during the early stages of *T. pallidum* infection is represented by dendritic cells (DCs). Specialized DCs called Langerhans cells are found in the skin, which is site where lesions mainly develop during primary and secondary syphilis; DCs are also found in the mucosa, the intestinal wall, and the heart, all sites that can be infected by *T. pallidum*.

Bouis and colleagues demonstrated that *T. pallidum* interacts with and is phagocytized by immature DCs, *in vitro,* promoting DCs maturation and leading to the production of pro-inflammatory cytokines such as interleukin-1β (IL-1β), IL-6, IL-12, and tumor necrosis factor alpha (TNF-α)

[32]. The production and secretion of pro-inflammatory cytokines significantly increases the immune response of the host enhancing endothelial cell activation, inflammatory cell migration, and promoting the maturation of immune system effectors. The activation of immune cells by *T. pallidum* is not likely to occur until treponemal antigens are released after bacterial distruption or autolysis [1]. Accordingly, the activation and maturation of DCs is delayed during *T. pallidum* infection, with respect to the time required in case of infection by other bacteria [32].

Such a delay could contribute to the early dissemination of *T. pallidum*, giving organisms the opportunity to penetrate organs and tissues before an active inflammatory response has been mounted by the host [1].

Of particular importance in the immune response against *T. pallidum*, is the strength of delayed-type hypersensitivity (DTH), which is mediated by CD4+ cells. DTH is a cell-mediated immune response, evoked since primary syphilis and characterized by an expanded population of antigen-specific T cells that produce cytokines, activating and recruiting additional lymphocytes and macrophages. Macrophages accumulate at the site of infection and, once activated by CD4+ Th-1 cell, protect against infection by destroying and clearing the organism. The primary chancre is consider to be a DTH reaction that is extremely effective in clearing infectious organisms from the site of infection. On the other hand, when the DTH response is insufficiently effective, the bacterium is not completely eliminated giving rise to secondary and tertiary disease. In this condition high, persistent, localized antigenic challenge can lead to an excessive inflammatory response producing immunopathology in the form of granulomatous inflammation and tissue destruction [10]. In primary chancres, CD4+ T cells (helper) and macrophages predominate, whereas in the lesions of secondary syphilis CD8+ T cells (cytotoxic) are the most abundant cell type. The role of CD8+ T cells in the immune response to *T. pallidum* is still not deeply understood, however it has been suggested that CD8 T cells may play a role in the antisyphilitic host response through a IL-17- and IL-22-mediated antibacterial effector functions and IFN-γ–dependent activation of macrophages involved in hte clearance of

opsonized treponemas. In addition, cytotoxic properties of CD8 T cells could be responsible for tissue destruction and inflammation characterizing the proggression of the disease [33].

Lesions of both primary and secondary syphilis are characterized by an abundant accumulation of Th1 cytokines such as IFN-γ and TNF-α. It was found that cells of syphilitic patients are able to produce IL-2, IFN-g, TNF-α, IL-10 and weakly IL-6 already in primary syphilis. The production of IL-6 reaches highest level in secondary syphilis when slightly increases the ability of cells to secrete IL-10 reaching the highest value in early latent syphilis. The growing ability of Th2 cells to produce IL-6 and IL-10 is accompanied with a diminished production of IL-2, IFN-γ and TNF-α [34].

Soon after the onset of primary syphilis circulating antibodies against *T. pallidum* start to be detectable and they reach the maximum level during secondary stage of the disease, when the infection disseminates throughout the body. IgM antibodies are usually the first that are produced, but they are shortly followed by IgG. The antibody response is specific for a broad range of *T. pallidum* molecules, including lipids of the surface, flagellar proteins and lipoproteins [1].

Although effective immune response against treponemal infection is mounted by the host, the organism often manages to evade total eradication. In this regard, it has been recently shown that *T. palldum* is able to promote the development of T regulatory cells (Treg). These cells represent a subpopulation of T cells responsible for the down-modulation of the immune response [35], [29]. This could be considered as an additional strategy for the bacterium to avoid the elimination by the host thus contributing to the chronicity of the disease.

1.5 Angiogenesis in syphilis disease

The role of angiogenesis is well established in a number of pathological processes such as in benign and malignant tumors, but also in a variety of

infectious and inflammatory diseases. Nevertheless, the role of bacteria in inducing angiogenesis in chronic infections is still poorly understood. It is well established that *T. pallidum* activates the endothelium during the early stages of the disease and, during syphilis progression, it promotes new vessels formations, but the bacterial factors involved in this process are still unknown. In this regard, Macaron and colleagues [36] observed that the number of microvessels was significantly increased in cutaneous lesions of patients affected by secondary syphilis with respect of normal skin. Moreover, *T. pallidum* was shown to cause activation of endothelial cells inducing the expression of intercellular adhesion molecule-I (ICAM-1) and procoagulant activity on the surface of endothelial cells of human source (HUVECs), and promoting the adherence of lymphocytes and monocytes to endothelium exposed to *T. pallidum* [30]. Treatment of human endothelial cells *with T. pallidum* results in increased expression of the endothelial activation markers; interestingly this activation does not occur with non-pathogenic Treponema species. The angiogenic process observed in the early stages of the disease could be a mechanism exploited by the bacterium to uptake nutrients directly from the host and to disseminate throughout the whole organism. Increased vascular permeability is indeed one of the first stages in angiogenesis [37] and the organism could take advantage of the vascular leakage to access to and egress from the bloodstream, resulting in systemic spread.

1.6 Angiogenesis

1.6.1 Angiogenesis: an overview

Angiogenesis (from the Greek word *Angêion*, vessel) is a physiopathological process involving the formation of new blood vessels and their subsequent growth, from a pre-existing vasculature. It occurs throughout life in both health and disease. A thick blood capillary network,

formed by angiogenic process, penetrates every metabolically active tissue in the body. Capillaries are crucial to ensure a correct and efficient exchange of nutrients and metabolites and they grow and regress in healthy tissues according to functional demands. Exercise stimulates angiogenesis in skeletal muscle and heart. A lack of exercise leads to capillary regression. Capillaries grow in adipose tissue during weight gain and regress during weight loss [38]. Leonardo da Vinci was the first to speculate about the cardiovascular system. His analytic method of the mechanism of organ formation was by analogy; he suggested that the vasculature developed like a tree from seed (the heart) by sprouting roots (the liver capillary network) and a trunk with major branches (aorta and arteries) (Figure 9) [39]. At the end of the 18th century John Hunter, the same Scottish scientist who studied syphilis pathogenesis [1], provided the first scientific insights in the field of angiogenesis. His observation, summarized in his *Treatise* published in 1794, suggested the existence of a relationship beetween vascularity and metabolic requirements [40]. A pioneer in the modern history of angiogenesis was Judah Folkman, a US oncologist: he formulated the hypothesis, included in a work published in 1971, that the tumor growth is angiogenesis-dependent.

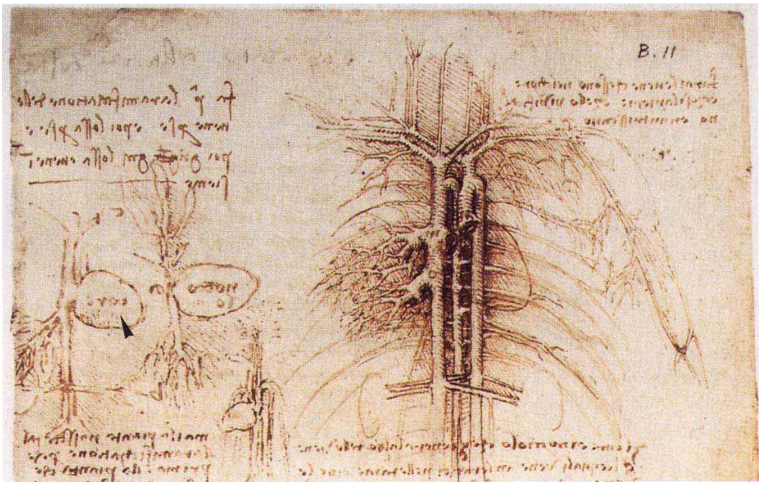

Figure 9. Analogy between the botanic and the vascular tree as drawn by Leonardo da Vinci (taken from: 'The anatomy of man: the cardiovascular system (ca. 1508)) [39]

The idea that the control of angiogenesis could lead to cancer therapies stimulated intensive research in the field with more than 5000 articles published within 40 years from 1970 to 2009 [38]. Nowadays is widely accepted that inhibiting angiogenesis can be therapeutic in cancer, ophthalmic conditions, rheumatoid arthritis, and many other diseases.
Conversely, stimulation of angiogenesis can be beneficial in several other pathological conditions such as ischemic heart disease, peripheral arterial disease, and wound healing.

1.6.2 The origin of blood vessels

The presence of a system which ensures a suitable and functional exchange of oxygen, nutrients and metabolites in all the tissues of a multicellular organism, is fundamental for a correct development and, in general, for the life. In all animals, the cardiovascular system is the first organ system that develops in the embryo [39]. In this regard, two physiological processes play a critical role in the formation, maintenance and remodeling of the vascular network: vasculogenesis and angiogenesis.

1.6.2.1 The origin of blood vessels: vasculogenesis

Vasculogenesis is a process occurring only during the embryonic development, in the extra-embryonic and intra-embryonic tissues of embryos, and it can be defined as *de novo* formation of new blood vessels from angioblast.
Angioblast are mesenchymal cells derived from hemangioblasts, multipotent cells, derived from mesodermal stem cells, which give rise to both hematopoietic stem cells, and angioblasts. Mesodermal-inducing factors belonging to the fibroblast growth factors family are crucial for the formation of both cell lines [39]. Angioblasts are a cell type with potency to differentiate into endothelial cells but have not yet acquired all

characteristic markers of endothelial cells; the first blood vessels are the result of their differentiation [41]. A first primitive proof of cardiovascular system appears in the middle of the third gestation week in human; angioblasts proliferate and coalesce into a primitive network of vessels known as the primary capillary plexus [42]. Once the latter is formed, further blood vessels are generated by angiogenesis, which are progressively remodeled into a functional adult circulatory system [39].

1.6.2.2 The origin of blood vessels: angiogenesis

Angiogenesis, consisting in the formation of new blood vessels from pre-existing ones, occurs in the embryo, where it establishes a primary vascular network as well as an adequate vasculature for growing and developing organs. Nevertheless, angiogenesis occurs, occasionally, also in the adult for example in the regulation of female reproductive system and during repair processes such as in the healing of wounds and fractures [43]. This process is regulated by several vascular growth factors, such as the vascular endothelial growth factor family of proteins (VEGF). These act on specific receptors in the vascular system to stimulate the growth of new vessels. VEGF also promotes vascular permeability to water and to large proteins and vasodilatation [37].

Angiogenesis occurs through two different processes: sprouting angiogenesis and non-sprouting angiogenesis. Sprouting angiogenesis is characterized by sprouts composed of endothelial cells which usually grow in response to an angiogenic stimulus, such as VEGF. This process is characterized by the proteolytic degradation of the extracellular matrix, driven by a class of enzymes named matrix metalloproteinases, followed by chemotactic migration and proliferation of endothelial cells from pre-existing vasculature (Figure 10) [39].

Sprouting angiogenesis is mainly induced by hypoxia condition; in poorly perfused tissues an oxygen sensing mechanism promotes the formation of new blood vessels in order to ensure a correct metabolic supply to parenchymal cells.

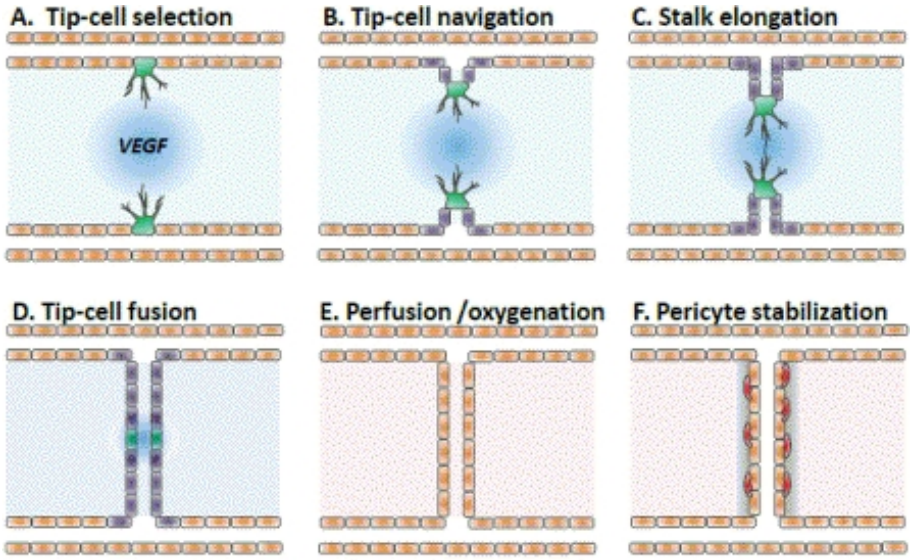

Figure 10. Schematic representation of VEGF-drived angiogenesis [38]

Many types of parenchymal cells such as monocytes, hepatocytes, and neurons respond to a hypoxic environment by secreting VEGF [44].

When the local tissue receives adequate amounts of oxygen, the concentration of VEGF returns to the basal level and this promotes the maturation and stabilization of the capillary [38].

Vessel maturation and stabilization require also the recruitment of pericytes and the deposition of extracellular matrix along with shear stress and other mechanical signals [42].

Non-sprouting, or intussuspective angiogenesis, discovered in post-natal lungs of rats and humans in 1986 [45], has a prominent role in vascular development in embryos and in the formation of artery and vein bifurcations.

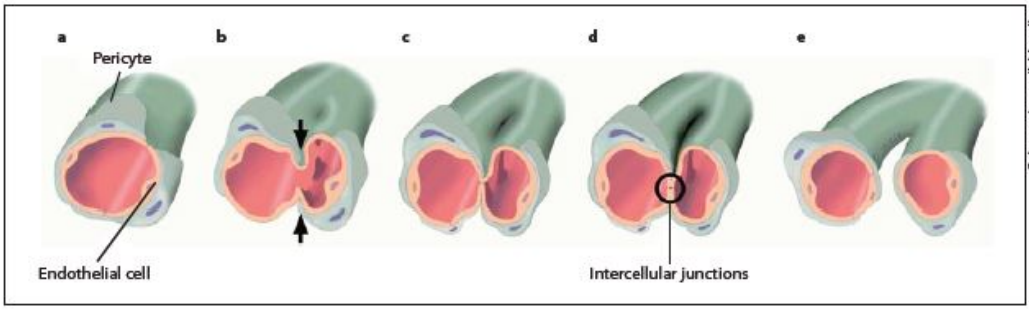

Figure 11. Schematic representation of non-sprouting angiogenesis [46]

This process starts with the migration of opposing endothelial walls within a vessel, followed by rearrangements of interendothelial junctions and invasion of pericytes and myofibroblasts, consequently leading to the splitting of the vessel (Figure 11) [46].
Non-sprouting angiogenesis is promoted by pro-angiogenic agents, such as VEGF, but also by mechanical stresses induced by the blood flow in some high-flow regions of the circulation [42]. Both types of angiogenesis are thought to occur in virtually all tissues and organs.

1.5.4 Angiogenesis: mechanisms of regulation

Angiogenesis is a fundamental and highly regulated biological process, not only in physiological conditions, but also in a number of disease including cancer, diabetic, retinopathy and rheumatoid arthritis.
Angiogenesis is tightly regulated and depends on the balance between different molecules ; specific angiogenic molecules can initiate the process and specific inhibitory molecules can stop it. Angiogenic factors and inhibitors have been discovered in the past two decades and the mechanism by which they regulate the process remain to be fully understood. Several inducers of angiogenesis have been identified, including the members of the VEGF family, angiopoietins, transforming growth factors (TGF), platelet-derived growth factor (PDGF), tumor necrosis factor-α, interleukins and the members of the fibroblast growth factor (FGF) family [47].
VEGF is a major angiogenic factor that regulates several endothelial cell functions, including mitogenesis, permeability, vascular tone and the production of vasoactive molecules [48]. Vascular endothelial growth factor-A (VEGF-A) is the best characterized and the most studied of the VEGF family members. VEGF-A exerts its biologic activity through the interaction with transmembrane tyrosine kinase receptors including the the neuropilin receptors (NP-1 and NP-2), expressed on neurons and vascular

endothelium, VEGF receptor-1 (VEGFR-1) and VEGFR-2, both expressed on vascular endothelial cells [49]; however VEGFR-2 appears to be the major receptor responsible for mediating the pro-angiogenic effects of VEGF-A. VEGF-A is the most potent pro-angiogenic factor identified so far. The engagement of VEGFR-2 triggers a phosphorilation cascade culminating in proliferation, sprouting and tube formation of endothelial cells. In addition, VEGF-A promotes vasodilatation by inducing the endothelial nitric oxide synthase, resulting in the nitric oxide production [47].

Angiopoietins 1 and 2 (Ang1 and Ang2) belong to a family of 70-kDa secreted proteins that promote maintenance, growth and stabilization of the vessels [47], [42].

Acidic and basic fibroblast growth factors (aFGF and bFGF, respectively) are heparin-binding proteins that trigger many processes involved in angiogenesis such as endothelial cell proliferation and MMPs production, following the engagement of tyrosine kinase FGF receptors (FGFRs). Nevertheless, unlike VEGF, which is mitogenic primarily for endothelial cells, FGF stimulates proliferation of most cells derived from embryonic mesoderm and neuroectoderm including pericytes, fibroblasts, myoblasts, chondrocytes, and osteoblasts.

The role of PDGF in angiogenesis is not yet fully understood [47], however it probably plays a role in recruiting pericytes to preformed capillaries or in inducing the proliferation of pericytes previously recruited by a PDGF-independent mechanism, thus helping the maintainance of capillary wall stability [42].

Conversely, the transforming growth factor-β (TGF-β) possesses both pro- and anti-angiogenic properties. In particular, at low doses, TGF-β1 seems to have pro-angiogenic activity by up-regulating angiogenic factors and proteinases, whereas, at high doses, TGF-β1 inhibits the proliferation of endothelial cells promotes basement membrane reformation and stimulates the differentiation and recruitment of smooth muscle cells [47].

1.5.4.1 IL-8: a pro angiogenic chemokine

In addition to the classical angiogenesis promoters, also some pro-inflammatory molecules are known to exert pro-angiogenic activities such as TNFα, IL-1β and chemokines of the CXC family.

Inflammation and angiogenesis are linked processes, but the precise relationship existing has not been well understood [50].

Chemokines are multifunctional mediators mainly responsible for leukocyte recruitment to inflamed tissues. Chemokines of the CXC family, however, have also a pivotal role in the control of angiogenesis: accordingly, not only leukocytes but also endothelial cells express specific receptors for these molecules [51].

IL-8, also known as CXCL8, is a pro-inflammatory CXC chemokine that promotes neutrophil chemotaxis and degranulation. Moreover, it is widely accepted that this chemokine plays also a crucial role in modulating angiogenesis by interacting directly with endothelial cells [52].

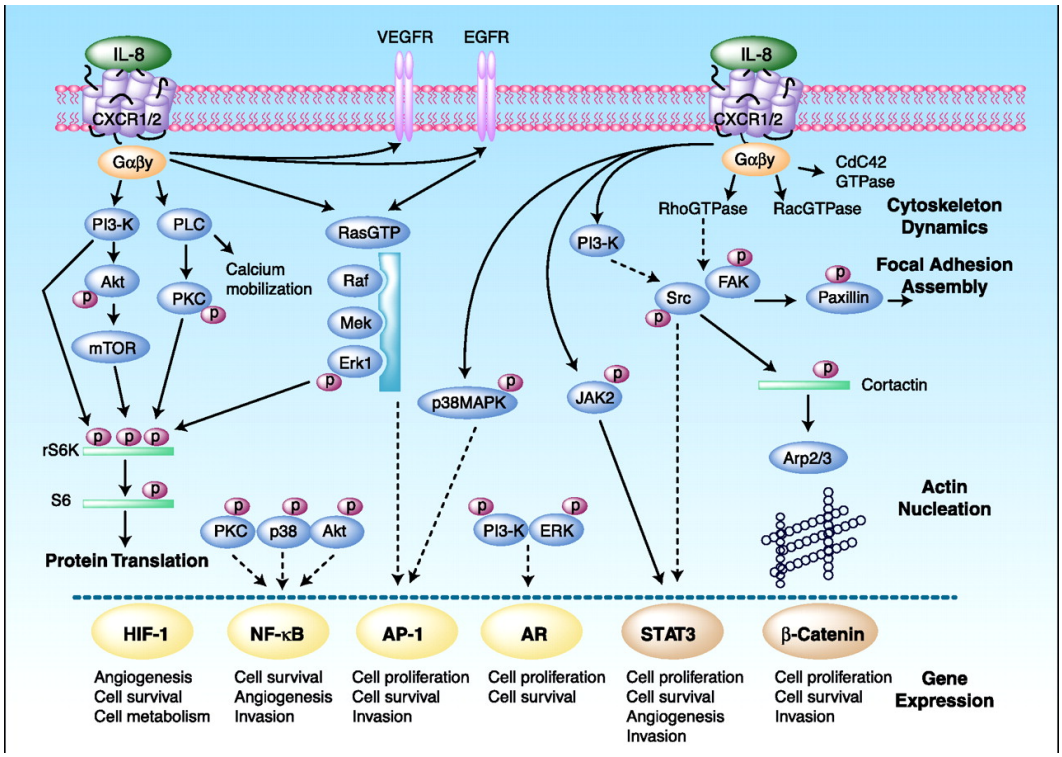

Figure 12. IL-8 signaling pathways. The schematic diagram illustrates the signaling pathways activated after the engagement of CXCR1and/or CXCR2 receptors with IL-8 [53].

IL-8 gene expression is regulated by several transcription factors (Figure 12): the activator protein 1 (AP1), the nuclear factor-κB (NF- κB) and the cAMP response element-binding protein (CREB) [53], [54].

IL-8 exerts its biological function through the engagement of two cell-surface G protein-coupled receptors known as CXCR1 and CXCR2. Signals are transmitted across the membrane through a ligand-induced conformational change that promotes the coupling to functional heterotrimeric G proteins. The activation of the G protein triggers a phosphorylation cascade culminating in the activation of several effectors, including the primary effector phosphatidil-inositol-3-kinase (PI3K), the phospholipase C (PLC) and/or the mitogen associated protein kinases (MAPK).

Furthermore, IL-8 signaling activates members of the RhoGTPase family, and a number of non-receptor tyrosine kinases involved in the regulation of cytoskeleton dynamics (Figure 12) [53]. Through the activation of these different signaling pathways, IL-8 promotes endothelial cell proliferation and migration as well as the production of matrix metalloproteinases such as MMP2 and MMP9. Recent studies also reveals that IL-8 inhibits spontaneous apoptosis of endothelial cells and modulate anti- and proapoptotic gene expression such as Bcl-2, Bcl-x_L, Bcl-x_s, and Bax [52].

Materials and Methods

1.Reagents

TpF1 was produced as described below; human umbilical vein endothelial cells (HUVECs) were isolated as described below; HEPES, human endothelial serum-free medium (HE-SFM), Fast DiI, Phalloidin-Alexa Fluor 546, rhodamine-labelled 10 kDa-dextran, Trizol Reagent, Superscript II, MMLV reverse transcriptase and protease inhibitor cocktail were from Life Technologies (Glasgow, Scotland, UK); Medium 199 (M199), foetal bovine serum (FBS), heparin, forskolin, LPS, NBT, BCIP, FITC-labeled 70 kDa-dextran, Tricaine and 1-phenyl-2-thiourea were from Sigma-Aldrich (St. Louis, MO, USA); L-glutamine, trypsin-EDTA, penicillin-streptomycin, were from Euroclone (Siziano, Italy). Fibronectin was from Roche (Basel, Switzerland). Matrigel was from Becton, Dickinson & Company (Franklin Lakes, New Jersey, USA). Goat anti-human IL-8 blocking antibody was from Abcam (Cambridge, UK); human recombinant VEGF was from Immunological Sciences (Roma, Italy). Monoclonal antibody anti anti-actin (clone C4), KG501 and QNZ were from Millipore (Bedford, MA, USA); rabbit policlonal antibodies anti-phospho-CREB (ser133) and anti-phospho-p65 were from Cell Signalling Technology (Danvers, MA, USA); Collagenase was from Worthington (Lakewood, NJ, USA).

2.2 Bacterial strains

Escherichia coli Top10 strain was used for cloning TpF1 gene. *Bacillus subtilis* SMS118 strain was used for expression and purification of TpF1 protein.

2.3 Construction of the plasmid pSM214G-tpf1

TpF1 was cloned and expressed in E. coliXl1blue. TpF1 gene was amplified by PCR starting from a preparation of *Treponema pallidum* genome. The PCR reaction was carried out using standard method.

The thermal cycling parameters were as follows:

- Initial denaturation: 94°C for 5 min
- Denaturation: 94°C for 1 seconds
- Annealing: 50° C for 1 seconds
- Extension: 72°C for 45 seconds
- Final extention: 72°C for 10 min

} For 45 cycles

Primers used were:
-Forward 5'-ccggaattcacgatgaacatgtgtaca-3'
-Reverse: 5'-cccaagcttctaggctttcagggtagc-3'

Primers contain restriction site for EcoRI and HindIII, respectively. The amplified fragment was excised by digestion with EcoRI and HindIII and ligated into EcoRI and HindIII sites of the expression vector pSM214G. pSM214G contains an artificial constitutive promoter, a chloramphenicol resistance cassette, and two origins of replication that allow expression of cloned genes both in *E. coli* and in B. subtilis.

2.4 Transformation of competent Escherichia coli

Top10 chemically competent cells (Invitrogen) which have been kept on freezer storage were thawed on ice. 100-200 ng of plasmid DNA were added to the competent cells and the transformation mix was kept on ice for 30 min. The cells were heat-shocked for 30-40 sec at 42°C and the cooled on ice for 2-3 min. The cells were incubated for 45 min at 37°C in 500 **µ**l of Luria-Bertani (LB) broth (10 g/l Bacto Tryptone, 5 g/l Bacto yeast

extract, 10 g/l NaCl) in agitation. The mix was plated on LB agar plates which contained the antibiotic chloramphenicol (20 **µ**g/ml) that selects for transformants. The plates were incubated overnight at 37°C. Bacterial colonies were colony-PCR analyzed.

2.5 Plasmid DNA isolation from bacteria (Miniprep)

E. Coli cells carrying the plasmid of interest were incubated overnight at 37°C at constant shaking (200-220 rpm) in 5 ml of LB broth supplemented with the appropriate antibiotic (chloramphenicol 20 **µ**g/ml). The cells were harvested by centrifugation at 13,000 x g (microcentrifuge Biofuge, Haeraeus) for 3 min and the plasmid DNA was isolated using the QIAprep Spin miniprep kit (Qiagen) following the manufacturer**'**s instruction. Briefly, cellular pellet was resuspended in 250 **µ**l of buffer P1 (Qiagen), then were added 250 **µ**l of buffer P2 (Qiagen) and the suspension was gently inverted 2-3 times; 350 **µ**l of neutralizing buffer N3 (Qiagen) were added, the suspension was gently inverted and centrifuged 10 min at 13,000 x g. Supernatants were applied in the Qiaprep spin column and centrifuged 1 minute at 13,000 x g; the column was washed two times by adding 750 **µ**l of buffer PE (Qiagen) and centrifuged 1 minute at 13,000 x g. The purified plasmid DNA was eluted from the column with 50 **µ**l of sterile water. The concentration and quality of the purified DNA was measured with a UV spectrophotometer at OD 260-280.

2.6 *Bacillus subtilis* transformation

Bacillus subtilis cells were chemically competent induced for the electroporation. *Bacillus subtilis* was grown overnight at 180 rpm at 37°C in 10 ml of LB broth; 1% of this culture was then grown 3 h at 150 rpm at 37°C in 50 ml of LB broth (to an OD of 0,6). Bacteria were centrifuged 10 min at 1,000 x g and washed two times with Hepes 1 mM 10 min at 1,000 x g. Cells were then treated two times with electroporation buffer (Hepes 1

mM pH 7.0, PEG 8000 25% v/v, mannitol 0.1 M). Bacteria were resuspended in 250 **µ**l of electroporation buffer to a final OD of 1.9. The bacteria were aliquoted in 70 **µ**l. Bacillus subtilis (70**µ**l) was electroporated using 1.5 **µ**g of DNA; one pulse of 200 **Ω**, 2.3 **κ**V, 2.5 **µ**F. Electroporated cells were incubated 10 min at 25°C in 500 **µ**l of LB and then incubated 2 h and 30 min at 37°C under slow agitation (at 120-140 rpm). The bacteria were plated on LB-agar plate supplemented with of chloramphenicol (20 **µ**g/ml) and incubated for 20 h at 37°C.

2.7 TpF1 purification

B. subtilis strain SMS 118 containing the plasmid pSM214G-TpF1 was grown for 15 h in 500 ml of YT medium (15 g/l yeast extract, 16 g/l bactotryptone, 5 g/l NaCl) supplemented with chloramphenicol (20 **µ**g/ml). The cells were pelleted by centrifugation at 4,000 x g and resuspended in 10 ml of fresh Tris-HCl 30 mM, pH 7.8 plus protease inhibitors (Roche). After three passages through a French Press and removal of debris by centrifugation at 32,000 x g, powder ammonium sulphate was added to the supernatant to a final concentration of 12.5 % w/v, at 4°C. At this percentage of ammonium sulfate most of the protein remained in solution. After 3 h at 4°C at slow stirring the sample was centrifuged at 32,000 x g for 30 min, the supernatant was recovered and ammonium sulfate was added to a final concentration of 22.5% w/v. The sample was kept for 3 h at at 4°C at slow stirring and then centrifuge at 32,000 x g for 30 min. The pellet containing the protein TpF1 was suspended in NaCl 0.1 M, Tris 30 mM, DTT 5 mM, pH 8.4 (buffer A) and dialyzed overnight in buffer A. The sample was fractionated by ion-exchange chromatography using a MonoQ column (Amersham Biosciences) equilibrated with buffer A. After the sample was applied, the column was eluted with a linear NaCl gradient in Tris 30 mM, DTT 5 mM, pH 8.4. Fractions were analysed by SDS-PAGE and TpF1-containing fractions were pooled. TpF1 was further purified by gel filtration chromatography using a superdex 200 HR 10/30 column (Amersham Biosciences) equilibrated with phosphate buffer saline, pH

7.8. Protein was concentrated using the ultrafiltration system Centricon® (Millipore), and the final product was checked for purity in a Comassie brilliant blue stained gel and analyzed by western blot with a specific polyclonal antibody.

2.8 Endothelial cell isolation

Human umbilical vein endothelial cells (HUVECs) were isolated from human cord of healthy donors. Cords were laid on a clean dish, excess blood was dabbed off and fresh cuts were made on both of the ends. The umbilical veins were cannulated with sterile cannulas; a cord has one vein and two arteries, usually the vein is the largest opening while the two smaller ones are arteries. Cannulas were clamped in place with a string and 20cc syringes were attached to each cannula. The vein was then perfused with phosphate buffered saline (PBS) using the syringe to wash out the excess of blood; the washings were performed until all the blood present in the vein was eliminated. The waste was collected in a beaker with bleach. Once cleaned the vein, about 10-12 ml of a 55 U/ml collagenase solution was poured inside a syringe and then pushed into the vein until the exit of a first amount from the opposite end. The cord was incubated for 20 minutes at 37°C, 5% CO_2. After 15 minutes, the cord was gently massaged with hands and then relocated into the incubator for the remaining time. After incubation the collagenase solution containing endothelial cells was flushed from the cord by perfusion with a wash medium (M199 20% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin) for a total volume of 50 ml. The effluent is collected in a sterile 50-ml tube. The cells were centrifuged 7 minutes at 100 G then medium was discarded, and endothelial cells were resuspended in 5 ml of fresh culture medium (described above). The cell suspension was placed in 25-cm flask coated with 2% gelatine and incubated over night at 37° C, 5% CO_2.
Next day the supernatant was removed, cells washed with PBS and then replaced with fresh medium.

2.9 Endothelial cell culture

HUVECs were kept in culture in 2% gelatin-coated cell culture flasks, in medium 199 supplemented with 20% foetal bovine serum (FBS), 50 μg/ml heparin, 100 U/ml penicillin, 100 μg/ml streptomycin, 68 μM L-glutamine, 10 mM Hepes and 20 ng/ml epithelial growth factor (EGF). Cells were used at passage 2–5 for all experiments. In all experiments stimuli were added in human endothelial serum free medium (HE-SFM) without any supplement.

2.10 Proliferation assay

HUVECs were seeded in 2% gelatin-coated 96-well tissue culture plate at 1×10^4 cells/well and maintained in M199 complete medium for 2 h before the application of stimuli in HE-SFM. Cells were exposed to 1 μM TpF1 or 20 ng/ml VEGF or vehicle (saline). For the inhibition experiments, cells were pre-treated or not with 1 μg/ml anti-IL-8 blocking antibody that was maintained during TpF1 treatment. After 12 and 24 h, cells were harvested and counted with electronic Coulter counter (Beckman Coulter). The proliferation rate is reported as the fold increase in cell number with respect to the number of plated cells.

2.11 Migration assay

Cell migration was evaluated by Transwell migration assay coating the lower side of the polycarbonate filter (8 μm pores) with fibronectin (5 μg/cm^2). Fast DiI labelled HUVECs (2×10^5 cells) were added to the upper compartment in HE-SFM and 1 μM TpF1 or 20 ng/ml VEGF, used as positive control, were added to the lower compartment. For the inhibition experiments supernatant of HUVECs, grown to confluence and stimulated in HE-SFM with 1 μM TpF1 for 24 h, was pre-incubated with 1 μg/ml anti-IL-8 blocking antibody for 45 min at 37°C before adding to the to the lower compartment. Migrated cells were quantified after 2 h, 6 h and 24 h by a

microplate reader (Infinite 200, Tecan), comparing to a standard curve and expressed as % of seeded cells.

2.12 In vitro tube formation

5.5×10^4 HUVECs, seeded on wells coated with Matrigel (12 mg/ml), were incubated for 10 h with 1 µM TpF1, in presence or absence of 1 µg/ml anti-IL-8 blocking antibody, to allow tube formation. VEGF was used as positive control. After fixation with 4% paraformaldehyde and staining with Phalloidin-Alexa Fluor 546, tubes (capillary-like structures) were counted under Leica AF6500 microscope using a LAS software (Leica).

2.13 Real-time PCR analysis on Endothelial cells

HUVECs were seeded in 2% gelatin-coated 24-well tissue culture plate at 4×10^4 cells/well and maintained in M199 complete medium until they reached 80% confluence. Cells were stimulated with 1 µM TpF1 in HE-SFM, harvested, and total RNA was isolated using RNeasy Kit according to the manufacturer's instructions (Qiagen, Venlo, Netherlands). RNA was reverse-transcribed using Superscript II and cDNA was amplified with the following protocol:

Sample mix was prepared as follows:

Power SYBR Green PCR Master Mix	5 µl
Primer Forward 50 µM	0.18 µl
Primer Reverse 50 µM	0.18 µl
H2O	2.64 µ

The PCR cycle for the Real-Time PCR was:

- Initial denaturation: 95°C for 15 min
- Denaturation: 95°C for 15 seconds
- Annealing: 58° C for 20 seconds
- Extension: 72°C for 10 seconds

} For 45 cycles

The primers used for Real-time PCR analysis were the following:

Gene		Primer sequence	AT (C°)
18S	F	5′-CGGCTACCACATCCAAGGAA-3′	58
	R	5′-GCTGGAATTAGCGCGGCT-3’	
VEGF-A	F	5′-GCCTTGCCTTGCTGCTCTA-3′	58
	R	5′-GATGTCCACCAGGGTCTCG-3′	
IL-8	F	5′-TTGGCAGCCTTCCTGATT-3′	58
	R	5′-AACTTCTCCACAACCCTCTG-3′	

After amplification, data analysis was performed using the second derivative algorithm by applying the $2^{-\Delta\Delta CT}$ method. For each sample, data were normalized to the endogenous reference gene (ribosomal subunit 18S). Cells harvested at time zero were taken as the reference value, set to 1 AU (arbitrary unit, as shown in the figures), and the relative expression levels for treated or untreated cells were calculated and shown.

2.14 ELISA

Culture supernatants of HUVECs, harvested for quantification of mRNAs, were collected and stored at -80°C for subsequent quantification of cytokine content by ELISA: specific kits for IL-8 (Affymetrix, High Wycombe, UK) and VEGF-A (Raybiotech, Norcross, GA, USA) were used

following the manufacturers' instructions. When required, cells were pre-incubated for 30 min with 100 nM KG501 or 10 μM QNZ before the exposure to TpF1.

2.15 Evaluation of the phosphorylation state of CREB and NF-κB p65 subunit

HUVECs were seeded in 2% gelatin-coated 6-well tissue culture plate at 3×10^5 cells/well and maintained in M199 complete medium until they reached 80% confluence. Cells were washed with HE-SFM and incubated in the same medium with 1 μM TpF1, 25 μM forskolin, 1 μg/ml LPS or saline for 30 min. Cells were then washed with ice-cold PBS, lysed in 1% Triton X-100, 20 mM Tris-HCl pH 8.0, 150 mM NaCl in the presence of protease inhibitor cocktail, resolved by SDS-PAGE and transferred to nitrocellulose. Phospho-proteins were revealed by specific policlonal antibodies. After band densitometry, actin was used to normalize the amount of the phospho-proteins.

2.16 Whole cell ELISA

HUVECs were seeded in 2% gelatin-coated 96-well tissue culture clear-bottom black plate (R&D Systems, Minneapolis, MN, USA) at 1×10^4 cells/well and maintained in M199 complete medium until they reached 80% confluence. Cells were exposed for 5, 15, 30 and 60 min to 1 μM TpF1, 1 μg/ml LPS, 25 μM forskolin or vehicle (saline) in HE-SFM. Phosphorylation of CREB on serine 133 and phosphorylation of IkB-α on serine 32 and 36 were evaluated by whole-cell ELISA according to the manufacturer's instructions (R&D). For each sample, data were normalized to the total CREB protein or to the GAPDH protein in case of IkB-α. Normalized fluorescence value of cells at time 0 was taken as reference and set as 1; CREB and IkB-α phosphorylation of treated cells was expressed as fold change with respect to the time 0.

2.17 Fluorescence Resonance Energy Transfer Imaging

HUVECs (5 × 10^5 cell/well), seeded on 24 mm diameter gelatin-coated coverslips in M199 complete medium 20% FBS, were transfected with 1 µg of mammalian expression plasmid encoding the cAMP biosensor Epac1-camps [55], using Amaxa Nucleofector Technology according to the manufacturer's instructions (Basic Nucleofector Kit for Primary Endothelial Cells, Lonza, Basel, Switzerland). FRET imaging experiments were performed 24–48 h after cell transfection. HUVECs were maintained at 37°C in HEPES-buffered Ringer-modified saline (125 mM NaCl, 5 mM KCl, 1 mM Na3PO4, 1 mM MgSO4, 2 mM $CaCl_2$, 5.5 mM glucose and 20 mM Hepes, pH 7.4) and imaged with an inverted microscope (Olympus IX50) equipped with a CellR imaging system and a beam-splitter optical device (Multispec Microimager; Optical Insights). Images were acquired every 5 s with a 603, 1.4 NA oil-immersion objective (Olympus) using the CellR software and processed using ImageJ (http://rsb.info.nih.gov/ij/). FRET changes were measured as changes in the background-subtracted 480/545 nm fluorescence emission intensities upon excitation at 430 nm and expressed as DR/R_0, where R is the ratio at time t and R_0 is the ratio at time = 0 s; $DR = R-R_0$.

2.18 Zebrafish embryos

All experiments, approved by the Ethical Committee of the University of Padua, were performed at the larval stage of zebrafish. Zebrafish (*Danio rerio*) embryos were obtained from natural spawning of wild-type and the transgenic zebrafish, Tg (Fli: eGFP) strain [56]. Embryos were raised and fishes were maintained as described [57]. Embryos were treated with 1-phenyl-2-thiourea to inhibit pigment formation [57]. This treatment regimen did not appear to have a significant effect on vascular development. For TpF1 injection, anesthetized living embryos at 2 dpf were first embedded in low-melt agarose in order to hold it in place. Embryos were injected via

glass capillaries into the yolk extension or the caudal artery with 10 μM TpF1, under a dissecting stereomicroscope and were allowed to develop. Control embryos were injected with the tracer and saline (vehicle). Larvae at 4 dpf were photographed live using a Nikon C2 H600L confocal microscope with water dripping objectives. Laser used to excite fluorophore was 488 nm for eGFP. The total volume and the intensity of fluorescence of vessels was calculated using VOLOCITY 6.0 software (Perkin Elmer, Waltham, MA) on eGFP-positive confocal acquired images. Embryos and larvae were anesthetized using Tricaine and mounted in 0.8% low melting agarose on a glass lid before photographing. Statistical analyses to compare results for injected samples and controls were performed using uncoupled Student's two-tailed t-test and Prism GraphPad software package.

2.19 RNA Isolation and real-time PCR in zebrafish

A group of 30 larvae were injected at 2 dpf into the yolk extension with 10 μM TpF1 and a second group of 30 larvae were injected with saline (vehicle). At 4 dpf, RNAs were extracted from the larvae of each group, using Trizol Reagent, and pooled. One microgram of total RNA was reverse transcribed using MMLV reverse transcriptase and cDNA was amplified with the following protocol:

The PCR cycle for the Real-Time PCR was:

- Initial denaturation: 95°C for 15 min
- Denaturation: 95°C for 15 seconds
- Annealing: 60°C for 20 seconds
- Extension: 72°C for 30 seconds

} For 45 cycles

The primers used for Real-time PCR analysis were the following:

Gene		Primer sequence	AT (C°)
β-actin	F	5'-CAGCAAGCAGGAGTACGATGAGT-3'	60
	R	5'-TTGAATCTCATTGCTAGGCCATT-3'	
IL-8 a	F	5'-AGCTTGAGAGGTCTGGCTGTAGA-3'	60
	R	5'-CGAGGCGTTGATAAGCTCTCTGCT-3'	
IL-8 b	F	5'-TGTTTTCCTGGCATTTCTGACC-3'	60
	R	5'-TTTACAGTGTGGGCT TGGAGGG-3'	
VEGF-A	F	5'-GATGTGATTCCCTTCATGGATGTGT-3'	60
	R	5'-GGATACTCCTGGATG ATGTCTACCA-3'	

After amplification, data analysis was performed using the second derivative algorithm by applying the $2^{-\Delta\Delta CT}$ method. For each sample, data were normalized to the endogenous reference gene (β-actin). Vehicle-injected larvae were taken as the reference value, set to 1 AU (arbitrary unit, as shown in the figures), and the relative expression levels for treated larvae were calculated and shown.

2.20 Vessel visualization

For yolk vessel observation, embryos were injected at 1 dpf into the yolk extension with 10 μM TpF1, or saline and 3 dpf, the embryos were fixed in 4% paraformaldehyde (PFA) and stained for endogenous alkaline phosphatase activity. Briefly, embryos were fixed in 4% PFA for 2 h at RT and washed 5–6 times in PBS 0.1% Tween-20 (PBST). Embryos were dehydrated and made permeable by successive washes in 25, 50, and 75% methanol (MeOH) in PBST, and finally suspended in 100% MeOH. This was followed by serial rehydration of the embryos in 75, 50, and 25% MeOH in PBST and suspended in PBST. Embryos were equilibrated with alkaline phosphatase buffer (100 mM Tris, pH 9.5, 50 mM MgCl, 100 mM NaCl and 0.1% Tween-20) at RT. Subsequently they were incubated in

staining solution (335 μg/ml NBT and 175 μg/ml BCIP in alkaline phosphatase buffer). Staining reaction was stopped by adding PBST. Embryos were immersed in a solution of 5% formamide and 10% hydrogen peroxide in PBS for 20 min to remove endogenous melanin in the pigment cells, allowing the visualization of the yolk vessels and stored in 80% glycerol in PBST for further analysis.

For the visualization of caudal vessel, 3 dpf embryos were anesthetized with 0.003% Tricaine and microangiografy was performed. A solution of FITC-labeled 70 kDa-dextran was injected into the cardiac venous sinus and images of live fishes were generated using a Leica inverted microscope connected to a Leica DFC490 camera.

The length of the vessel and the ectopic processes in the yolk and the distance from CA (caudal artery) and CV (caudal vein) were measured using Sigma Scan Pro software.

2.21 Statistical analysis

Data are reported as the mean ± S.D. Student *t* test was used for statistical analysis of the differences between experimental groups. The *p* values ≤ 0.05 were considered significant.

Results

Syphilis is a progressive and multistage disease with diverse and wide-ranging manifestations. How the bacterium triggers the clinical manifestations characterizing the different stages of the disease has not been fully elucidated; however it is established that *T. pallidum* is able to invade and survive in a wide variety of tissues and organs [1] and that it promotes the formation of new blood vessels [36]. Angiogenesis could have a crucial role in syphilis pathogenesis, at least for two reasons: i) the bacterium has limited metabolic capabilities [58], thus probably it requires support from the host and it may derive most essential macromolecules from the blood. ii) Increased vascular permeability is one of the first stages in angiogenesis [37] and the organism could take advantage of the vascular leakage to access to and egress from the bloodstream, resulting in systemic spread.

Although syphilis is a highly inflammatory disease, is well established that, in untreated patient, it becomes chronic and this probably reflects the ability of the bacterium in eliciting a T regulatory response, which could be associated with fading of the host effector immune responses against the pathogen [59], [60]. In this regard, it has been recently demonstrated in our lab that the bacterioferritin TpF1, a major antigen of *T. pallidum*, in addition to promote the secretion of proinflammatory cytokines via inflammasome, it has a central role in driving this suppressive immune response, by modulating the development of regulatory T cell [29].

TpF1 is a protein structurally related to another immunomodulant antigen produced by *Helicobacter pylori* and called HP-NAP. Both these proteins belong to the Dps-like family, a versatile group of bacterial stress miniferritins with a nearly spherical dodecameric structure.

HP-NAP, besides interacting with neutrophils, monocytes and dendritic cells and modulating their activity (as TpF1 does), also binds to endothelial cells, in which it is internalized by transcytosis at the basal side before being exposed on the surface exposed to the lumen of blood vessels.

Considering the pivotal role of TpF1 in syphilis pathogenesis, the homology existing between HP-NAP and TpF1, and the fact that the angiogenic process is a hallmark of the secondary syphilis, we wondered whether TpF1 could interact with endothelial cells and promote new blood vessels formation.

To investigate the angiogenic activity of TpF1 we took advantage of *in vitro* assays using human humbelical vein endothelial cells (HUVECs) as cell model. The majority of assays on endothelial cell utilize HUVECs, because they are easy to obtain and manipulate and because they maintain the capacity to proliferate preserving, on the same time, their normal *in vivo* physiological characteristics.

However, endothelial cells are highly heterogeneous (depending on the blood vessel type, organ, host) *in vivo* and they are mostly in a quiescent state of the established vasculature rather than in a proliferative state. For these reasons, and because of the complex interactions among different cell types (smooth muscle cells, pericytes, fibroblast, macrophages) forming functional blood vessels, *in vitro* angiogenesis assay must be considered a starting point of the research and the *in vitro* findings have to be confirmed *in vivo* using an animal model.

3.1 TpF-1 induces an angiogenic phenotype in cultured endothelial cells

Endothelial cell proliferation and migration, as well as extracellular matrix remodeling, are considered to be key steps of sprouting angiogenesis.

Proliferation of endothelial cells is indeed essential for developing capillaries in the organism; behind tip cells, endothelial stalk cells proliferate elongating the new forming capillary. In order to verify whether TpF1 could be involved in the angiogenic process during syphilis progression, we first tested the ability of TpF1 in inducing human endothelial cell proliferation in vitro. To this aim, endothelia cells, seeded on a gelatin-coated 24-well plate, were treated with TpF1, VEGF (as positive control) or vehicle (saline) and the proliferation of the cells was

evaluated, after 12 and 24 h of stimulation.

As shown in Figure 13, TpF1 induces cell proliferation after both 12 and 24 h in a manner, which is superimposable to that of VEGF.

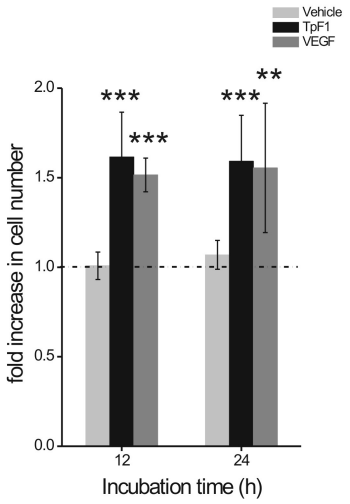

Figure 13. TpF1 induces proliferation of endothelial cells.
Endothelial cells were exposed to 1 μM TpF1,20 ng/ml VEGF (positive control) or vehicle (saline). After 12 and 24 h, cells were counted and normalized by the number of plated cells (set as 1, dotted line). The graph shows the fold increase in cell number under the different conditions. Significance was determined by student's t-test for data of TpF1 treated cells and VEGF treated cells versus vehicle-treated cells. **, $p < 0.01$ ***, $p < 0.001$.

In sprouting angiogenesis, endothelial cells degrade the extracellular matrix and, along chemical gradients of pro-angiogenic stimuli, cells extend, contract, and throw their rear toward the front and progress forward [61].

Therefore, we moved to address whether TpF1 was able to stimulate endothelial cells migration by taking advantage of a transwell system: in this system endothelial cells are placed on the upper layer of a cell permeable membrane and a solution containing the test agent is placed below the cell permeable membrane. The filter pores are small enough (~8 μm) to allow passage of actively migrating cells into the lower chamber, avoiding the passive membrane transition driven by gravity.

Endothelial cells were added to the upper chamber and the number of cells migrated into the lower chamber, containing either TpF1 or VEGF or

vehicle, was counted at different time intervals. The results showed a pro-migratory effect of TpF1 similar to that of VEGF (Figure14).

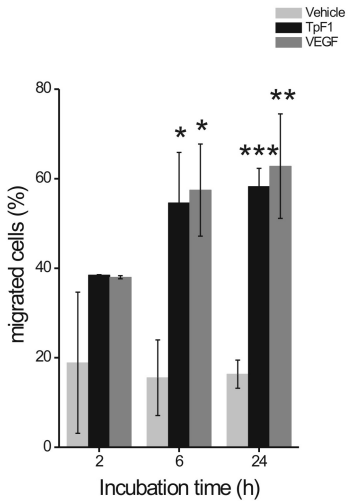

Figure 14. **TpF1 induces migration of endothelial cell.** Fast DiI-labeled endothelial cells were seeded on the upper chambers of a 24-transwell plate in HE-SFM. 1 µM TpF1, 20 ng/ml VEGF or vehicle were added to the lower chambers. Migrated cells were quantified after 2, 6 and 24 h by a microplate reader (Infinite 200, Tecan) and expressed as % of seeded cells. Data are presented as mean ± S.D. of three independent experiments. Significance was determined by student's t-test for data of TpF1 treated cells and VEGF treated cells versus vehicle-treated cells. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

As clearly depicted in the graph, TpF1 stimulated migration of approximately 38% cells after 2 h, and mobilized more than 54% cells after 6 h, reaching 58% after 24 h. During angiogenesis, subsequently to proliferation and migration, endothelial cells begin to form organized tubules with defined lumens in order to permit a correct blood flow.

Since TpF1 stimulated proliferation and migration of endothelial cell, we next evaluated the ability of TpF1 to induce vessel formation using an *in vitro* tube formation assay in matrigel.

Matrigel is a solubilized basement membrane preparation extracted from the Engelbreth-Holm-Swarm (EHS) mouse sarcoma, a tumor rich in extracellular matrix proteins that include laminin (a major component), collagen IV, heparan sulfate proteoglycans, and entactin/nidogen (BD Falcon). When endothelial cells are seeded on matrigel, in the presence of a pro-angiogenic factor, they attach within one hour, migrate toward each

other, tend to form tubes with a lumen, and develop tight cell–cell and cell–matrix contacts [62].

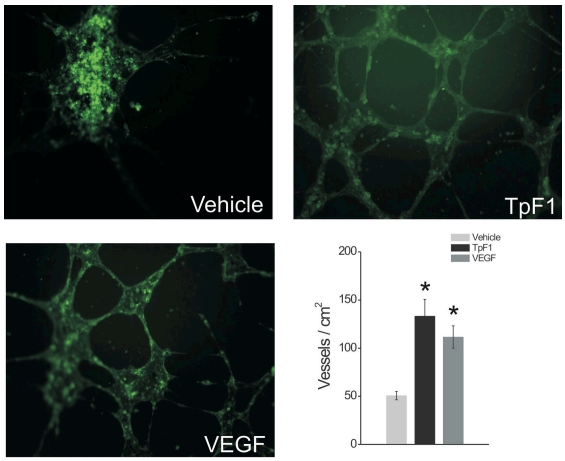

Figure 15. TpF1 induces endothelial cell organization in microcapillary-like structures. Endothelial cells were seeded on matrigel-coated coverslips (area=0.69 cm^2) and exposed to 1 μM TpF1, 20 ng/ml VEGF or vehicle, for 12 h. Cells were fixed, stained with Phalloidin-Alexa Fluor 546 and analyzed by confocal microscopy at 200× magnification. Microcapillary-like structures were counted from three different coverslips for each experimental condition and expressed as the average of the number of vessels per cm^2. Data are presented as mean ± S.D. Significance was determined by student's t-test for data of TpF1 treated cells and VEGF treated cells versus vehicle-treated cells. *, $p < 0.05$.

We grew endothelial cells in matrigel and stained the cells for actin to visualize tube formation. Confocal microscopy images clearly reveal that endothelial cells incubated with no agonists tended to cluster together forming large aggregates without any tubular organization. On the contrary, TpF1 induced marked changes in the cell pattern with the formation of tubules assembled by elongation and joining of endothelial cells; a similar pattern was observed with VEGF. Quantitative analysis of the data showed that the effect of TpF1 was even higher than that of VEGF (Figure 15).

3.2 TpF1 promotes synthesis and secretion of IL-8 in endothelial cells

To gain insights into how TpF1 induces angiogenesis *in vitro*, we decided to investigate about the molecular pathway that could be involved in this

process. To this aim we have first evaluated gene synthesis and protein release of VEGF, the most potent angiogenesis inducer produced by the endothelium [63].

Endothelial cells, isolated from human cord, were treated with TpF1, VEGF or vehicle (saline), harvested at different time points and total RNA was isolated for RT-PCR analysis. Culture supernatants of the same cells were collected for subsequent quantification of VEGF content by ELISA.

Interestingly TpF1 did not affect both the expression and the release of VEGF (Figure 16, left panels).

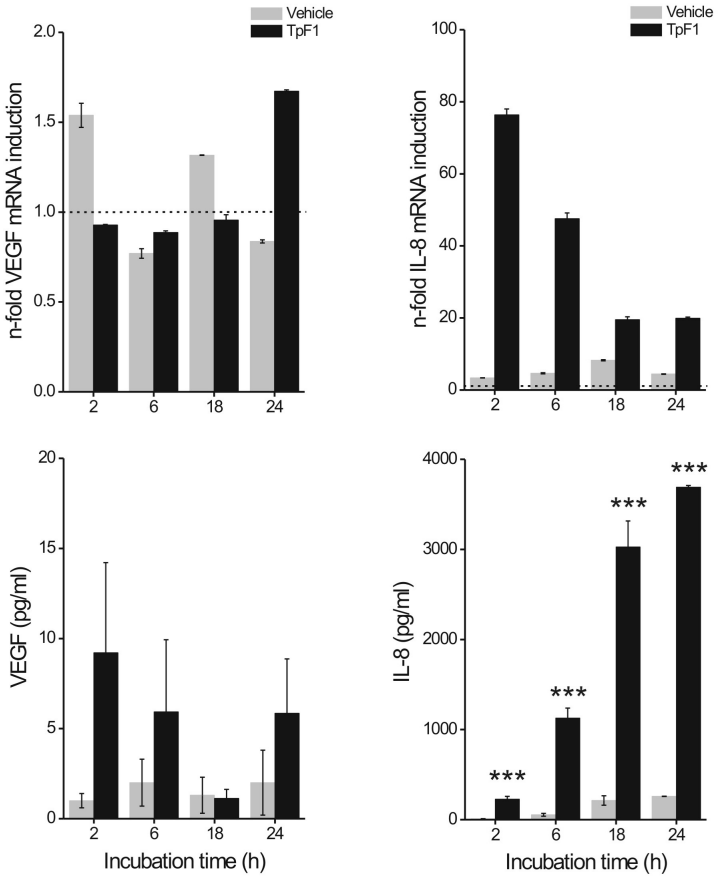

Figure 16. TpF1 stimulates IL-8 expression. Endothelial cells were exposed to 1 μM TpF1 or vehicle (saline) for 2, 6, 18, 24 h and the expression of VEGF (left top panel) or of IL-8 (right top panel) was evaluated by real-time PCR. Data were normalized to an endogenous reference gene (ribosomal subunit 18S). Values at T_0 cells were taken as reference and set as 1 (dotted line) and the expression levels for treated cells were expressed as *n*-fold change relative to the expression of T_0 cells. Culture supernatants from endothelial cells, harvested for quantification of mRNA, were collected and the VEGF (left bottom panel) and IL-8 (right bottom panel) protein content was quantified by ELISA. Data are expressed as mean ± S.D. of four independent experiments. Significance was determined by student's t-test for data of TpF1 treated cells versus vehicle-exposed cells (***, $p < 0.001$).

We next evaluated whether TpF1 promoted the production of IL-8, a pro-inflammatory chemokine with pro-angiogenic properties, also produced by endothelial cells [52].

In contrast to what observed for VEGF, TpF1 strongly induced both expression and secretion of IL-8 (Figure 16, right panels) in a time-dependent manner suggesting a role for the cytokine in the TpF1-induced angiogenic process.

3.3 The pro-angiogenic activity of TpF1 *in vitro* is mediated by IL-8

The role of the chemokine in the TpF1-induced angiogenesis was verified by applying an IL-8-blocking antibody to endothelial cells exposed to the bacterial protein. Endothelial cells were treated with TpF1, IL-8 or vehicle (saline) in presence or absence of an anti-IL-8 blocking antibody and cell proliferation was evaluated after 12 and 24 h of stimulation.

As shown in Figure 17A the presence of the blocking antibody almost completely abrogated endothelial cell proliferation, both at 12 and 24 h.

For the migration assay, we stimulated endothelial cells with TpF1 for 24 h; the supernatant was then collected and incubated 45 min at 37°C with the anti-IL-8 antibody before adding to the lower chamber of the transwell system, where fresh endothelial cells seeded on the filter of the upper chamber. By using this experimental set-up, we verified that TpF1-induced migration was also reduced upon the blockage of IL-8, slightly after 2 h, but significantly after 6 and 24 h (Figure 17B).

Finally, in order to unravel the IL-8-dependency of the angiogenic process due to TpF1, we repeated the *in vitro* tube formation assay. We grew endothelial cells in matrigel in the presence of TpF1 with or without the IL-8-blocking antibody, before proceeding with the staining of the cells for actin to visualize tube formation.

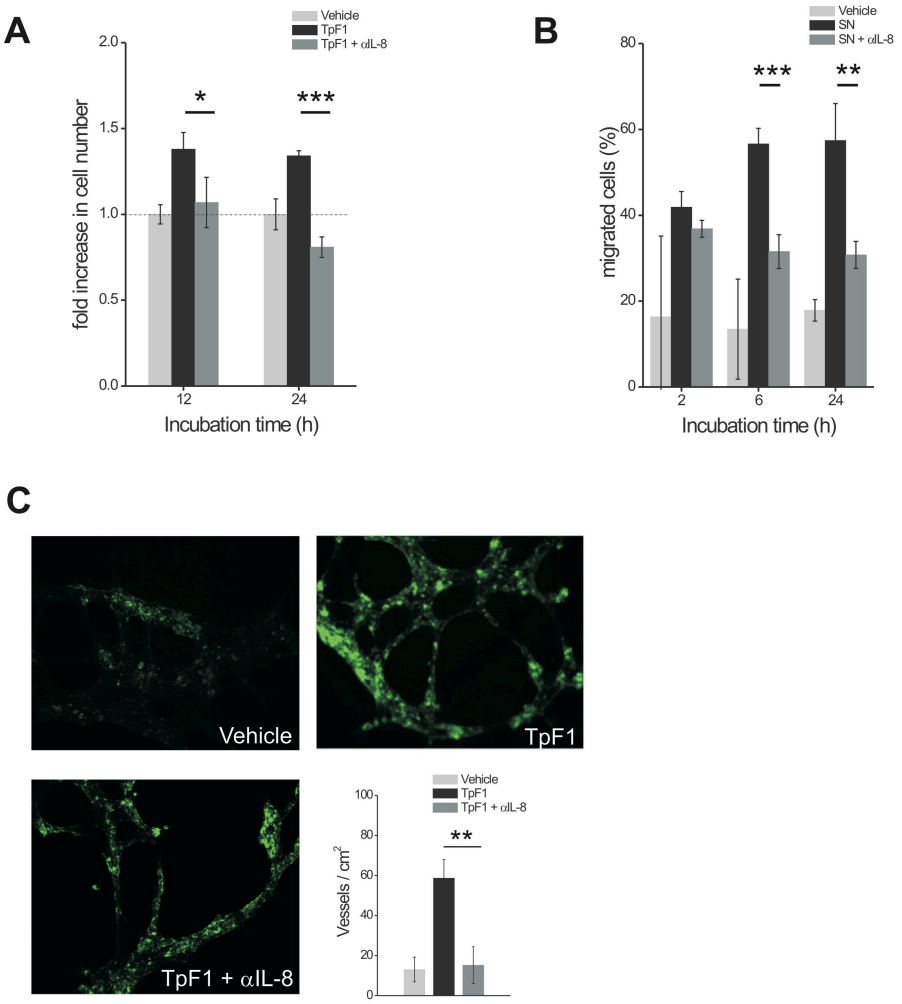

Figure 17. IL-8 is required for the pro-angiogenic activity of TpF1. (**A**) Endothelial cells were exposed to TpF1, as in Figure 1A, in presence or absence of 1 μg/ml anti-IL-8 blocking antibody; after 12 and 24 h cells were counted and normalized by the number of plated cells (set as 1, dotted line). The graph shows the fold increase in cell number under the different conditions. (**B**) Endothelial cells, grown to confluence in a 24-well plate, were exposed to TpF1 for 24 h. Supernatant was collected and incubated or not with 1 μg/ml anti-IL-8 blocking antibody for 45 min at 37°C, before transferring to the lower chamber of a 24 transwell plate with Fast DiI-labeled endothelial cells seeded on the upper chamber. Migrated cells were quantified after 2, 6 and 24 h as before. (**C**) Endothelial cells seeded on matrigel-coated coverslips (area=0.69 cm^2) were exposed to 1 μM TpF1 for 12 h, in presence or absence of 1 μg/ml IL-8 blocking antibody. Microcapillary-like structures were counted from three different coverslips for each experimental condition and expressed as the average of the number of vessels per cm^2. Data are expressed as mean ± S.D. of four independent experiments. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Confocal microscopy analysis confirmed the ability of TpF1 to promote the capillary formation; conversely, when IL-8 was blocked, cells clustered together forming large aggregates, showing a morphology resembling the negative control where cells were clustered and no elongations and joining

of endothelial cells were detectable; quantitative analysis of the formed tubules confirmed the inhibitory effect of the anti-IL-8 antibody (Figure 17C).

3.4 TpF1 increases cAMP in endothelial cells

Established the involvement of the chemokine in the TpF1-induced angiogenesis we decided to focus our study on the signaling pathway evoked by TpF1 leading to the IL-8 gene transcription.

It is known that under inflammatory conditions, especially in presence of IL-1β, the expression of CXC chemokine, and in particular of IL-8, is promoted and it follows the activation of two transcription factors: NF-κB and CREB (cAMP response element-binding protein) [54]. Accordingly, the consensus sequence of both CREB and NF-κB are located in the IL-8 promoter [64], [65].

Since also TpF1 had pro-inflammatory properties [29], and it induced IL-8 secretion, it was plausible that it modulated the same transcription pathway involved in the IL-1β signaling. Therefore, we sought to determine whether CREB and NF-κB mediated the TpF1-induced IL-8 expression in endothelial cells.

Recent evidence suggest that CREB and NF-κB may share an upstream common activator, the adenylate cyclase [66], [67].

Adenylate cyclases is an enzyme, located in the plasma membrane, which catalyzes the conversion of adenosine triphosphate (ATP) into 3',5'-cyclic AMP (cAMP) and pyrophosphate. cAMP is a second messenger, which in turn, through a number of different signal pathways, leads to the activation of both CREB and NF-κB thus promoting the expression of the IL-8 gene (Figure 18).

The latter consideration prompted us to investigate the cAMP dynamics in living endothelial cells exposed to TpF1. Endothelial cells were transfected with the genetically encoded, FRET-based cAMP sensor Epac1 [55], and imaged under a fluorescence microscope.

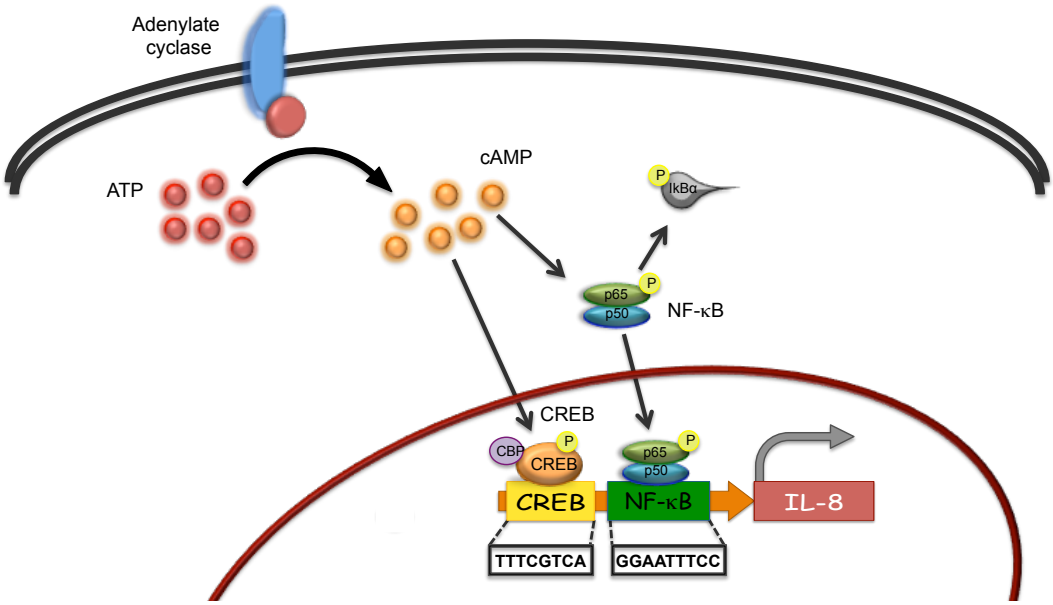

Figure 18. Schematic representation of c-AMP-triggered activation of CREB and NF-κB.

Epac1 sensor consists of a single cAMP-binding site from human Epac1 protein flanked by YFP and CFP. Binding of cAMP to the sensor leads to a conformational change of the protein, resulting in a decrease of the YFP fluorescence due to FRET and a concomitant increase in CFP fluorescence [68].

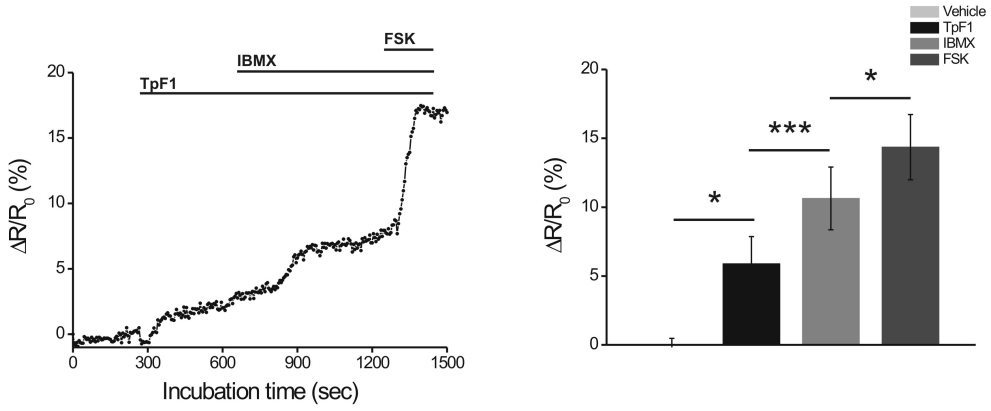

Figure 19. TpF1 increases cytosolic cAMP in endothelial cells. (**A**) Representative kinetics of cAMP changes recorded in a Epac1-expressing endothelial cells, upon addition of 1 µM TpF1, followed by 100 µM IBMX and by 25 µM forskolin. cAMP variations are presented as DR/R_0. The average $\Delta R=R-R_0$ increases (mean ± SEM, n=9) are presented in (**B**). *, $p < 0.05$; ***, $p < 0.001$

TpF1 application induced a cAMP increase in endothelial cells, which was potentiated by the inhibition of phosphodiesterases by 3-isobutyl-1-methylxanthine (IBMX).

The TpF1-induced cAMP accounted for about 30% of the maximal cAMP generated in endothelial cells, when adenylate cyclases were maximally activated by the direct activator forskolin and phosphodiesterases were maximally inhibited.

Figure 19A shows a typical kinetic of $\Delta R/R_0$ in an endothelial cell challenged with TpF1, followed by the application of IBMX and forskolin. Figure 19B summarizes the average $\Delta R/R_0$ changes induced by TpF1, alone or plus the drugs, in Epac1-expressing endothelial cells (n=9).

3.5 NF-κB and CREB are activated by TpF1 in endothelial cells.

Since CREB is activated via phosphorylation at Ser133 by several different kinases, including MAPK, but mainly PKA (cAMP-dependent protein kinase), we monitored the phosphorylation level of CREB in endothelial cells exposed for 30 min to TpF1. Figure 20A shows that TpF1 promoted the phosphorylation of CREB to a similar extent than the adenylate cyclase activator forskolin.

This result was confirmed by a quantitative kinetic performed with a phospho-CREB cell-based ELISA (Figure 20B): phosphorylation was detectable as early as after a 5 min-exposure to the bacterial protein and it remained high until 30 min, before dropping down to the basal level after 60 min. As before, the extent of phosphorylation induced by TpF1 was similar to that triggered by forskolin. For addressing whether TpF1 activated NF-κB too, we first evaluated the phosphorylation of the NF-κB p65 subunit, an event required for transactivation of target genes [69].

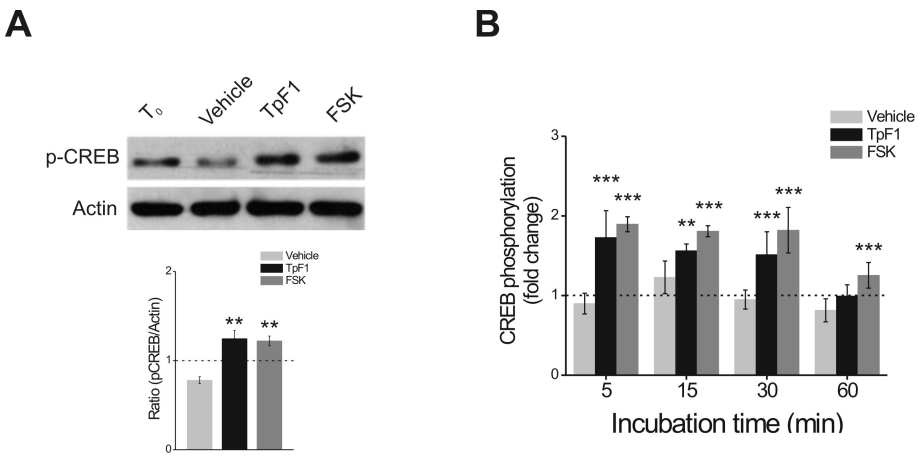

Figure 20. TpF1 activates CREB in endothelial cells. (**A**) **Endothelial cell**s were exposed to 1 μM TpF1, 25 μM forskolin or vehicle (saline). After 30 min, cells were lysed and processed for western blot; p-CREB and actin were revealed with specific antibodies. Graph shows the ratio between p-CREB and actin (n=4). (**B**) **Endothelial cell**s were exposed to 1 μM TpF1, 25 μM forskolin or vehicle; at the indicated time points, the phosphorylation of CREB was evaluated by ELISA assay. For each sample, data were normalized to the total CREB protein. Normalized fluorescence value of cells at time 0 (T_0) was taken as reference and set as 1 (dotted line) and CREB phosphorylation of treated cells was expressed as fold change of T_0 cells. Data are expressed as mean ± S.D. of three independent experiments. Significance was determined by student's t-test for data of agonist-treated cells versus vehicle-exposed cells. **, $p < 0.01$; ***, $p < 0.001$.

As shown in Figure 21A, depicting the activation state of p65 after a 30 min-incubation with agonists, TpF1 promoted the phosphorylation of the subunit similarly to LPS, adopted as positive control.

A quantitative kinetic of the phosphorylation of the NF-κB inhibitor κBα (IκBα), an event that precedes its proteasomal degradation and the translocation of the active form of NF-κB to the nucleus [70], was also performed.

Figure 21B shows that the exposure of endothelial cells to TpF1 increased by 1.5 folds the phosphorylation state of IκBα, similarly to what observed in case of LPS intoxication. The phosphorylation of IκBα which remained high at 15 min of incubation, decreased to the basal level thereafter, in accordance to the rapid degradation of the inhibitor by the proteasome.

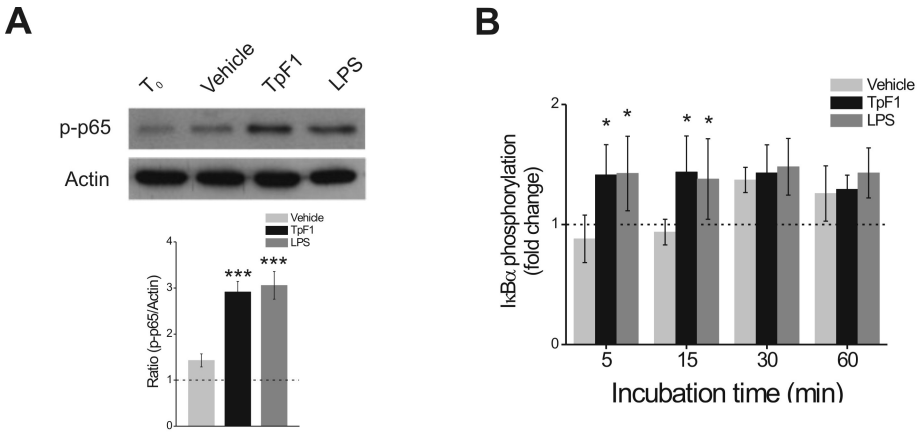

Figure 21. TpF1 activates NF-κB in endothelial cells. (**A**) **Endothelial cell**s were exposed to 1 μM TpF1, 1 μg/ml LPS or vehicle. After 30 min, cells were processed for western blot; p-p65 and actin were revealed with specific antibodies. Graph shows the ratio between p-p65 and actin (n=4). (**B**) **Endothelial cell**s were exposed to 1 μM TpF1, 1 μg/ml LPS or vehicle; at indicated time point the phosphorylation of IκBα was evaluated by ELISA assay. For each sample, data were normalized to the GAPDH protein. Normalized fluorescence value of cells at time 0 (T_0) was taken as reference and set as 1 (dotted line) and IκBα phosphorylation of treated cells was expressed as fold change of T_0 cells. Data are expressed as mean ± SD of three independent experiments. Significance was determined by student's t-test for data of agonist-treated cells versus vehicle-exposed cells. *, $p < 0.05$.

3.6 The activation of CREB and NF-κB is essential for TpF1-induced IL-8 production

Based on the evidence that TpF1 activated both CREB and NF-κB, we next evaluated whether these two transcription factors were those responsible for the increased expression and secretion of IL-8, observed in endothelial cells exposed to the bacterial protein.

To this aim, we took advantage of two inhibitors: 2-naphthol-AS-E-phosphate (KG501) and N4-[2-(4-phenoxyphenyl)ethyl]-4,6 quinazolinediamine (QNZ). KG501 is a small molecule that binds to the transcription co-activator CREB-binding protein (CBP) and blocks its interaction with the active form of CREB, p-CREB [71]. QNZ, also called EVP4593, is antagonist of the NF-κB pathway activation, acting by inhibiting store-operated calcium (Ca^{2+}) entry (SOC) [72].

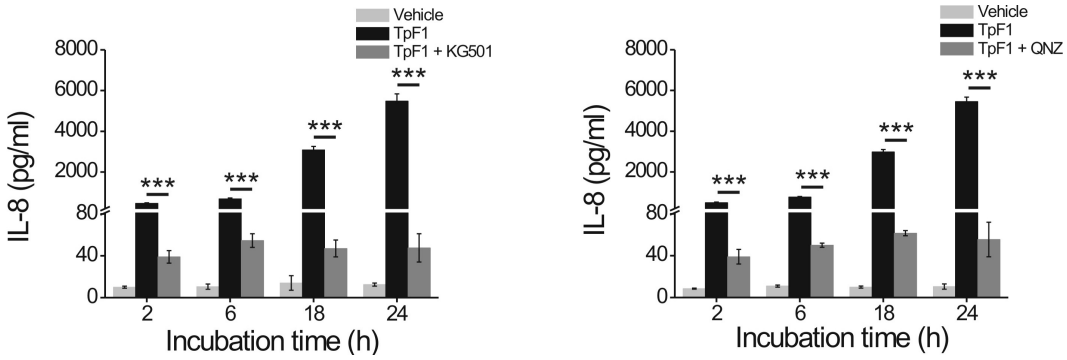

Figure 22. CREB is NF-κB **are essential for the production of IL-8 by endothelial cells exposed to TpF1.** Endothelial cells were pre-incubated or not pre-incubated for 30 min with the CREB inhibitor KG501 (left panel) or the NF-κB inhibitor QNZ (right panel), before the exposure to 1 μM TpF1 or vehicle. At the indicated time points, culture supernatants were collected and evaluated for their IL-8 content by ELISA. Data are expressed as mean ± SD of three independent experiments. ***, $p < 0.001$.

Figure 22 shows that a 30 min pre-incubation of endothelial cells with each inhibitor results in a significant reduction of the secretion of IL-8 triggered by TpF1; this result definitively supports the conclusion that CREB and NF-κB, which are activated by TpF1, are essential for IL-8 gene expression induced by the bacterial protein.

3.7 TpF1 has angiogenic activity in living zebrafish

We previously said that in vitro angiogenesis studies should be considered as a starting point rather than an endpoint of our research; due to the complexity of the angiogenic process, the in vitro findings must be confirmed in an animal model. Zebrafish has recently gained much attention as model to study the *in vivo* angiogenesis. Zebrafish embryos develop outside of the uterus, and this permits to follow the animals during its development. Moreover, the possibility to inhibit the pigmentation by a chemical treatment, guarantees that the embryos remain transparent. Zebrafish is relatively easy and cheap to maintain, compared to rodents. They produce many eggs per each breeding cycle, and the embryonic development is fast [56]. All these characteristics make zebrafish a useful tool for studying angiogenesis *in vivo*. In the last decades many kinds of transgenic lines expressing fluorescent proteins in a cell-type specific

manner have been generated. Among them, the fli1a:EGFP transgenic line has become a very useful tool for examining vascular development in zebrafish. Fli1:EGFP transgenic line expresses EGFP (enhanced green fluorescent protein) under the control of the promoter for fli1, a known endothelial cell marker in mouse [73], which is also expressed during vascular development in zebrafish embryos [74]. Application of this vascular-specific transgenic zebrafish, in conjunction with confocal microscopy analysis, allows a precise and detailed *in vivo* observation of the embryonic vasculature in a vertebrate animal model [56].

3.5.1 TpF1 induces new blood vessels formation in zebrafish

In a first set of experiments we based on the angiogenic activity assay developed by Serbedzija and colleagues [56] and consisting in measuring the length of SIVs (sub intestinal veins) in animals injected with potential angiogenic molecules in the yolk.

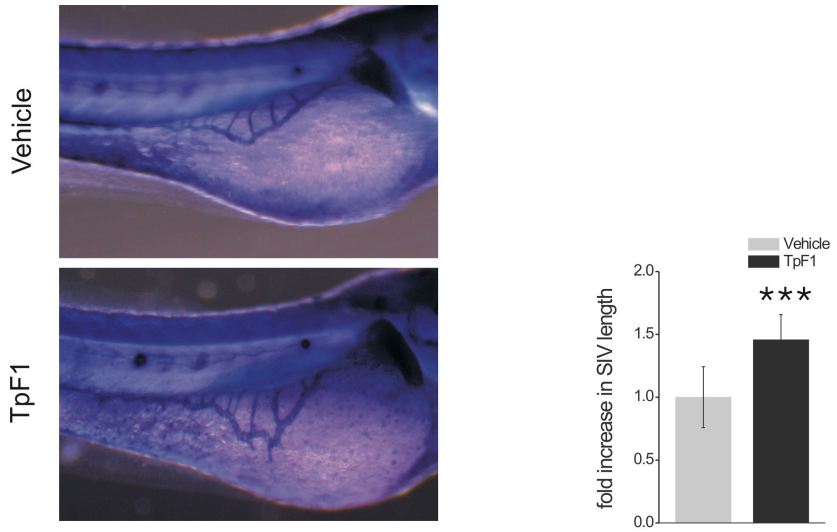

Figure 23. TpF1 promotes SIVs sprouting in zebrafish Comparison of the blood vessel patterning, at the sub intestinal area (SIVs), between vehicle- and TpF1-treated larvae. Animals were injected in the yolk 1 dpf and the alkaline phosphatase-based assay was performed at 3 dpf. Graph represents the fold increase in SIV length referring to the value of vehicle-treated animals, set as 1. Data are expressed as mean ± S.D. (n= 20 for each treatment). Significance was determined by student's t-test for data of TpF1-treated fish versus vehicle-exposed fish. ***, $p<0.001$.

The SIVs assay has the advantage of being low tech; on the other hand, it has high variability and works *ex vivo*.

With this first screening, we observed, 3 dpf (days post fertilization), a significant change in the shape and length of SIVs in 1 dpf TpF1-injected-fishes (Figure 23). In order to obtain further insights into TpF1 activity *in vivo*, we performed a zebrafish microangiography. Microangiography is a simple technique in which a fluorescent dye is injected directly into the Sinus Venosus and/or the Posterior Cardinal Vein, allowing the rapid labeling and easy detection of vessels [75]. We injected zebrafish early larvae at 1 dpf, with TpF1 or vehicle (saline); at 3 dpf a solution of FITC-labeled 70 kDa-dextran into the cardiac venous sinus was injected and live fish were then analyzed using a Leica inverted microscope.

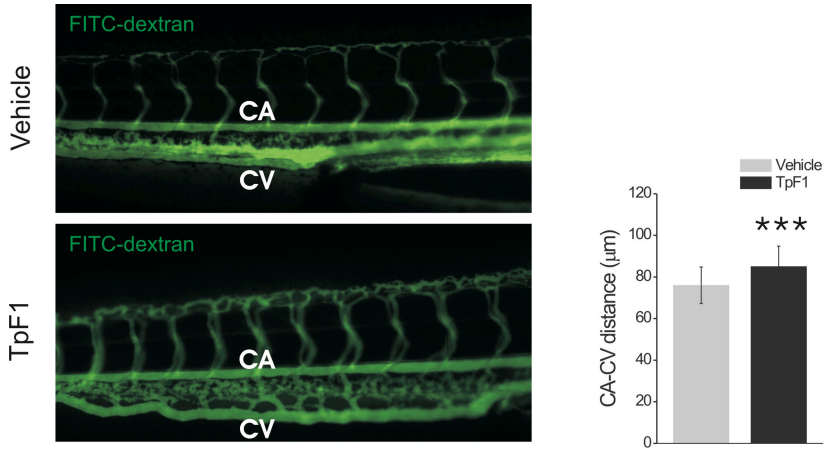

Figure 24. TpF1 increases the CA-CV distance in zebrafish Comparison of the blood vessel patterning at the trunk-tail region, between vehicle- and TpF1-treated larvae. The circulatory tree was revealed by FITC-labelled 70 kDa-dextran, injected into the cardiac venous sinus of 3 dpf old larvae. Animals were injected with vehicle or TpF1 in the yolk at 1 dpf and images were taken at 3 dpf. Graph shows the mean ± S.D. of the distance between the caudal aorta (CA) and the caudal vein (CV) (n= 12 for each treatment). Significance was determined by student's t-test for data of TpF1-treated fish versus vehicle-exposed fish. ***, $p<0.001$.

In accordance to previous data, microangiography of TpF1-injected fish revealed a significant increase of the distance between the Caudal Aorta (CA) and the Caudal Vein (CV) (Figure 24). Based on these results, we decided to fully exploit the *in vivo* potential of zebrafish by measuring the total level of integrated density of the intestinal circulation in the sector

from the 8th to the 14th intersomitic vessel (Figure 25A, red box). The idea of focusing the analysis on the intestinal circulation relied on the fact that TpF1, injected in the yolk sac, was able to diffuse posterior in its own extension, as revealed by using a rhodamine-conjugated TpF1 (data not shown).

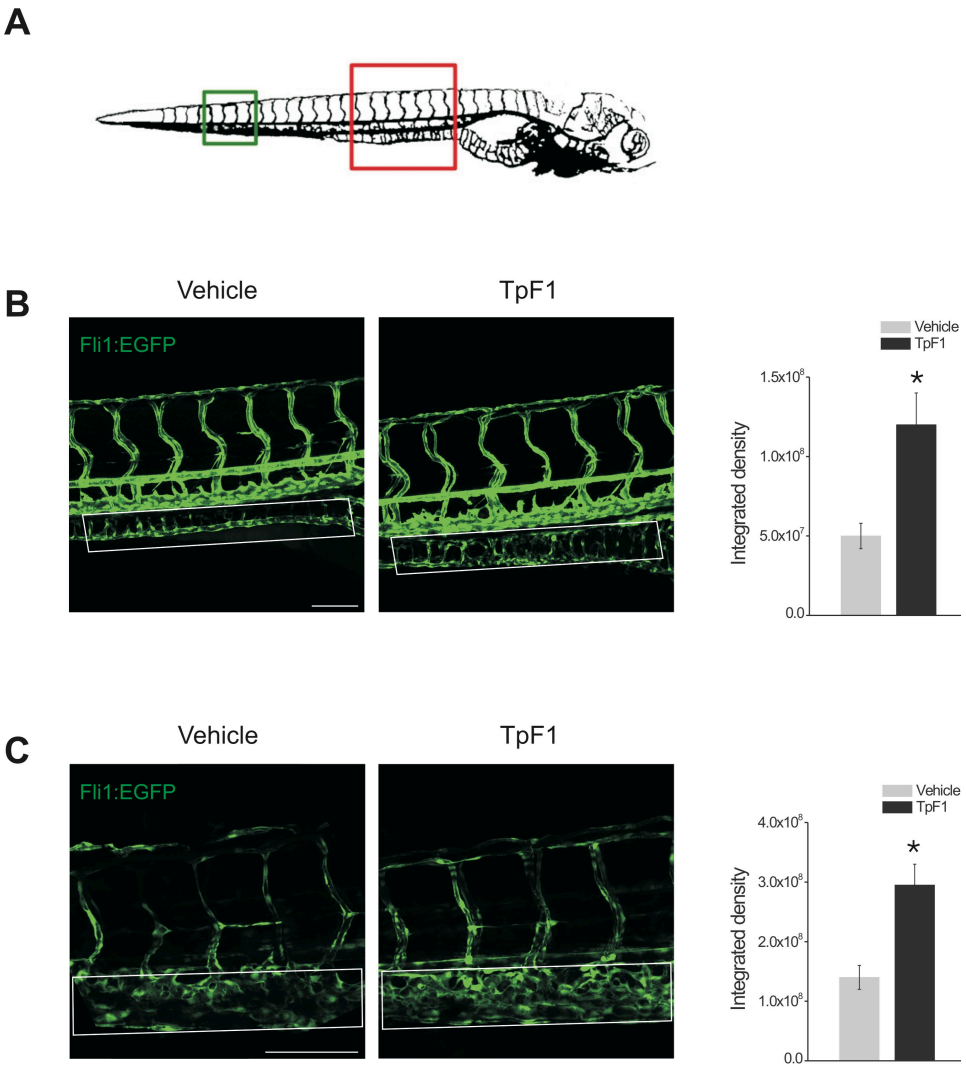

Figure 25. New blood vessels formation in zebrafish after TpF1 treatment. (**A**) Illustration of the vascular system of a 4 dpf larva; regions for image acquisition are indicated by the colored boxes (panel A). Embryos at 2 dpf were injected with 10 μM TpF1 or vehicle. Injections were performed either in the yolk (**B**) or in the caudal vein plexus (**C**). Representative images were taken at 4 dpf by confocal microscopy at 20× and 40× magnification, respectively. Scale bar: 100 microm. Graphs summarize data of six independent experiments and are expressed as mean ± S.D of integrated density of fluorescence in the green channel. Significance was determined by student's t-test for data of TpF1-treated fish versus vehicle-exposed fish. *, $p < 0.05$. Squares indicates the region of interest analyzed by VOLOCITY 6.0 software.

To this aim, we took advantage from the transgenic fluorescent zebrafish Tg(fli1:EGFP)^{y1} [55]. Fli1 is a transgenic zebrafish line expressing enhanced GFP in the entire vasculature, under the control the fli1 promoter.

Two dpf transgenic fli1:EGFP larvae were injected with TpF1 at the concentration of 10 μM in the yolk sac. After injection, normally shaped and positively injected larvae were processed for imaging studies. As shown in Figure 25B, embryos injected with TpF1 revealed a significant fluorescence increase in the intestinal circulation at two days post injection when compared with vehicle-injected controls.

To confirm the angiogenic effect, we also injected 2 dpf larvae in the caudal plexus. Then, the integrated fluorescence of the caudal plexus, in the segment between the 21st and 25th intersomitic vessel (Figure 25A, green box), was computed in fli1:EGFP fish injected with TpF1 and the measure compared with vehicle-injected fish (Figure 25C). Results confirmed a consistent and significant angiogenic activity by TpF1 also in the caudal plexus.

3.5.2 TpF1 promotes synthesis of IL-8 in zebrafish

Finally, we evaluated the expression of the angiogenic factors VEGF and IL-8 in zebrafish exposed to the bacterial protein, in order to further corroborate with the *in vivo* model, the *in vitro* evidence.

We found that the mRNA level of the two isoforms of the IL-8 gene was up-regulated after treatment with TpF1 at 2 days post-injection, while the expression level of VEGF was comparable to that of vehicle-injected controls (Figure 26). These results, demonstrating that the angiogenic effect of TpF1 *in vivo* is paralleled by the increase of IL-8 expression, support the conclusion that this cytokine is crucial for the pro-angiogenic activity of TpF1.

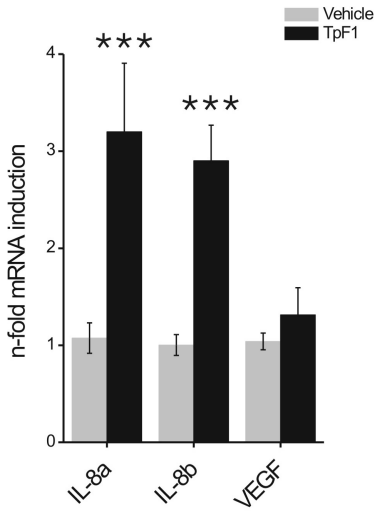

Figure 25. TpF1 induces IL-8 gene expression in zebrafish. Wt animals were injected with 10 µM TpF1 or vehicle. The injection was applied in the yolk at 2 dpf. RNAs were extracted at 4 dpf and the expression of VEGF, IL-8a and IL-8b was evaluated by real-time PCR. For each sample data were normalized to the endogenous reference gene β-actin. Vehicle-treated fish were taken as reference and set as 1; expression levels of TpF1-exposed fish were expressed as *n*-fold change relative to the expression level of vehicle-treated fish. Data represent mean ± S.D. (n=30 larvae for each treatment). Significance was determined by student's t-test for data of TpF1-treated fish versus vehicle-exposed fish. ***, $p < 0.001$.

4. Discussion

Syphilis is a chronic and multistage disease caused by the spirochete Treponema pallidum. Despite it could be cured with a simple and inexpensive antibiotic treatment, syphilis is worldwide spread and represent a global health problem; even today it is considered the major sexual transmitted disease. Nowadays none vaccine to prevent syphilis has been formulated yet, in particular due to the difficulties in cultivating *T. pallidum in vitro,* because of its peculiarities, (striking lack of metabolic capabilities, sensitivity to oxygen and temperature) which makes more difficult to study this pathogen, as well as the identification of its virulence factors. Although little is known about the mechanism of syphilis pathogenesis and how the bacterium causes the typical clinical manifestations that accompany the different stages of the disease, it is widely accepted that T. pallidum is able to invade and survive in a wide variety of tissues and organs [1] and that it activates the endothelium promoting new blood vessels formation [36]. Angiogenesis, in this contest, could be exploited by the bacterium to uptake nutrients directly from the host and, taking advantage of the increased vascular permeability characterizing first stages of angiogenesis [37], to access to and egress from the bloodstream, resulting in systemic spread. Despite the pivotal role that angiogenesis could play in syphilis progression, bacterial factors involved in this process are still unknown. Another distinctive feature of syphilis relies on the capacity of *T. pallidum* to elicit a strong inflammation and, at the same time, to avoid the complete clearance exerted by the immune response of the host leading, thus, to chronic disease. In this regard it has been recently demonstrated by our research group, that the mininferritin TpF1, a major treponemal antigen, promotes inflammasome activation with the subsequent release of pro-inflammatory cytokines. Moreover, we observed that TpF1 promotes the development of T regulatory cells, the main effectors in the down-modulation of immune response [29].

TpF1 is a dodecameric glycoprotein of 190 kDa belonging to the Dps-like family, highly immunogenic, localized in the periplasmic space of the bacterium and probably released after *Treponema* autolysis [22]. TpF1 is homolog to another immunomodulant Dps-like protein, HP-NAP, produced by *Helicobacter pylori*.

HP-NAP is known to interact with different cell types (neutrophils, monocytes, dendritic cells) and to modulate their activity; moreover, it also binds to endothelial cells, in which it is internalized by transcytosis.

Considering the role played by TpF1 in syphilis pathogenesis, the homology existing between HP-NAP and TpF1, and the fact that angiogenesis is characteristic of secondary syphilis, we decided to verify if TpF1 could interact and activate the endothelium promoting the formation of new blood vessels.

In a series of pilot experiments, we investigated whether TpF1 promoted proliferation and migration of endothelial cells. To this purpose we used human umbilical vein-derived endothelial cells (HUVECs), isolated from umbilical cords of healthy donors.

We found that TpF1 promoted cell proliferation, as well as cell migration. In accordance, endothelial cells, seeded on matrigel,, tend to form microcapillar-like structures in the presence of TpF1.

Notably, the effect of TpF1 on endothelial cells was superimposable to that observed in the presence of the vascular endothelial growth factor (VEGF), the best characterized angiogenesis promoter [76]

In order to gain insights into how TpF1 induces angiogenesis *in vitro*, we evaluated the production of VEGF and IL-8, a chemokine with a potent pro-angiogenic activity [52]; both of the factors are produced by endothelial cells. Interestingly RT-PCR and ELISA analysis revealed that VEGF production was not affected upon TpF1 stimulation; conversely, TpF1 strongly induced both IL-8 gene expression and protein release in a time dependent manner. The fact that the chemokine was crucial for the pro-angiogenic activity of TpF1 was proven by the inhibitory effect exerted by a specific IL-8 blocking antibody: it inhibited endothelial cell proliferation and migration as well as the formation of micro capillary-like structures.

Is known that under inflammatory conditions, especially in presence of IL-1β, the expression of IL-8 is promoted and it follows the activation of two transcription factors: NF-κB and CREB (cAMP response element-binding protein) [54]. Accordingly, the promoter of IL-8 gene contains the binding sites for both the factors [77].

Since also TpF1 has pro-inflammatory properties [29], it was plausible that it modulated the same transcription pathway involved in the IL-1β signaling. Emerging findings suggest that cAMP, produced by adenylate cyclases, is able to induce gene transcription through the activation of several protein kinases, with the subsequent phosphorylation of both CREB and NF-κB [66], [67]. The analysis of cAMP dynamics in living endothelial cells revealed that TpF1 promotes cAMP production. Moreover, we found that TpF1 triggered the phosphorylation of CREB, NF-κB and IκBα (a direct negative regulator of NF-κB); notably, the inhibition of the two transcription factors resulted in a significant reduction in IL-8 secretion. This evidence definitively supports the conclusion that CREB and NF-κB, which are activated by TpF1, are essential for the IL-8 gene expression induced by the bacterial protein.

Finally, to corroborate the *in vitro* findings, we evaluated the angiogenic properties of TpF1 *in vivo*, using zebrafish as animal model. Results obtained by both wild type and transgenic fli1:EGFP zebrafish, injected with TpF1, confirmed the ability of the treponemal toxin to induce, also *in vivo*, the formation of new blood vessels.

Finally, we found that the expression of the two isoforms of the IL-8 gene was up-regulated in zebrafish after TpF1 injection, while the mRNA level of VEGF was not affected. The fact that TpF1 triggers the formation of new blood vessels and, simultaneously, it stimulate IL-8 expression and secretion, supports the conclusion that this chemokine play a pivotal role in the pro-angiogenic activity of TpF1, also *in vivo*.

In conclusion, with this study we have identified a treponemal factor endowed with pro-angiogenic activity that could be involved in the angiogenic process that accompanies the early stages of syphilis. It is conceivable that angiogenesis plays a central role in syphilis progression,

probably because it provides *T.pallidum* of nutrients but also because it facilitates the dissemination of the bacterium within the host, resulting in systemic spread.

Our evidence adds a new piece to the complicated puzzle of a disease still affecting worldwide approximately 12 million of people every year.

1. Lafond, R.E. and S.A. Lukehart, *Biological basis for syphilis.* Clin Microbiol Rev, 2006. **19**(1): p. 29-49.

2. Peeling, R.W. and E.W. Hook, 3rd, *The pathogenesis of syphilis: the Great Mimicker, revisited.* J Pathol, 2006. **208**(2): p. 224-32.

3. Morton, R.S., *Syphilis in art: an entertainment in four parts. Part 2.* Genitourin Med, 1990. **66**(2): p. 112-23.

4. Farhi, D. and N. Dupin, *Origins of syphilis and management in the immunocompetent patient: facts and controversies.* Clin Dermatol, 2010. **28**(5): p. 533-8.

5. Arba, F., et al., *Stroke in Renaissance time: the case of Francesco I de' Medici.* Cerebrovasc Dis, 2012. **33**(6): p. 589-93.

6. Johnson, H.A., *Gerard de Lairesse: genius among the treponemes.* J R Soc Med, 2004. **97**(6): p. 301-3.

7. Kent, M.E. and F. Romanelli, *Reexamining syphilis: an update on epidemiology, clinical manifestations, and management.* Ann Pharmacother, 2008. **42**(2): p. 226-36.

8. de Melo, F.L., et al., *Syphilis at the crossroad of phylogenetics and paleopathology.* PLoS Negl Trop Dis, 2010. **4**(1): p. e575.

9. Fenton, K.A., et al., *Infectious syphilis in high-income settings in the 21st century.* Lancet Infect Dis, 2008. **8**(4): p. 244-53.

10. Carlson, J.A., et al., *The immunopathobiology of syphilis: the manifestations and course of syphilis are determined by the level of delayed-type hypersensitivity.* Am J Dermatopathol, 2011. **33**(5): p. 433-60.

11. Goh, B.T., *Syphilis in adults.* Sex Transm Infect, 2005. **81**(6): p. 448-52.

12. Mattei, P.L., et al., *Syphilis: a reemerging infection.* Am Fam Physician, 2012. **86**(5): p. 433-40.

13. Gayet-Ageron, A., et al., *Use of Treponema pallidum PCR in Testing of Ulcers for Diagnosis of Primary Syphilis(1.).* Emerg Infect Dis, 2015. **21**(1): p. 127-9.

14. Singh, A.E. and B. Romanowski, *Syphilis: review with emphasis on clinical, epidemiologic, and some biologic features.* Clin Microbiol Rev, 1999. **12**(2): p. 187-209.

15. Kohl, P.K. and I. Winzer, *[The 100 years since discovery of Spirochaeta pallida].* Hautarzt, 2005. **56**(2): p. 112-5.

16. Klingmuller, G., *[Treponema pallidum or Spirochaeta pallida?].* Hautarzt, 1983. **34**(12): p. 628-31.

17. Porcella, S.F. and T.G. Schwan, *Borrelia burgdorferi and Treponema pallidum: a comparison of functional genomics, environmental adaptations, and pathogenic mechanisms.* J Clin Invest, 2001. **107**(6): p. 651-6.

18. Hazlett, K.R., et al., *TP0453, a concealed outer membrane protein of Treponema pallidum, enhances membrane permeability.* J Bacteriol, 2005. **187**(18): p. 6499-508.

19. Turner, T.B. and D.H. Hollander, *Biology of the treponematoses based on studies carried out at the International Treponematosis Laboratory Center of the Johns Hopkins University under the auspices of the World Health Organization.* Monogr Ser World Health Organ, 1957(35): p. 3-266.

20. Norris, S.J., *Polypeptides of Treponema pallidum: progress toward understanding their structural, functional, and immunologic roles. Treponema Pallidum Polypeptide Research Group.* Microbiol Rev, 1993. **57**(3): p. 750-79.

21. Liu, J., et al., *Cellular architecture of Treponema pallidum: novel flagellum, periplasmic cone, and cell envelope as revealed by cryo electron tomography.* J Mol Biol, 2010. **403**(4): p. 546-61.

22. Thumiger, A., et al., *Crystal structure of antigen TpF1 from Treponema pallidum.* Proteins, 2006. **62**(3): p. 827-30.

23. Penn, C.W. and J.G. Rhodes, *Surface-associated antigens of Treponema pallidum concealed by an inert outer layer.* Immunology, 1982. **46**(1): p. 9-16.

24. Fehniger, T.E., et al., *Purification and characterization of a cloned protease-resistant Treponema pallidum-specific antigen.* Infect Immun, 1984. **46**(2): p. 598-607.

25. Walfield, A.M., et al., *Primary structure of an oligomeric antigen of Treponema pallidum.* Infect Immun, 1989. **57**(2): p. 633-5.

26. Radolf, J.D., et al., *Role of disulfide bonds in the oligomeric structure and protease resistance of recombinant and native Treponema pallidum surface antigen 4D.* J Bacteriol, 1987. **169**(4): p. 1365-71.

27. Cunningham, T.M., et al., *Selective release of the Treponema pallidum outer membrane and associated polypeptides with Triton X-114.* J Bacteriol, 1988. **170**(12): p. 5789-96.

28. Coates, S.R., et al., *Serospecificity of a cloned protease-resistant Treponema pallidum--specific antigen expressed in Escherichia coli.* J Clin Microbiol, 1986. **23**(3): p. 460-4.

29. Babolin, C., et al., *TpF1 from Treponema pallidum activates inflammasome and promotes the development of regulatory T cells.* J Immunol, 2011. **187**(3): p. 1377-84.

30. Riley, B.S., et al., *Virulent Treponema pallidum activates human vascular endothelial cells.* J Infect Dis, 1992. **165**(3): p. 484-93.

31. Chung, K.Y., et al., *Treponema pallidum induces up-regulation of interstitial collagenase in human dermal fibroblasts.* Acta Derm Venereol, 2002. **82**(3): p. 174-8.

32. Bouis, D.A., et al., *Dendritic cells phagocytose and are activated by Treponema pallidum.* Infect Immun, 2001. **69**(1): p. 518-28.

33. Stary, G., et al., *Host defense mechanisms in secondary syphilitic lesions: a role for IFN-gamma-/IL-17-producing CD8+ T cells?* Am J Pathol, 2010. **177**(5): p. 2421-32.

34. Podwinska, J., et al., *The pattern and level of cytokines secreted by Th1 and Th2 lymphocytes of syphilitic patients correlate to the progression of the disease.* FEMS Immunol Med Microbiol, 2000. **28**(1): p. 1-14.

35. Belkaid, Y., *Regulatory T cells and infection: a dangerous necessity.* Nat Rev Immunol, 2007. **7**(11): p. 875-88.

36. Macaron, N.C., et al., *Cutaneous lesions of secondary syphilis are highly angiogenic.* J Am Acad Dermatol, 2003. **48**(6): p. 878-81.

37. Bates, D.O. and S.J. Harper, *Regulation of vascular permeability by vascular endothelial growth factors.* Vascul Pharmacol, 2002. **39**(4-5): p. 225-37.

38. Adair, T.H. and J.P. Montani, in *Angiogenesis*. 2010: San Rafael (CA).

39. Risau, W., *Mechanisms of angiogenesis.* Nature, 1997. **386**(6626): p. 671-4.

40. Williams, J.K., et al., *Tumor angiogenesis as a prognostic factor in oral cavity tumors.* Am J Surg, 1994. **168**(5): p. 373-80.

41. Schmidt, A., K. Brixius, and W. Bloch, *Endothelial precursor cell migration during vasculogenesis.* Circ Res, 2007. **101**(2): p. 125-36.

42. Papetti, M. and I.M. Herman, *Mechanisms of normal and tumor-derived angiogenesis.* Am J Physiol Cell Physiol, 2002. **282**(5): p. C947-70.

43. Klagsbrun, M. and P.A. D'Amore, *Regulators of angiogenesis.* Annu Rev Physiol, 1991. **53**: p. 217-39.

44. van Hinsbergh, V.W. and P. Koolwijk, *Endothelial sprouting and angiogenesis: matrix metalloproteinases in the lead.* Cardiovasc Res, 2008. **78**(2): p. 203-12.

45. Caduff, J.H., L.C. Fischer, and P.H. Burri, *Scanning electron microscope study of the developing microvasculature in the postnatal rat lung.* Anat Rec, 1986. **216**(2): p. 154-64.

46. De Spiegelaere, W., et al., *Intussusceptive angiogenesis: a biologically relevant form of angiogenesis.* J Vasc Res, 2012. **49**(5): p. 390-404.

47. Otrock, Z.K., et al., *Understanding the biology of angiogenesis: review of the most important molecular mechanisms.* Blood Cells Mol Dis, 2007. **39**(2): p. 212-20.

48. Hopkins, N., et al., *Chronic airway infection leads to angiogenesis in the pulmonary circulation.* J Appl Physiol (1985), 2001. **91**(2): p. 919-28.

49. Dvorak, H.F., *Vascular permeability factor/vascular endothelial growth factor: a critical cytokine in tumor angiogenesis and a potential target for diagnosis and therapy.* J Clin Oncol, 2002. **20**(21): p. 4368-80.

50. Imhof, B.A. and M. Aurrand-Lions, *Angiogenesis and inflammation face off.* Nat Med, 2006. **12**(2): p. 171-2.

51. Romagnani, P., et al., *CXC chemokines: the regulatory link between inflammation and angiogenesis.* Trends Immunol, 2004. **25**(4): p. 201-9.

52. Li, A., et al., *IL-8 directly enhanced endothelial cell survival, proliferation, and matrix metalloproteinases production and regulated angiogenesis.* J Immunol, 2003. **170**(6): p. 3369-76.

53. Waugh, D.J. and C. Wilson, *The interleukin-8 pathway in cancer.* Clin Cancer Res, 2008. **14**(21): p. 6735-41.

54. Sun, H., et al., *Cyclic AMP-responsive element binding protein- and nuclear factor-kappaB-regulated CXC chemokine gene expression in lung carcinogenesis.* Cancer Prev Res (Phila), 2008. **1**(5): p. 316-28.

55. Nikolaev, V.O., et al., *Novel single chain cAMP sensors for receptor-induced signal propagation.* J Biol Chem, 2004. **279**(36): p. 37215-8.

56. Lawson, N.D. and B.M. Weinstein, *In vivo imaging of embryonic vascular development using transgenic zebrafish.* Dev Biol, 2002. **248**(2): p. 307-18.

57. Westerfield, M., *The zebrafish book. A guide for the laboratory use of zebrafish (Danio rerio)*, E. Univ. of Oregon Press, Editor. 2000.

58. Fraser, C.M., et al., *Complete genome sequence of Treponema pallidum, the syphilis spirochete.* Science, 1998. **281**(5375): p. 375-88.

59. Tabor, D.R., D.P. Kiel, and R.F. Jacobs, *Cyclophosphamide-sensitive activity of suppressor T cells during treponemal infection.* Immunology, 1987. **62**(1): p. 127-32.

60. Maizels, R.M. and K.A. Smith, *Regulatory T cells in infection.* Adv Immunol, 2011. **112**: p. 73-136.

61. Lamalice, L., F. Le Boeuf, and J. Huot, *Endothelial cell migration during angiogenesis.* Circ Res, 2007. **100**(6): p. 782-94.

62. Benton, G., et al., *Matrigel: from discovery and ECM mimicry to assays and models for cancer research.* Adv Drug Deliv Rev, 2014. **79-80**: p. 3-18.

63. Takahashi, H. and M. Shibuya, *The vascular endothelial growth factor (VEGF)/VEGF receptor system and its role under physiological and pathological conditions.* Clin Sci (Lond), 2005. **109**(3): p. 227-41.

64. Iourgenko, V., et al., *Identification of a family of cAMP response element-binding protein coactivators by genome-scale functional analysis in mammalian cells.* Proc Natl Acad Sci U S A, 2003. **100**(21): p. 12147-52.

65. Roebuck, K.A., *Oxidant stress regulation of IL-8 and ICAM-1 gene expression: differential activation and binding of the transcription factors AP-1 and NF-kappaB (Review).* Int J Mol Med, 1999. **4**(3): p. 223-30.

66. Gerlo, S., et al., *Cyclic AMP: a selective modulator of NF-kappaB action.* Cell Mol Life Sci, 2011. **68**(23): p. 3823-41.

67. Sassone-Corsi, P., *The cyclic AMP pathway.* Cold Spring Harb Perspect Biol, 2012. **4**(12).

68. Borner, S., et al., *FRET measurements of intracellular cAMP concentrations and cAMP analog permeability in intact cells.* Nat Protoc, 2011. **6**(4): p. 427-38.

69. Ghosh, S. and M. Karin, *Missing pieces in the NF-kappaB puzzle.* Cell, 2002. **109 Suppl**: p. S81-96.

70. Beg, A.A., et al., *Tumor necrosis factor and interleukin-1 lead to phosphorylation and loss of I kappa B alpha: a mechanism for NF-kappa B activation.* Mol Cell Biol, 1993. **13**(6): p. 3301-10.

71. Best, J.L., et al., *Identification of small-molecule antagonists that inhibit an activator: coactivator interaction.* Proc Natl Acad Sci U S A, 2004. **101**(51): p. 17622-7.

72. Wu, J., et al., *Neuronal store-operated calcium entry pathway as a novel therapeutic target for Huntington's disease treatment.* Chem Biol, 2011. **18**(6): p. 777-93.

73. Melet, F., et al., *Generation of a novel Fli-1 protein by gene targeting leads to a defect in thymus development and a delay in Friend virus-induced erythroleukemia.* Mol Cell Biol, 1996. **16**(6): p. 2708-18.

74. Thompson, M.A., et al., *The cloche and spadetail genes differentially affect hematopoiesis and vasculogenesis.* Dev Biol, 1998. **197**(2): p. 248-69.

75. Schmitt, C.E., M.B. Holland, and S.W. Jin, *Visualizing vascular networks in zebrafish: an introduction to microangiography.* Methods Mol Biol, 2012. **843**: p. 59-67.

76. Tammela, T., et al., *The biology of vascular endothelial growth factors.* Cardiovasc Res, 2005. **65**(3): p. 550-63.

77. Bezzerri, V., et al., *Mapping the transcriptional machinery of the IL-8 gene in human bronchial epithelial cells.* J Immunol, 2011. **187**(11): p. 6069-81.