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Summary

Syphilis is a chronic disease caused by the spirochaete *Treponema pallidum*. Syphilis is acquired by direct contact, usually sexual, with active primary or secondary lesion. The infection also occurs when *Treponema* crosses the placenta to infect fetus in a pregnant woman. Of particular importance is the recognition that syphilis infection greatly increases the transmission and acquisition of HIV (Ficarra & Carlos, 2009). After the Second World War, the introduction of penicillin, serological controls of pregnant women and treatment of infected partners resulted in a decrease of the disease incidence. Subsequently, periods of increased incidence alternated with decreased ones occurred, in relation to funds committed to programs to control public health, socioeconomic level of population and behaviours of infected people. Nowadays, the syphilis still constitutes an important global health problem and it remains a major sexually transmitted disease, particularly in developing countries, although an increase in new cases has also been recently noted in USA as well as in Europe (Golden *et al.*, 2003; Fenton *et al.*, 2008).

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, the treponemes can also proliferate in the chancre and can be transported via lymphatics to the bloodstream, from which they disseminate throughout the body. Secondary syphilis is a systemic disease. The most common manifestation of this stage is a disseminated muco-cutaneous 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; this is subdivided into gummatous syphilis, neurosyphilis and cardiovascular syphilis. The third stage can be extremely disfiguring and can lead to patient death (Lafond & Lukehart, 2006). During early infection *T. pallidum* is able to elicit a vigorous inflammation and the ensuing adaptive immune response. The latter is a typical cell-mediated immune response, characterized by Th1 predominance. T helper cells are believed to mediate bacterial clearance primarily through the production of cytokines (such as IFN-γ), which then activate macrophages. The latter then engulf and kill opsonised bacteria (Leader *et al.*, 2007). Nevertheless, although cell-mediated immune response take part in the course of syphilis, *T. pallidum* often manages to evade eradication and, in untreated individuals, may trigger chronic infection.

Efforts to elucidate the molecular mechanisms of *T. pallidum* virulence have been hampered by some of its characteristics, which make it difficult to be cultured *in vitro.* Little is know about how *T. pallidum* causes the syphilis, and in particular how *T. pallidum* survives within the host despite the immune response, thus giving a chronic disease, remains an unexplored issue. In the present study, we investigated the role of a *T. pallidum* protein, called antigen 4D or TpF1 in the pathogenesis of syphilis. TpF1 is an oligomeric protein of 190 kDa localised in the periplasm of *T. pallidum* (Cunningham *et al.*, 1988) . TpF1 has been identified and purified in 1984 (Fehniger *et al.*, 1984), but its function is still unknown. We have selected TpF1 as a potential candidate to have a role in syphilis for different reasons. In 1986, Coates and colleagues reported that TpF1 induces an important seroconversion particularly in primary syphilis patients (Coates *et al.*, 1986). Borestein *et al.* demonstrated that the *intra vena* administration of TpF1 in subsequently treponema-challenged rabbits reduced the mean incubation periods for lesion development, suggesting a role for this protein in inducing an inflammatory and immune response (Borenstein *et al.*, 1988). Moreover, TpF1 belongs to the Dps-like protein family and it is homolog to two proteins of this family, HP-NAP from *Helicobacter pylori* and NAPA from *Borrelia Burgdorferi.* These proteins have been studied in our lab and their pro-inflammatory and immune-modulatory properties have been demonstrated to be crucial in sustaining chronic diseases (Amedei *et al.*, 2006; Codolo *et al.*, 2008).
Therefore, the aim of our study was to verify whether TpF1 might have a role in triggering inflammation and, eventually, in down-modulating the adaptive immune response, thus permitting the survival of the bacterium in the host. Accordingly, several years ago it has been proposed the involvement of T lymhocytes endowed with immune suppressive activity to explain the inability of the host to clear the bacterium (Tabor *et al.*, 1984; Tabor *et al.*, 1987) .

Firstly, we examined the pro-inflammatory potential of TpF1 and we demonstrated that the antigen activates monocytes to express and release the pro-inflammatory cytokines IL-1β, IL-6 and TNF-α, suggesting that TpF1 may contribute to inflammation. Considering that TpF1 induces the production of IL-1β, a cytokine that follows a peculiar way of secretion which involves the activation of the so-called “inflammasome” complex (Martinon *et al.*, 2002), we investigated the mechanism by which TpF1 activates inflammasome. We demonstrated that TpF1 induces endogenous ATP release by monocytes. The engagement of $P2X_7$ receptor by ATP results in the activation of inflammasome, leading, as a final effect, to the release of IL-1β. In addition, we observed that TpF1 contributes to maintenance of inflammation by preventing the natural occurring apoptosis in monocytes, via the induction of the expression of the anti-apoptotic proteins Mcl-1 and Bcl-X_L.

Despite the fact that a strong specific immune response is evoked after establishment of bacterial infection, in absence of antibiotic treatment *T. pallidum* survives in the host and then syphilis evolves as chronic disease. Several mechanisms have been suggested to ensure the persistence of the microorganism in the host. As reported above, several years ago, Tabor and colleagues suggested that *T. Pallidum* infection induces the production of "suppressor T cells", which, they showed, suppress the activity of macrophages (Tabor *et al.*, 1984; Tabor *et al.*, 1987).

We demonstrated that, upon TpF1 stimulation, peripheral blood monocytes produce also IL-10 and TGF-β, key cytokines in the differentiation of T cells towards the T reg phenotype. In our work, we reported for the first time that $CD4^{+}CD25^{+high}$ T reg cells are increased in the peripheral blood of patients with secondary syphilis. Furthermore, we found that these cells are Foxp3 positive and are able to produce TGF-β. Finally, we demonstrated that TpF1 is able to promote the expansion of T reg cells isolated from secondary syphilis patients, as well as the release by these cells of TGF-β.

In conclusion, TpF1 of *T. pallidum* displays peculiar activities on inflammatory and immune responses, which are relevant for the pathogenesis of secondary syphilis. On one hand, TpF1 stimulates inflammation and tissue destruction by triggering both the production and release of several pro-inflammatory cytokines, such as TNF-a, IL-6 and IL-1β; on the other hand, however, TpF1 inhibits the immune response, *i.e.* the clearance of the pathogens, via the induction of the a T reg response.

Sommario

La sifilide è una malattia cronica causata dalla spirocheta *Treponema pallidum.* Essa è acquisita per contatto diretto, usualmente sessuale, attraverso una lesione primaria o secondaria attiva. L'infezione può trasmettersi anche per via verticale, nel caso in cui *Treponema* attraversi la placenta in una donna gravida recante l'infezione. Di particolare importanza è la scoperta che la sifilide aumenta il rischio di trasmettere e di acquisire il virus dell'HIV (Ficarra & Carlos, 2009). Dopo la seconda Guerra mondiale, l'introduzione dell'uso della penicillina, i test sierologici alle donne incinta ed il trattamento dei partner infetti hanno diminuito di molto l'incidenza della patologia. Successivamente, si è assistito ad un alternarsi di aumento e diminuzione dei casi, in rapporto alle risorse destinate ai programmi di sorveglianza della malattia, al livello socioeconomico del paese e a variazioni nei comportamenti delle persone infette. Oggi, la sifilide costituisce ancora un importante problema di salute pubblica e rimane una delle maggiori malattie trasmissibili sessualmente, in particolar modo nei paesi in via di sviluppo, anche se un aumento dei casi è stato registrato negli ultimi anni anche negli Stati Uniti e in Europa (Golden *et al*., 2003; Fenton *et al*., 2008).

La sifilide è caratterizzata da tre stadi evolutivi. Inizialmente appare una papula rossa nel sito d'infezione, in genere rappresentato dai genitali. Entro pochi giorni la papula si ulcera, producendo la tipica cancrena della sifilide primaria, che è indolore e ricca di batteri. La cancrena è accompagnata da una linfoadenopatia regionale. In assenza di trattamento antibiotico, i batteri possono proliferare a livello della cancrena e possono raggiungere con il sistema linfatico il circolo sanguigno, attraverso il quale disseminano per tutto il corpo. Questo stadio, che definisce la sifilide secondaria è dunque una tipica malattia sistemica. La manifestazione più ricorrente di questo stadio è un eritema disseminato, che in genere si risolve spontaneamente. Nei pazienti non trattati i sintomi sono assenti per un periodo di tempo variabile, che viene chiamato fase latente. In circa un terzo dei pazienti non trattati si può sviluppare la sifilide tardiva sintomatica. Questa include la gomma sifilitica, la neurosifilide e la sifilide cardiovascolare. Il terzo stadio può portare a lesioni molto deturpanti ed anche alla morte del paziente (Lafond & Lukehart, 2006). Nelle prime fasi dell'infezione *T. pallidum* è in grado di attivare una potente risposta infiammatoria ed immunitaria adattativa. Quest'ultima è tipicamente una risposta di tipo cellulo-mediata, caratterizzata da una predominanza di cellule T helper 1 (Th1). Si ritiene che i linfociti Th medino l'eliminazione del batterio attraverso la produzione di citochine come l'INF-γ, che potenzia il potere microbicida dei macrofagi (Leader *et al*., 2007). I macrofagi poi fagocitano e uccidono i batteri opsonizzati. Nonostante ciò, sebbene nel corso della sifilide intervenga risposta immunitaria di tipo

cellulo-mediata, *T. pallidum* spesso riesce ad evitare di essere eliminato e, in pazienti non trattati, può portare ad un'infezione cronica.

Gli sforzi fatti per chiarire i meccanismi molecolari di virulenza di *T. pallidum* sono stati ostacolati da alcune sue caratteristiche, che lo rendono difficile da coltivare *in vitro*. Dato che poco si conosce riguardo ai meccanismi attraverso cui *T. pallidum* causa la sifilide, abbiamo deciso di indagare il ruolo di una proteina, prodotta dal batterio, chiamata antigene 4D o TpF1, nell'insorgenza della patologia. TpF1 è una proteina oligomerica di 190 kDa, localizzata nel periplasma (Cunningham *et al*., 1988). Nonostante TpF1 sia stata identificata e purificata nel 1984, la funzione non è ancora nota (Fehniger *et al*., 1984). Abbiamo deciso di studiare TpF1 per diverse ragioni, qui di seguito brevemente riassunte. Nel 1986, Coates e colleghi hanno riportato che TpF1 induce una notevole seroconversione, in particolare nei pazienti con sifilide primaria (Coates *et al*., 1986). Borenstein *et al*, hanno dimostrato che la somministrazione *intra vena* di TpF1 in conigli, che venivano successivamente esposti a *Treponema,* riduceva il tempo dello sviluppo delle lesioni, suggerendo un ruolo per questa proteina nell'induzione di una risposta infiammatoria e/o immunitaria (Borenstein *et al*., 1988). Inoltre, TpF1 appartiene alla famiglia delle Dps-like (*DNA-binding proteins from starved cells*) ed è omologa a due proteine di questa famiglia, HP-NAP di *Helicobacter pylori* e NAPA di *Borrelia Borgdoferi* (Amedei *et al*., 2006; Codolo *et al*., 2008). Queste due proteine sono state studiate nel nostro laboratorio e le loro proprietà immunomodulanti sono state dimostrate essere cruciali nel sostenere patologie croniche. Alla luce di queste considerazioni abbiamo dunque voluto verificare se TpF1 potesse avere un ruolo nello scatenare l'infiammazione ed, eventualmente, nel reprimere la risposta immunitaria adattativa, permettendo così al batterio di sopravvivere nell'ospite. In accordo, alcuni anni fa è stato proposto il coinvolgimento dei linfociti T dotati di attività immunosoppressiva per spiegare l'incapacità dell'ospite di eliminare il batterio (Tabor *et al*., 1984; Tabor *et al*., 1987) .

Inizialmente, abbiamo indagato il potenziale pro-infiammatorio di TpF1 ed abbiamo dimostrato che l'antigene attiva i monociti ad esprimere e rilasciare le citochine pro-infiammatorie quali IL-1β, IL-6 e TNF-α, suggerendo che TpF1 contribuisca all'infiammazione. Considerando la capacità di TpF1 di indurre la produzione di IL-1β, abbiamo indagato il meccanismo molecolare attraverso cui TpF1 promuove questo effetto. Abbiamo dimostrato che TpF1 induce il rilascio di ATP endogeno dai monociti. Il legame dell'ATP al recettore $P2X_7$ determina l'attivazione dell'inflammasoma, portando, come effetto finale, al rilascio di IL-1β. Inoltre, abbiamo osservato che TpF1 contribuisce al mantenimento dell'infiammazione prevenendo il naturale decorso apoptotico dei monociti, attraverso l'induzione dell'espressione di proteine anti-apoptotiche, quali Mcl-1 e Bcl-X_L.

Nonostante la specifica e potente risposta immunitaria che si instaura in seguito all'infezione batterica, in assenza di un trattamento antibiotico, *T. pallidum* può sopravvivere nell'ospite e la sifilide può evolvere in una malattia cronica. Sono stati suggeriti diversi meccanismi che potrebbero garantire la persistenza del microrganismo nell'ospite. Come riportato sopra, diversi anni fa Tabor e colleghi suggerirono che l'infezione con *T. pallidum* inducesse la produzione di cellule T soppressorie, inibenti l'attività dei macrofagi (Tabor *et al.*, 1984; Tabor *et al.*, 1987).

Abbiamo dimostrato che monociti, isolati da sangue periferico e stimolati con TpF1, producono IL-10 e TGF-β, citochine cruciali nel differenziamento dei linfociti T verso il fenotipo T regolatorio. Nel nostro lavoro, abbiamo riportato, per la prima volta, che nel sangue periferico di pazienti con sifilide secondaria c'è un aumento dei linfociti T regolatori $CD4^{+}CD25^{+high}$. Queste cellule sono positive per il fattore di trascrizione Foxp3 e sono in grado di produrre TGF-β. Inoltre, abbiamo dimostrato che TpF1 promuove l'espansione dei linfociti T regolatori isolati da pazienti con sifilide secondaria ed induce la produzione da parte degli stessi di TGF-β.

In conclusione, la proteina TpF1 di *T. pallidum* possiede una peculiare attività immunomodulante che è attesa ripercuotersi *in vivo* sia sulla risposta infiammatoria che immunitaria adattativa. Da un lato, TpF1 stimola l'infiammazione e potrebbe contribuire alla distribuzione del tessuto inducendo la produzione e il rilascio di citochine pro-infiammatorie, come TNF-α, IL-6 e IL-1β; d'altra parte, TpF1 inibisce la risposta immunitaria, ovvero l'eliminazione del batterio, attraverso l'induzione di una risposta T regolatoria.

Introduction

1.1 Syphilis and Treponema pallidum

1.1.1 The syphilis: historical background

The syphilis is a chronic infectious disease, sexually transmitted and caused by the spirochaete *Treponema pallidum* subspecies *pallidum*. Theoretically, syphilis should be an eradicable disease as it has no animal reservoir, in fact the only known natural host of *T. pallidum* is the human. Despite syphilis can be diagnosed and cured with simple inexpensive tests and antibiotics, it remains a global health problem. Social impediments and resource limitations have contributed to the persistence of syphilis until now; moreover, because of its prolonged clinical course and its protean manifestations, the syphilis has earned the name of the “Great Mimicker” (Peeling & Hook, 2006). Since its recognition in the 15th-century Europe as a new disease, syphilis has been the subject of a great mystery and legend (Lafond & Lukehart, 2006). The name syphilis has been used for the first time by Fracastoro in his Latin poem "*Syphilis sive de morbo gallico*", describing an Italian shepherd boy, who contracted the "French disease" sweeping through Europe in the early 16th century (Peeling & Hook, 2006). On the origin of syphilis there are different hypotheses. The Columbian hypothesis states that it came from the Americas and was spread to Europe by Columbus’ seamen; the rapid spread of syphilis in Europe was probably facilitated by the era's wars and movement of troops. The Pre-Columbian hypothesis argues that syphilis and the other treponemal diseases were present throughout the New as well as the Old World in pre-Columbian times, but was misdiagnosed as leprosy in Europe. Recently, the Unitarian hypothesis states that treponematoses always had a worldwide distribution, where every social group had the kind of treponematosis appropriate to its geographic and climatic conditions and its stage of cultural development. Thus, according to this hypothesis, yaws, bejel, pinta and venereal treponematosis (or syphilis) are seen as adaptive responses of *Treponema pallidum* to peculiarities of different environments, cultures, and contacts with other populations (de Melo *et al.*, 2010).

1.1.2 The syphilis: epidemiology

Syphilis is distributed worldwide, but it is particularly problematic in developing countries, where the disease is a leading cause of genital ulcers. Globally, most cases occur in sub-Saharan Africa and southeast Asia. Syphilis is usually transmitted by sexual

contact, from mother to fetus, but also by transfusion of blood from donors in the incubation stage of the disease. During the Second World War the incidence of syphilis was over 500,000 infections per year; between the years 1945 and 2000 the number of infections reported declined to 31,575 because of effective therapies. In fact, in 1928 Fleming discovered penicillin which in 1943 was firstly introduced as a treatment for syphilis, with substantial improvements in prognosis. More recently, the incidence declined to a low level that has been sustained by outbreaks. Similar patterns in disease incidence were observed in many European countries (Fenton *et al*., 2008). In the late 1980s and early 1990s, syphilis re-emerged in the USA and became more concentrated in urban and rural southern regions and in large urban centers throughout the country. The demographics of the disease changed from an infection affecting predominantly homosexual men to one affecting mainly heterosexual African-Americans. An increase in syphilis cases among men who have sex with men in the USA occurred between 2000 and 2005, characterized by high rates of HIV co-infection and high-risk sexual behavior (Fenton *et al*., 2008). Since 1996, syphilis has again increased in many northern and western EU countries. In Denmark, diagnoses of infectious syphilis increased by more than 50% between 1999 and 2002, and in Belgium, between 2000 and 2002, a 3 to 5-fold increase in the number of laboratory-diagnosed syphilis cases was detected by sentinel networks of laboratories. In Austria, the notified number of syphilis cases steadily increased from 124 in 1993 to 420 in 2002, with about 70% of cases reported in Vienna. Other major urban centers such as London, Dublin, Berlin, Paris, and Rotterdam all showed huge increases in syphilis reports during this period, predominantly among populations of men who have sex with men (Fenton *et al*., 2008).

1.1.3 The syphilis: Clinical manifestations and pathogenesis

Syphilis is a multistage disease with diverse and wide ranging manifestations; the distinct stages of syphilis were first described by Philippe Record in the mid-1800s (Lafond & Lukehart, 2006). The disease is classified as:

- Acquired: early (primary, secondary and early latent< 2 years of infection) and late (late latent>2 years of infection and tertiary)
- Congenital: early (diagnosed in the first 2 years of life) and late (presenting after the age of 2 years) (Lee & Kinghorn, 2008).

1.1.3.1 Primary syphilis

Infection is initiated when *T. pallidum* penetrates dermal micro abrasions or intact mucous membranes. Primary syphilis is characterised by an ulcer (the chancre) and

regional lymphoadenopathy. The incubation period is 9-90 days, but the primary lesion most often appears about three weeks after transmission. At 14 to 21 days after penetration of *T. pallidum* into a dermal site, a red papule 0.5 to 2 cm in diameter appears at the site of inoculation. Within the few days the papule ulcerates, producing the typical chancre of primary syphilis, an ulcerated area sometimes covered by a slight yellowish or grayish exudate and surrounded by a slightly indurated margin. The chancre is classically in the anogenital region, is single and painless, but filled with treponemes and is, therefore, highly contagious. The chancre is accompanied by regional lymphadenopathy. Because the chancre is painless and may be located in an inconspicuous anatomical site, diagnosis of syphilis in women and homosexual men is sometimes delayed until later disease manifestations become apparent. Clinical evaluation can also be complicated by the fact the appearance of primary chancres in some individuals does not fit the classic description. Varied presentations can be explained by the number of spirochetes inoculated, the immune status of the patient, concurrent antibiotic therapy, and the possible impetiginization of the chancre. If untreated, the primary chancre heals spontaneously within 4 to 6 weeks, but may still be discernible in about 15% of patients at the onset of secondary syphilis. A local immune reaction, rather than generalized one, accounts for the healing, because secondary lesions regularly appear during or after the regression of the primary one (Lafond & Lukehart, 2006).

1.1.3.2 Secondary syphilis

In untreated individuals, treponemes proliferate in the chancre and are carried via lymphatics to the bloodstream, from which they disseminate throughout the body. The time at which the secondary lesions make their appearance basically depends on two factors: the virulence of the treponema and the systemic response of the host. Manifestations of secondary syphilis usually occur within 3 months of initial infection and are sometimes quite subtle. Secondary syphilis is typically a systemic disease, with the patient often presenting a variety of symptoms, such as sore throat, muscle aches, malaise, mad weight loss, low-grade fever and pruritus. Lymph nodes enlargement is present in the great majority of patients. The most common manifestation of secondary syphilis is a disseminated mucocutaneous rash. Pale and discrete macular lesions appear initially on the trunk and proximal extremities, on the palms of the hands and soles of the feet. The most common types of secondary syphilis lesions are maculopapular (in 50% to 70% of patients), papular (12%), macular (10%) and annular papular (6% to 14%). In rare individuals, lesions may be necrotic, a condition called “lue maligna” (Lafond & Lukehart, 2006). *T. pallidum* infection of the hair follicles results in alopecia of the scalp. Concurrent with the appearance of secondary lesions, about 10%

of patients develop condyloma lata, more large white or grey lesions in warm, moist areas. These lesions reflect a local breakdown of secondary lesions with extension of infection in areas of tissue trauma, most frequently involving the axilla and groin. Infrequently, secondary syphilis can be accompanied by gastric and renal involvement and hepatitis (Baughn & Musher, 2005).

1.1.3.3 Latent Syphilis

Disseminated lesions and other manifestations of secondary syphilis usually resolve spontaneously within 3 months of appearance, and symptoms are absent for a variable period of time in untreated individuals. Latent syphilis is divided in two stages, based upon an approximation of the time of infection. For the first year after infection, patients are considered to have early latent syphilis; up to 25% may have recurrent secondary manifestations. Organism may seed the bloodstream intermittently during latent syphilis and can infect the developing fetus during the pregnancy. Latent syphilis ends when curative antibiotic therapy is administrated or when manifestations of tertiary disease develop (Lafond & Lukehart, 2006).

1.1.3.4 Tertiary Syphilis

About one-third of untreated patients will subsequently develop symptomatic late syphilis; this is categorised into gummatous syphilis, neurosyphilis and cardiovascular syphilis but they may coexist. Tertiary syphilis is a term often used synonymously with late symptomatic syphilis.

Gumma: Granulomatous, nodular lesions with variable central necrosis may develop as early 2 years after initial infection, although they usually appear much later. These destructive lesions most commonly affect the skin, the bones, although they may also occur in the liver, heart, brain, stomach and upper respiratory tract. Gummas usually do not cause serious complications, thus the term “late benign syphilis”.

Cardiovascular syphilis: complications occur in approximately 10% of untreated patients between 10 and 30 years after the initial infection. Syphilitic aortitis is the most common manifestation and typically involves the ascending aorta; the most serious complication of untreated syphilitic aortitis is aortic regurgitation.

Neurosyphilis: After initial invasion of the cerebrospinal fluid (CSF) by spirochetes, untreated or inadequately treated infection may follow one of several courses: spontaneous resolution, asymptomatic meningitis or acute syphilitic meningitis. The most common symptoms are vertigo, insomnia and personality changes (Lafond & Lukehart, 2006).

1.1.3.5 Congenital Syphilis

T. pallidum can be transmitted from bloodstream of the infected woman to her developing foetus at any time during pregnancy, although risk of foetal infection is much higher during early maternal syphilis than during later stages. Antibiotic treatment of the mother during the first two trimesters is usually 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. Congenital syphilis is divided into stages: early manifestations (appearing in the first 2 years of life) and late manifestations (appearing after 2 years). Early manifestations are infectious and resemble severe symptoms of adult secondary syphilis; they usually become evident after 2 or 10 weeks after delivery. The first symptom seen in up to 50% of newborns with congenital syphilis is “snuffles”. *T. pallidum* can invade the bones and cartilage of the nose and palate, leading to gummatous destruction later in life. The infants commonly have skin lesions, sometimes accompanied by desquamation of the skin of palms and soles, and jaundice. Between the ages of 5 and 25, interstitial keratitis may cause damage to the cornea and iris, and eighth-nerve deafness may be apparent (Lafond & Lukehart, 2006).

1.1.4 Diagnosis and treatment

The diagnosis of syphilis is based on the analysis of clinical manifestations, identification of treponemes in the lesion material and serologic test for syphilis.
Dark-field microscopy and direct fluorescent-antibody testing for *T. pallidum* (DFA-TP) are the main diagnostic methods for primary syphilis. However, neither dark-field microscopy nor DFA-TP can distinguish *T. pallidum* from the other species of *Treponema*. Serological testing remains the mainstay for laboratory diagnosis for secondary, latent and tertiary syphilis. These test are in turn divided into treponemal and nontreponemal, however tests alone are not sufficient for diagnosis. Nontreponemal tests are useful for screening, while treponemal tests are used to confirm the diagnosis. Non treponemal tests include the Venereal Disease Research Laboratory (VDRL) and the Rapid Plasma Reagin (RPR) card test. These tests use an antigen comprising lecithin, cholesterol and purified cardiolipin to detect an antibody that is present in the sera of many syphilis patients. These test are available and relatively inexpensive, but they are limited by their lack of sensitivity in early and late syphilis and by false-positive reactions. Treponemal tests include the serum fluorescent treponemal antibody absorption test (FTA-ABS) and microhemagglutination test for *T. pallidum* (MHA-TP), which use lyophilized *T. pallidum* or a lysate of pathogenic *T. pallidum*, respectively. DNA amplification by PCR provides an easy method of diagnosing early syphilis on oral or other lesions where contamination

with commensal treponemes is likely. In certain circumstances, PCR may be helpful in diagnosis by demonstrating *T. pallidum* in tissue sample, vitreous fluid and cerebral spinal fluid. In the recent years, enzyme immunoassay (EIA) tests have become established as the screening test of choice in syphilis.

After 60 years of use, intramuscular penicillin still remains the drug of choice in syphilis treatment. Standard syphilis therapy with penicillin rarely fails to cure the disease. As alternative treatments, non-penicillin antibiotics that have been evaluated include doxycycline, erythromycin and azithromycin. In many studies immunization with *T. pallidum* molecules was conducted and it stimulated the production of strongly reactive antibodies, but these immunization regimens induced only partial protection, shown by attenuated development and rapid healing of lesions. Therefore, until now there is not a vaccine able to counteract the diffusion of syphilis (Singh & Romanowski, 1999).

1.2 Biology of *Treponema pallidum*

On March 3rd 1905, Schaudinn examined a fresh preparation of a material obtained by Hoffman from an eroded papula in the vulva of a woman with secondary syphilis and identified *Treponema pallidum* as the etiological agent of syphilis (Kohl & Winzer, 2005).
T. pallidum subsp. *pallidum* a member of the *Spirochaetaceae* (spirochetes), is a biomedically important bacterial phylum that includes the etiological agents of Lyme disease (*Borrelia Burgdoferi*) and leptospirosis (*Leptospira interrogans*). A striking features of *T. pallidum* and other spirochetes is their capacity to swim efficiently in a highly viscous gel-like environment, such as connective tissue, where most externally flagellated bacteria are slowed or stopped. Therefore, motility is likely to play an important role in the widespread dissemination of spirochetal infections and in the establishment of chronic disease. *T. pallidum* is related to other pathogenic treponemes that cause nonvenereal diseases. *T. pallidum* subsp. *endemicum* (bejel), *T. pallidum* subsp. *pertenue* (yaws), and *T. carateum* (pinta) can be differentiated from *T. pallidum* by the clinical manifestations of their respective diseases and by genetic differences (Singh & Romanowski, 1999).

T. pallidum varies from 6 to 25 μm in length and from 0.1 to 0.18 μm in diameter (Fig.1), making it invisible by light microscopy; in fact, dark-field microscopy is generally used in clinical practice for visualization (Singh & Romanowski, 1999). It consists of a central protoplasmic cylinder bounded by a cytoplasmic membrane, an overlying layer of peptidoglycan (PGN), which provides structural stability, and an outer membrane. The flagella are located in the periplasmic space, between the cytoplasmic membrane and the outer membrane. Bundles of flagella originate from flagellar motors at both ends of the

organism, wind around the flexible protoplasmic cell cylinder, and overlap in the middle (Lafond & Lukehart, 2006).

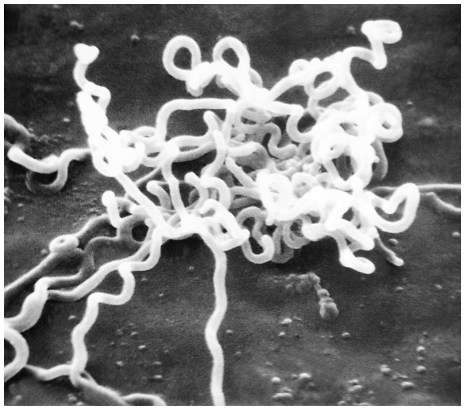


Fig.1 Electron micrograph of T. pallidum subsp pallidum Nichols (from PHIL, Centers for Disease Control and Prevention). Figure taken from Izard *et al*., 2009.

The complete genome sequence of *T. pallidum* was published in 1998 and is a circular chromosome of about 1.14 Mb and contains 1041 open reading frame (ORFs), making it a relatively small bacterial genome. This relatively small genome strengthens the assumption that *T. pallidum* relies in host biosynthetic pathways for many of its metabolic needs. Biological function have been predicted for about 55% of *T. pallidum* ORFs and further 17% show homology with hypothetical proteins from other species, and a high proportion of *T. pallidum* ORFs (28%) have no database match and presumably represent novel genes (Fraser *et al*., 1998).

Efforts to elucidate molecular mechanisms of *T. pallidum* virulence have been hampered by certain characteristics of the organism. *T. pallidum* does not survive outside of the organism; infectious capability is lost within a few hours or days of harvest. The organism has a striking lack of metabolic capabilities, it is able to carry out the glycolysis but lacks tricarboxylic acid cycle enzymes and electron transport chain. Analysis of the genome suggests an absence of pathways for the use of alternative carbon sources for energy and for *de novo* synthesis of enzymes cofactors and nucleotides. Amino acid and fatty acid synthesis pathways are also lacking, but *T. pallidum* does carry enzymes for the interconversion of amino acids and fatty acids. To take up macromolecules from the host environment, specific transporters may be utilized by *T. pallidum*. The organism has a broad spectrum of amino acid transporters, it contains 57 ORFs (5% of the total) that encode 18 distinct transporters with predicted specificity for amino acids, carbohydrates and cations; the most extensively studied of these transporters is the ATP-binding cassette (ABC) transporter (Fraser *et al.*, 1998). To obtain sufficient organisms for experimental manipulation *T. pallidum* must be propagated in rabbits. It cannot be cultured more than 100-fold using artificial media, an equivalent of about 7 generations. The generation of *T. pallidum* is unusually slow and several biological factors may

contribute to sluggish replication rate of *T. pallidum*. The organism lacks of enzymes such as catalase and oxidase that detoxify reactive oxygen species. In addition to its sensitivity to oxygen, *T. pallidum* may have a limited stress response, in fact the typical heat shock response regulated by σ32 is lacking (Lafond & Lukehart, 2006).

1.3 Polypeptides of *Treponema pallidum*

Despite its fragility to environmental factors, *T. pallidum* readly causes chronic infection and varied disease manifestations in the host.

A nomenclature has been developed to aid in the identification of *T. pallidum* polypeptides. The format consists of the prefix TpN (for *T. pallidum* Nichols, the reference strain) followed by a consensus Mr (relative molecular mass, based on SDS-PAGE results) and, if necessary, a letter to distinguish between polypeptides with similar Mrs. The corresponding gene can be indicated by lower case italics. *T. pallidum* lacks lipopolysaccharide (LPS), the endotoxin found in outer membranes of many gram-negative bacteria that cause fever and inflammation. However, the organism does produce a number of lipoproteins which may induce expression of inflammatory mediators via toll-like receptor 2 (TLR2) recognition. The endoflagellar filament of *T. pallidum* is composed of multiple polypeptides arranged into an outer sheath and a central core (Norris, 1993). Six flagellum-associated polypeptides have been described in *T. pallidum*, corresponding to TpN37a, TpN34.5, TpN33, TpN30, TpN29 and TpN27.5 and have been subdivided into class A and class B flagellins on the basis of their N-terminal amino acid sequences, gene sequences and antigenic relatedness. *T. pallidum* endoflagella are ~17nm in diameter and can extend more than half length of the organism within the periplasmic space. Two to four endoflagella originate at each end and entwine the cell body as they extend toward the middle of the cell. TpN29 and TpN27.5 are two additional polypeptides which have been detected in some *T. pallidum* preparations. Little information on these proteins is available, and their structural and functional roles are unknown. The composition of the outer membrane of *T. pallidum* has been investigated intensively, because of its perceived importance in the immune clearance of the organism. The outer membranes of *T. pallidum subsp. pallidum and T. pallidum subsp. pertenue* contain extremely small numbers of intramembranous particles corresponding to integral membrane proteins. The putative proteins corresponding to the intramembranous particles have been termed treponemal rare outer membrane proteins (TROMPs). It has been postulated that the apparently low content of surface-localized proteins could decrease the reactivity of antibodies and immune cells with intact *T. pallidum*, permitting the organism to evade the immune response (Norris, 1993).

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 in 1984, it has an Mr of 190 kDa and consists of a ring-like structure 6 nm in diameter formed of 19-kDa subunits (Fehniger *et al.*, 1984; Fehniger *et al.*, 1986). The deduced amino acid sequence contains two cysteine residues, which are presumably involved in interchain disulfide bonds whose reduction is required for dissociation of the oligomeric structure (Walfield *et al.*, 1989). Giving that disulfide-bonded proteins have been demonstrated in the outer membranes of a number of bacterial pathogens, it has been suggested that TpF1 could fulfill a structural role in the outer membrane (Radolf *et al.*, 1987). In the 1988 Cunningham *et al.* demonstrated using Triton X-114 that the protein derived from the periplasmic space (Cunningham *et al.*, 1988). In the 2006 the structure of TpF1 was resolved by (Thumiger *et al.*, 2006) (Fig. 2).

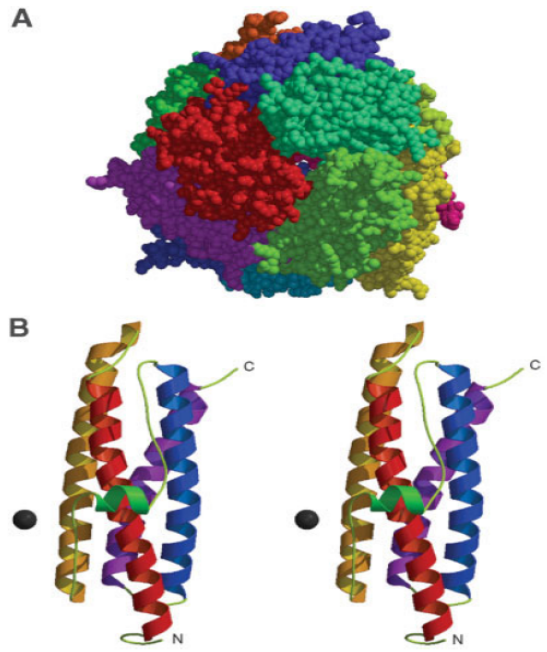


Fig. 2 Crystal structure of antigen TpF1.

(A) Van der Waals representation of the dodecamer of TpF1. Each subunit is colored differently. One of the threefold axis is running, approximately perpendicular to the plane of the paper in the center of the image, through one of the putative tunnel for the iron entrance. (B) Stereoview of the ribbon representation of TPF1 monomer. Each α-helix is shown in different colours, Fe(II) ion as a black sphere. N- and C-terminus are labelled N and C, respectively. Figure taken from Thumiger *et al.*, 2006.

TpF1 is a dodecamer, about 90 Å in diameter, displaying 32 symmetry. Each TpF1 subunit folds in a way very similar to the other miniferritins: a four-helix bundle, with

helices B and C connected through a long stretch that includes a short helix. The arrangement of the 12 monomers of TpF1 results in the nearly spherical shell typical of miniferritins, with an internal cavity where the iron is stored. Immunogenic properties of the protein are more difficult to rationalize on the basis of the crystal structure, they largely depend from the flexible N-terminal portion, which protrudes from the surface of the spherical shell (Thumiger *et al.*, 2006).

TpF1 is highly immunogenic and specific antibody was detected in 95% of the sera by radioimmunoassay (Coates *et al.*, 1986), while no detectable antibody to the 19-kd *T. pallidum* peptide was observed in human syphilitic serum (Fehniger *et al.*, 1984). The *intra vena* (i.v.) administration of TpF1 in subsequently *Treponema*-challenged rabbits, significantly reduced the mean incubation periods for lesion development, in accordance to TpF1-specific proliferation *in vitro* of isolated splenocytes from animals infected intratesticularly with *Treponema* (Borenstein *et al.*, 1988).

1.5 The immune system and inflammation

During evolution two complementary systems, the innate and the adaptive immune systems, have evolved to detect and clear pathogens in vertebrates. The innate immune system is dynamic and complex network for recognizing and responding to cellular insult or tissue damage. The innate immune system is the first to be activated by pathogens and is usually sufficient to clear the infection, but when it is overwhelmed, it triggers and directs the adaptive arm, thus activating specific B and T cells for pathogen clearance. The adaptive immunity employs somatic recombination of the genes encoding for T-cell receptor and the immunoglobulin heavy and light chains to generate diversity in receptors for microbial and antigens. This process enables the generation of a virtually infinite repertoire of antigen receptors, allowing the adaptive immunity to specifically recognize any type of microorganism. In contrast, innate immunity is characterized by its ability to recognize a wide range of pathogens such as viruses, bacteria, and fungi. A limited number of well-conserved microbial structure, which are known as pathogen-associated molecular patterns (PAMPs), are recognized by a limited number of germline-encoded receptors known as pattern-recognition receptors (PRRs). This smaller requirement for specificity allows a limited number of highly active effector genes to induce rapid and efficient defensive response to different pathogen. PRRs can be found in “classical” immune and non immune cells, including macrophages, monocytes, dendritic cells (DCs), neutrophils, and epithelial cells, and they allow the early detection of pathogens directly at the site of infection. PRRs in the innate immune system function at both extracellular and intracellular levels and include soluble, membrane-bound and cytosolic molecules. Once

activated, the innate immune system initiates the inflammatory response by secreting cytokines and chemokines, inducing the expression of adhesion and costimulatory molecules in order to recruit immune cells to the site of infection and to trigger the adaptive immune response. Pathogens can evolve rapidly and, in principle, could avoid detection by the innate immune system by simply altering the targeted PAMPs. By doing so, the pathogen would not only escape the recognition by the innate immune system but would also avoid the adaptive immune response. However, the immune system has evolved to recognize PAMPs that are essential for the viability of microbes and are thus less prone to modifications. PAMPs can be of diverse origins; sugars, flagellin, and the cell wall components peptidoglycan (PGN) and lipopolysaccharide (LPS) are all recognized by the innate immune system. Interestingly, it has been suggested that some of these receptors are also able to sense various endogenous cellular products associated with tissue injury or self “danger” signals, such as toxic compound, defective nucleic acids, or the presence of normal cell components in atypical esxtracellular or intracellular locations (Matzinger, 2002). These signals are commonly referred to as danger-associated molecular patterns (DAMPs). The release of DAMPs is a common event, as tissue damage and cell lysis are often associated with infections which lead to the release of host molecules. Recognition of these DAMPs by the immune system not only allows the sensing of an ongoing infection and subsequent recruitment of more immune cells, but also can initiate the repair of the damaged tissue. It seems then that the innate immune pathway not only scans the cellular environment for signs of invading pathogens, but also recognizes the damage caused by them (Matzinger, 2002).

Our innate immune system has evolved at least four major PRR families that cooperatively operate to recognize microbial pathogens or endogenous danger signals: the toll-like receptor system (TLRs), the RIG-I-like receptors (RLRs), the C-type lectin receptors (CLRs), and the nucleotide-binding domain leucine-rich repeats (NLRs). The prototypical sensors, TLRs, once activated by PAMPs, induce different signaling cascades depending on the adaptor protein, ultimately leading to the activation of the transcription factors NF-κB, AP-1, and interferon-regulatory factor (IRF)-3. TLR activation results in the production of antimicrobial peptides, inflammatory cytokines and chemokines, tumor necrosis factor (TNF)-α, and costimulatory and adhesion molecules, as well as in the upregulation of major histocompatibility complexes (MHCs). One given pathogen does not trigger only one specific TLR, but rather a set of TLRs leading to the expression of different proteins depending on the nature of the activated TLRs; therefore, the immune system is instructed on the type of the invading microorganism and induces the most appropriate response to fight it (Martinon *et al.*, 2009). In comparison, the NLRs regulate innate immunity in response to recognition of microbial products in the cytosol (Fritz & Girardin, 2005).

1.6 The host immune response to *T. pallidum*

Compared to the wealth of information concerning the disease-causing mechanisms of many bacterial pathogens, little is known about how *T. pallidum* causes the protean manifestations of syphilis. In the absence of cytotoxins and other known virulence factors, it is probable that inflammation and the ensuing adaptive immune response to *T. pallidum* cause the tissue destruction characteristic of syphilis infection. At all stages of the disease, syphilitic lesions are characterized by vasculopathic changes and local cellular infiltrates consisting of lymphocytes, macrophages and plasma cells; the importance of cellular immune response in containing the infection is shown by the presence of granulomata, which, in the case of gummatous disease, assume a necrotizing character. *T. pallidum* quickly gains access to deeper tissues and the bloodstream, probably by traversing the junctions between endothelial cells (Riley *et al.*, 1992). It has been reported that *T. pallidum* induces in dermal cells the production of matrix metalloproteainase-1 (Chung *et al.*, 2002), which is involved in breaking down collagene, which may help the bacterium to penetrate tissues. Virulent *T. pallidum* induces cultured endothelial cells to express the adhesion molecules ICAM-1, VCAM-1 and E-selectin (Lee *et al.*, 2000), promoting the leakage of serous fluids and migration of leukocytes out of blood vessels into infected tissues. During acute bacterial infection, polymorphonuclear lymphocytes (PMNs) are often the first cells to infiltrate the site of infection, in fact PMNs are seen in very early syphilis lesions (Musher *et al.*, 1983), but the inability of PMNs to adequately control *T. pallidum* is demonstrated by the progression of infection. Specialized DCs called Langerhans cells are found in skin, the site of the majority of primary and secondary lesions; DCs are also found in the mucosa, the intestinal wall and the heart, all potential sites of *T. pallidum* infection. It has been shown that *T. pallidum* interacts with and is phagocytized by immature DCs in cell culture. In response to pathogenic stimulation mature DCs produce inflammatory cytokines interleukin 1β (IL-1β), IL-6, IL-12 and tumor necrosis factor alpha (TNF-a) (Bouis *et al.*, 2001).

In primary chancres, $CD4^+$ T cells and macrophages predominate, whereas in the lesions of secondary syphilis there is a majority of $CD8^+$ Tcells. The role of $CD8^+$ Tcells in the immune response to *T. pallidum* is not clear but could include lysis of cells, harboring intracellular bacteria or production of IFN-γ to enhance phagocytosis and killing of *T. pallidum* by macrophages (van Voorhis *et al.*, 1996), which infiltrate the site of infection after 6 to 10 days, ingest and kill organism, and reach the maximal number at approximately day 13. Between days 13 and 17 post infection, the number of detectable *T. pallidum* organisms declines. In lesions of both primary and secondary syphilis, there occurs an increase of the expression of the Th1 cytokines IL-2, IFN-γ and TNF-α; in

secondary early syphilis the ability of Th1 lymphocytes to secrete cytokines is slightly decreased, whereas it increases the ability of cells to produce IL-6 and IL-10, to reach high level of Th2 cytokines (IL-6 and IL-10) and low level of Th1 cytokines in late syphilis (Podwinska *et al.*, 2000). Circulating antibodies to *T. pallidum* can be found soon after the onset of primary syphilis and reach high titres as the infection disseminates in the secondary stage. IgM antibodies are usually the first to develop after establishment of bacterial infection, shortly followed by IgG. The antibody response is specific for a broad range of *T. pallidum* molecules, including lipids of the surface, flagellar proteins, lipoproteins and various other proteins (Lafond & Lukehart, 2006).

Despite effective immune defences to treponemal infection, the organism often manages to evade total eradication. In 1984 Tabor *et al.* have shown that syphilitic T cells can suppress macrophage activity following intradermal treponemal inoculation (Tabor *et al.*, 1984). This kind of T cells are different from Th1 and Th2 cells; initially they were called suppressor T cells and more recently T regulatory cells (T reg). Moreover, in 1987 *Tabor et al.* also demonstrated that treponemal infection induced the production of cyclophasphamide-sensitive suppressor T cells, which are responsible for reducing macrophages C3b receptor-mediated ingestion (Tabor *et al.*, 1987). This could be a strategy for the bacterium to alter and avoid the elimination by the host response and contributes to the chronicity of the disease.

1.7 Inflammasome

1.7.1 General features of Inflammasome

In 2002 Tschopp and colleagues coined the term “inflammasome” to describe a high molecular weight complex that activates inflammatory caspases and the pro-inflammatory cytokines, such as IL-1β, IL-18 and IL-33; the term derived from the word inflammation, to reflect the function of this complex, and from the Greek “soma” meaning body (Martinon *et al.*, 2002). The inflammasome was first described as being composed of the intracellular receptor NALP1 and its adaptor ASC. NALPs belong to the NOD-like receptor (NLR) family of pattern recognition receptors which sense “danger” signals and microbial motifs, and activate inflammatory and antimicrobial response. NLRs comprise a large family of intracellular proteins with a tripartite architecture containing: an amino-terminal protein–protein interaction domain such as a CARD (caspase recruitment domain) or PYD (pyrin domain) or BIR (baculovirus inhibitor of apoptosis repeat domain); an intermediary NACHT domain (also referred to as NOD domain) that is required for nucleotide binding and self-oligomerization; and a carboxy-terminal region characterized

by a series of LRRs (leucine-rich repeat motifs). The LRR domain has been implicated in ligand sensing and autoregulation of NLRs, yet the precise mechanism on how NLR LRRs sense their ligands is largely unknown. No experimental data have convincingly demonstrated a direct interaction between the LRRs of NLRs and their respective activators, suggesting that sensing of pathogens and other signals by NLRs may be indirect. The crucial step in NLR activation lies in the oligomerization of the NACHT domain, forming active, high molecular weight complexes that characterize inflammasomes and NOD signalosomes. NLR subfamilies differ in their N-terminal effector domains, which mediate signal transduction to downstream targets, leading to activation of inflammatory caspases by inflammasomes or NF-kB by NOD signalosome. Expression patterns of most NLRs in various cell populations and tissues have not yet been studied in detail; the importance of NLRs in defence strategies of the body is supported by the fact that several NLRs are expressed in cells and tissues that have a role in immunity such as phagocytes (Martinon *et al.*, 2009). Most NLRs may be induced by other branches of the innate immunity as a part of regulatory network. Until now four different kinds of inflammasome have been identified and described: NALP1, IPAF, AIM2 and NALP3/cryopyrin (Fig. 3) (Stutz *et al.*, 2009).

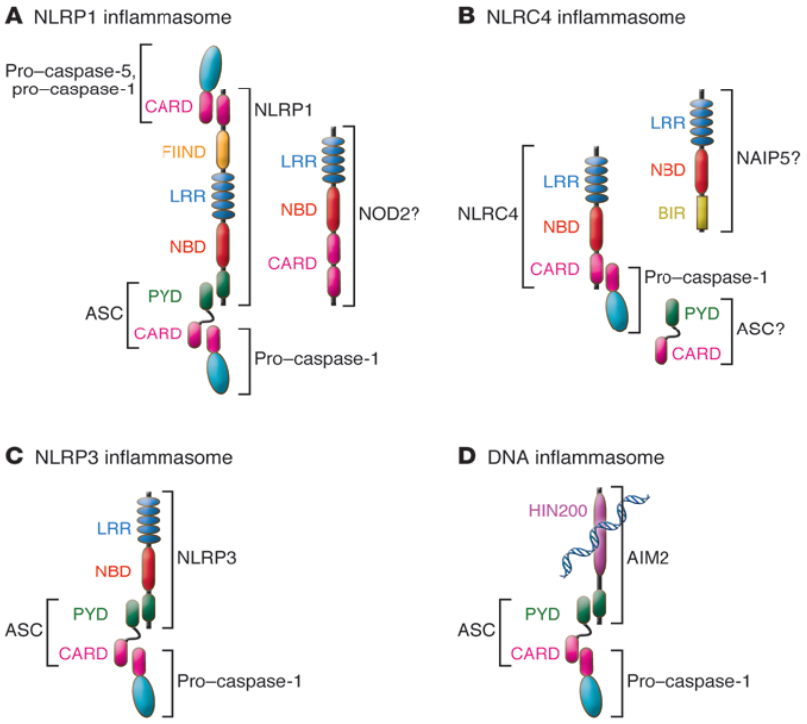


Fig. 3 Graphic rappresentation of known infalmmasomes.

(A) NLRP1 can recruit pro-caspase-1 and -5 and possibly forms a complex with NOD2. Recruitment of ASC enhances activation of pro-caspase-1. (B) NLRC4 contains a CARD that can directly recruit pro-caspase-1. (C) NLRP3 activates pro-caspase-1 via recruitment of ASC. (D) AIM2 is aa bipartite protein and recognizes cytoplasmic double-stranded DNA and assembles the DNA inflammasome with ASC and pro-caspase-1. Figure taken from Stutz *et al.*, 2009.

NALP1, also known as NLRP1, differs from the other member of the NLR family in its organization domain; in fact, it has a C-terminal extension consisting of a FIIND motif that harbours a CARD, which can directly recruit caspase-1 and caspase-5, without the need of ASC (apoptosis-associated speck-like protein containing a CARD), an adaptor protein. Two well-known stimuli for NALP1 inflammasome are the cytosolic anthrax lethal toxin (LT) and the muramyl dipeptide (MDP), a fragment of bacterial PGN.

IPAF, also known as NLRC4, with its CARD was shown to recruit directly caspase-1, such as NALP1. The oligomerazation of IPAF is triggered by cytosolic flagellin or other stimuli possibly delivered by a bacterial secretion system (type III or IV) of *Salmonella typhimurium*, *Legionella pneumophila*, *Shigella flexneri* or *Pseudomona aeruginosa*.

AIM2 is the first example of inflammasome composed by proteins that do not belong to NLR family. AIM2 is a bipartite protein consisting of a DNA-binding HIN200 domain, which recognizes double-strand DNA, and a PYD domain, which allows for recruitment of ASC and a formation of a caspase-1-activating inflammasome (Stutz *et al.*, 2009).

1.7.2 Mechanism of inflammasome activation

Specific mechanisms underlying the regulation and activation of inflammasomes are not completely elucidated. Recent advances suggest that the inflammasome is a sophisticated and dynamic collection of entities in which constituent components are selectively employed according to the nature of the primary trigger. It is believed that upon stimulation the inflammasome oligomerizes and recruits pro-caspase 1 directly via CARD-CARD homotypic interaction (e.g. NLRP1 or NLRC4) or indirectly via an adaptor protein, ASC or Pycard. The latter is the case of NALP3 and AIM2, which interact with ASC via PYD-PYD homotypic interaction. In turn, ASC interacts with pro-caspase 1 through their CARD domains. In resting cells, pro-caspase 1 is present in a catalitically pro-form. The formation of inflammasome induces the autocatalytic activation of caspase-1 by means of assembling of two or more of caspase-1 monomers, leading to the cleavage of the caspase zymogen into two subunits of 20-kDa (p20) and 10-kDA (p10) (Stutz *et al.*, 2009). The active enzyme then assembles into two heterodimers of p20 and p10 subunits, containing two actives sites (Wilson *et al.*, 1994). Caspase-1 is a member of a large family of intracellular aspartate specific cysteine proteases. Caspase-1, the first caspase to be discovered in mammalians, was initially known as IL-1-converting enzyme (ICE), since its first known substrate, pro-IL-1β, is proteolytically converted into the bioactive cytokine IL-1β. Caspase-1 can also cleave other members of the IL-1 family, pro-IL-18 and IL-33; this latter cytokine is inactivated by caspase-1 cleavage and activated by calpain. Mature IL-1β is a potent pyrogen with pleiotropic functions, involved

in the generation of systemic and local responses to infection, injury, and immunological challenges by generating fever, activating lymphocytes, and by promoting leukocyte infiltration at sites of injury or infection. Due to this repertoire of highly potent pro-inflammatory activities, accidental release of IL-1β would be quite deleterious. IL-1β is produced and secreted by monocytes, macrophages and dendritic cells and circulates systematically. The synthesis, processing and release of this cytokine are tightly regulated. Synthesized as a cytosolic 31 kDa precursor, pro-IL-1β is processed by caspase-1 into a 17 kDa product, which represents its mature biologically active form, and then secreted. These events require at least two distinct stimuli. An initial microbial stimulus, propagated via PRRs (e.g TLR4), induces accumulation of intracellular stores of pro-IL-1β. The molecular mechanisms underlying pro-IL-1β transcription are not well elucidated, although the transcriptional factor NF-kB and mitogen activated protein (MAP) kinase signalling have both been implicated. A second signal is necessary to induce the assemblation of the inflammasome to enhance the proteolytic maturation and secretion of IL-1 β (Dinarello, 2009). The cytokine lacks a secretory signal sequence, and is released through a non classical pathway that avoids the ER-Golgi route. The mechanisms leading to secretion of the mature cytokines are not well elucidated. Several models for their export have been proposed, such as secretion via multivescicolar bodies containing exosomes, via shedding of microvesicles from the plasma membrane or directly through the plasma membrane via unidentified transporters (Andrei *et al*., 1999). IL-18 is expressed by macrophages and dendritic cells as well as epithelial cells, such as keratinocytes. While the production of IL-1 typically requires an inducing stimulus, pro-IL-18 is constitutively expressed, but needs processing by caspase-1 to be activated. IL-18 induces IFN-γ production by activated T cells and NK cells and contributes to the differentiation of T cells helper type I (Th1). After the release, this cytokine binds IL-18 receptor (IL-18R) leading the activation of NF-kB, AP-1, MAPK and STAT3. In certain situations a process of caspase-1-dependent cell death, called pyropoptosis, is closely linked to inflammasome complex formation. In contrast to apoptosis, which is an immunologically "silent" form of cell death, pyroptosis is highly inflammatory form of cell death and is often observed during infection with cytosolic pathogens. NLRP1, NLRP3, NLRC4 and AIM2 inflammasomes all appear to trigger pyroptosis in cells of monocyte/macrophage lineage. The mechanism triggering pyroptosis involve cleavage and activation of caspase-1, rapid pore formation in the plasma membrane to allow water to enter the cell, causing osmotic lysis and release of pro-inflammatory cellular contents, along with the release of the caspase-1-associated cytokines such as IL-1β and IL-18. DNA cleavage also occurs in pyroptosis by a unidentified, caspase-1-activated, nuclease. This process does not result in the DNA laddering typical of apoptosis, but does cause nuclear condensation while maintaining nuclear integrity (Fink & Cookson, 2006).

1.7.3 The NALP3 inflammasome

The inflammasome NALP3, known also as “NLRP3”, “PYPAF1” or “cryopyrin”, is the best characterized of the inflammasomes. It consists of NLRP3 protein, ASC and caspase-1. NLRP3 recruits the adaptor protein ASC by PYD interactions. ASC, in turn, is responsible of the recruitment of caspase-1. Many stimuli can trigger the assembly of NALP3 inflammasome, leading to the formation of only one large complex, up to 2 μm in diameter, per cell; it consists mainly of the adaptor ASC. The stimuli reported are of diverse physico-chemical natures and include many microbes (viruses, bacteria and fungi), a range of PAMPs, bacterial toxins and aggregated substances. Live bacteria and viruses are reported to activate the NLPR3 inflammasome. Microbes express many products that can activate innate immune receptors such as TLRs, RLRs or other NLRs; in fact, many microbes-derived substances such as MDP, bacterial RNA, LPS, Pam2CysK4 were all reported to activate the NLRP3 inflammasome when administrated in the presence of ATP.

In addition to these pathogen-associated stimuli, a number of endogenous stress signals have been reported to activate the NLRP3 inflammasome. The first danger signal described was ATP (Mariathasan *et al.*, 2006). Mammalian cells contain up to 5-10 mM ATP in their cytosol, while in the extracellular space the concentration is maintained low by the activity of the ectoATPases (Di Virgilio, 2000). ATP is released from dying cells and it is likely that ATP is present locally in abundant quantities in stressed tissue. Stimulated cytotoxic lymphocytes, microglia and human monocytes have been shown to release ATP. In addition to ATP, cells release other danger signals to activate the immune system. NALP3 inflammasome is able to recognize also signals of metabolic stress, such as high concentration of extracellular glucose and monosodium urate (MSU) crystals. Uric acid is the end product of the cellular catabolism of purines and assembles in the joints of the hyperuricemic patients, forming MSU. High levels of circulating uric acid have been associated with various inflammatory diseases, including multiple sclerosis, hypertension and cardiovascular diseases. Similar to what was found for MSU, asbestos and silica microparticles activate the NALP3 inflammasome. The proinflammatory activity of crystalline materials has long been exploited as an adjuvant for vaccines. Alum, the most commonly used vaccine adjuvant, triggers caspase-1 via the NLRP3 inflammasome. Also peptides aggregates can cause NLRP3 activation; β-amyloid aggregates, related to Alzheimer disease, appear to be sensed in the CNS by microglia via NLRP3. The skin is the first barrier of the body against external threats and is often damaged by various insults; ultraviolet irradiation, for example, was recently shown to

activate the NALP3 inflammasome and promote IL-1β maturation in keratinocytes. One of the features of the NALP3 inflammasome is the broad range of activating molecules; this pleiotropism suggests that NALP3 activation is likely to be indirect and complex (Schroder & Tschopp, 2010).

1.7.4 Potential mechanisms of NLRP3 inflammasome activation

The sequence of molecular events that lead to the NLRP3 activation has not been completely elucidated. In the literature, three distinct models of activation have been proposed (Fig. 4) (Jin & Flavell, 2010; Tschopp & Schroder, 2010).

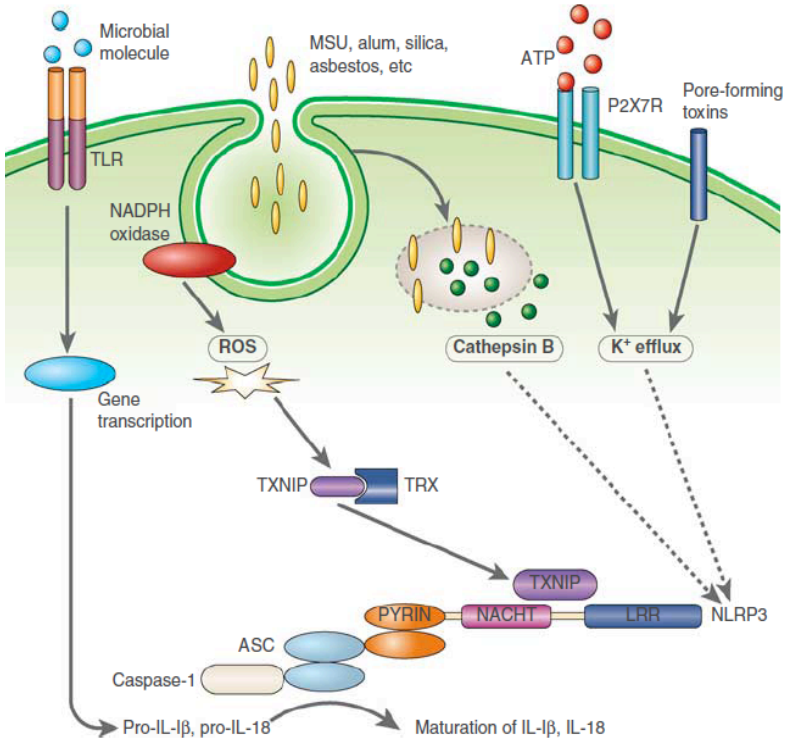


Fig. 4 Three proposed signalling pathways for activation of NALP3 inflammasome. Figure taken from Jin & Flavell, 2010.

The first model suggests that the main stimulus for activation is the low intracellular potassium concentration of potassium (K^+). Potassium efflux can be achieved as a consequence of the binding of extracellular ATP, released from damaged tissue, to purinergic receptor $P2X_7$. $P2X_7R$ activation results in plasma membrane depolarization, cell swelling, loss of cytoskeletal organization and K^+ efflux, by means of the formation of larger pore mediated by the protein pannexin-1. Potassium efflux leads to calcium influx and activation of various phospholipases involved in the sequence of events culminating in IL-1β maturation and secretion (Ferrari *et al.*, 2006; Pelegrin & Surprenant, 2006).

Moreover, it has been suggested that pores formed by pannexin-1 might deliver activators directly into cytosol, where they bind to NALP3 or other upstream factors. According to this, MDP is released from endosomal compartments into the cytosol upon ATP stimulation requiring pannexin-1 (Marina-Garcia *et al.*, 2008). In the 2008, Piccini *et al.* have demonstrated the main role of ATP in the inflammasome activation and release of IL-1β and IL-18. In human monocytes, a variety of activators can trigger the exocytosis of ATP, which may explain the ability of TLR ligands to activate IL-1β maturation and release in the absence of second stimulus. The release of ATP is followed by a paracrin and autocrin stimulation of $P2X_7$ receptor. The involvement of $P2X_7R$ in the maturation and release of IL-1β is confirmed by the observation that using receptor antagonist, such as oATP, and enzyme the hydrolyze ATP, such as apyrase, the maturation and release of IL-1β are inhibited. This model suggests that the process of IL-1β secretion can be split into two major steps. Firstly, the gene expression and synthesis of pro-IL-1β are induced by inflammatory signals, such as LPS. Then, the second signal, a danger signal such as ATP, is necessary to induce inflammasome activation and secretion of mature cytokine (Piccini *et al.*, 2008).

The second model has been proposed for activators forming crystalline or particle structure, such as the above mentioned, MSU, silica, asbestos, alum and β-amyloid aggregates (Hornung *et al.*, 2008). Based on this model, the inefficient elimination of these particles, internalized by phagocytes, leads to phagosomal destabilization and release of lysosomal contents into the cytosol. NLRP3 can sense phagosomal or lysosomal disruption as a danger signal and thereby indirectly sense excessive crystal phagocytosis or escape of microbes from endo-lysosomal compartments into the cytosol. The exact nature of ligand formed after lysosomal damage remains unknown. Lysosomal contents could be a danger signal sensed by NLRP3, or lysosomal proteases released in the cytosol could cleave a substrate, which, in turn, could be a ligand for the inflammasome. A role in this process for cathepsin B, a lysosomal protease, has been suggested (Hentze *et al.*, 2003). The inhibition of this protease led to a substantial decrease of IL-1β maturation and cathepsin B-deficient macrophages show partially reduced IL-1β secretion upon NLRP3 activation. In addition, the activation of pannexin-1 pores might, not only induce K^+ efflux or microbial products access in the cytoplasm, but also destabilize lysosomes and lead to release of lysosomal content into the cytoplasm (Stutz *et al.*, 2009).

The third model proposed that reactive oxygen species (ROS) act on a target upstream of the NALP3 inflammasome and indirectly cause its activation. The involvement of redox reactions in inflammasome activation has been suggested by several studies. Many known NLP3-activating stimuli, such as ATP, MSU and alum, generate ROS. Although consensus exists on the importance of the redox pathway in

inflammasome activation, how and by which ROS are generated and their role in specific activation of caspase-1 by pathogen- or damage–associated molecular patterns remain unclear. It is believed that NADPH oxidase or mitochondria could be involved. ROS generation is frequently associated with potassium efflux, demonstrating that the two models can be connected (Stutz *et al.*, 2009; Tschopp & Schroder, 2010). ROS might induce directly the assembly of inflammasome or indirectly via cytoplasmatic proteins that modulate inflammasome activity. It has been demonstrated that the ROS production, ATP mediated, led to PI3K-pathway activation and that pharmacological inhibition of PI3K results in prevention of caspase-1 activation induced by ATP (Cruz *et al.*, 2007; Martinon, 2010). Recently, Zhou *et al.* have identified thioredoxin (TRX) -interacting protein (TXNIP) as a redox-sensitive regulator of inflammasome activation induced by MSU. In normoxic homeostasis TXNIP is bound to TRX and inhibits the reducing activity of TRX; after oxidative stress, the TXNIP-TRX complex dissociates and the unbounded TXINP interacts with NALP3. This interaction is necessary for inflammasome activation in response to oxidative stress *in vitro*. Accordingly, Txnip$^{-/-}$ macrophages treated with ATP or MSU crystals show less caspase-1 maturation and IL-1β production (Zhou *et al.*, 2010). The role of ROS in the activation of inflammasome and IL-1β maturation and release is controversial. Tassi *et al.* proposed that IL-1β maturation and release are regulated by a biphasic redox response, characterized by two stages: the oxidative stress and then the antioxidant response. They demonstrated that different PAMPS induce the generation of ROS by NADPH oxidase in human monocytes. Subsequently, an antioxidant pathway is engaged to countermand the oxidative stress, via up-regulation of enzymes, such as thioredoxin, and a high increase of activity of cystine/cysteine redox cycle. Antioxidant response is slightly delayed compared to ROS generation and is concomitant to IL-1β release. The oxidative stress could be necessary to induce the antioxidant response and is essential for IL-1β cleavage and release; in fact, the reduction of extracellular medium with exogenous reducing agents (e.g. DTT) rescues IL-1β release in the presence of inhibitors of ROS production (Tassi *et al.*, 2009). In contrast, other authors deny the ROS involvement in the activation of inflammasome and in the subsequent events. Meissner *et al.* suggest that ROS can in fact inhibit directly ATP-induced activation of caspase-1 by oxidation and glutathionylation and that the reduction of ROS by SOD1 is needed in order to recover IL-1β production (Meissner *et al.*, 2008). In addition, van de Veerdonk *et al.* evaluated the activation of the IL-1β in cells of patients with chronic granulomatous disease (CGDs), which have defective NADPH activity and, consequently, defective NADPH-dependent ROS generation. Activation of CGD monocytes by uric acid crystals induced a higher level of IL-1β secretion compared to unaffected subjects, and this increase was not due to increased synthesis of IL-1β precursor. Moreover, they found no decrease in either caspase-1 activation or secretion

of IL-1β using diphenylene iodonium (DPI), as ROS inhibitor (van de Veerdonk *et al.*, 2010).

These three theories for NALP3 inflammasome activation are different, but considering these as being exclusive of one another is probably an oversimplification, as crosstalks among these pathways probably occur. It is possible that ROS generation contributes to lysosomal destabilization and that bacterial products indirectly contributes to NLRP3 activation (Antunes *et al.*, 2001). It is also reasonable that the activation of NALP3 is a multistep process that requires more than one factor.

Recent studies have demonstrated that priming via transcriptionally active pattern recognition receptors (e.g. TLRs or NODs) or via cytokines is required for subsequent activation of NLRP3 inflammasome by ATP, pore-forming toxins, or crystals in macrophages. These studies have demonstrated that cell stimulation via PRRs or cytokine receptors leads to high cytosolic levels of NLRP3; when a critical NLRP3 concentration was reached, NLRP3 inflammasome assembly was induced by stimulation with ATP, nigericin or crystalline activators. Many of the reported NALP3 stimuli are not direct activators, but rather they are required for the transcriptional regulation of the inflammasome components. Thus, the range of NALP3 activators could be likely less broad than previously expected (Stutz *et al.*, 2009).

1.8 Apoptosis

The term “apoptosis” was used for the first time in a paper by Kerr, Wyllie and Currie 1972 to describe a form of cell death, which is morphologically distinct from necrosis (Kerr *et al.*, 1972). Apoptotic phenomena occur normally during development and aging as a homeostatic mechanism to tightly control cell numbers and tissue size. Apoptosis is also involved in defence mechanism such as in immune reactions and when cells are damaged by disease or noxious agents (Norbury & Hickson, 2001). When the cell death program is activated, apoptotic cells shrink and are rapidly eaten by neighbouring cells, before there is any leakage of their contents. By contrast, cells that die in response to an acute injury swell and burst, spilling their contents over their neighbours and eliciting a damaging inflammatory response. This uncontrolled process of cell death is called necrosis (Raff, 1998). The characteristics of the apoptotic cell include chromatin condensation and nuclear fragmentation (pyknosis), plasma membrane blebbing and cell shrinkage. Most of the morphological changes that were observed by Kerr *et al.* are caused by a set of cysteine proteases that are activated specifically in apoptotic cells. These proteases are homologous to each other and are part of a large

protein family known as caspases. Caspases are highly conserved during evolution. Over a dozen of caspases are identified in humans, and about two-third of these have been suggested to function in apoptosis. Caspases are present in the cells as enzymatically inert zymogens, but can be triggered to have active states and, in turn, activate other caspases, leading to proteolytic cascade and amplifying pro-apoptotic signals. All known caspases possess an active-site cysteine, and cleave their substrates at aspartic acid (Asp) residues. These zymogens are composed of three domains: an N-terminal prodomain, and the p20 and p10 domains, which are found in the mature enzyme. The mature enzyme is a heterotetramer containing two p10/p20 heterodimers and two active sites. From functional perspectives, they can be subgrouped in “apoptotic” and “proinflammatory” (caspase-1, -4 and -5). Within the apoptotic group, the caspases are distinguished in “initiators” (caspase-8, -9 and -10) and “effectors” (caspase-3, -6 and -7). Initiator caspases activate effector caspases, which, in turn, have different targets. These substrates of effector caspases include protein-kinases and other signal transduction proteins, cytoskeletal and nuclear matrix proteins, chromatin modifying (eg, PARP poly-ADP ribosyl polymerase) and DNA repair proteins and inhibitory subunits of endonucleases (CIDE family proteins). Pro-inflammatory caspases are involved in processing of pro-inflammatory cytokines (Reed, 2000). Programmed cell death can be activated via two pathways: extrinsic and intrinsic.

The extrinsic pathway involves transmembrane receptor-mediated interactions. These involve death receptors that are members of the tumor necrosis factor (TNF) receptor gene superfamily. Several members of this family of receptors, such as Fas, share similar cysteine-rich extracellular domain and have a citoplasmatic domain of about 80 amino acids, called the “death domain” (DD). This domain plays a key role in transmitting the death signal from the cell surface to the intracellular signalling pathway. The sequence of events that define the extrinsic phase of apoptosis are best characterized with FasL/FasR and TNF-α/TNFR1 models. In these models, there is clustering of receptors and binding with the homologous trimeric ligand. Upon ligand binding, cytoplasmic adapter proteins are recruited which exhibit corresponding death domains that bind with the receptors. The binding of ligand (FasL) to receptor (FasR) results in the binding of adapter protein FADD (Fas-associated death domain protein) and the binding of TNF ligand to TNF receptor results in the binding of TRADD. Subsequently, FADD associates with pro-caspase 8 through dimerization of DD domains, leading to the formation of a complex, called DISC (death-inducing signalling complex) and auto-catalytic activation of pro-caspase-8. After caspase-8 activation, the execution phase of apoptosis is triggered. This pathway can be inhibited by a protein called c-FLIP, which binds both FADD and caspase-8, rendering them ineffective. The extrinsic pathway is used also by cytotoxic T lymphocytes (CTLs) to kill virus-infected cells or tumor cells. This

pathway involves secretion of the transmembrane pore-forming molecule perforin with a subsequent release into the target cell of cytoplasmic granules composed of granzyme B and granzyme A. Granzyme B cleaves proteins at aspartate residues and therefore activate pro-caspase-10 and can cleave factors like ICAD (Inhibitor of Caspase Activated DNAse).

The intrinsic pathway involves a diverse array of non-receptor-mediated stimuli that produce intracellular signals. The stimuli produce intracellular signals that may act in either a positive or negative fashion. Negative signals involve the absence of certain growth factors, hormones and cytokines, that can lead to failure of suppression of death programs, thereby triggering apoptosis. Positive signals comprise radiation, toxins, hypoxia, hyperthermia, viral infection and free radicals. All of these stimuli cause changes in the inner mitochondrial membrane that results in an opening of the mitochondrial permeability transition pore (MPT), loss of the mitochondrial transmembrane potential and release of two main groups of normally sequestred pro-apoptotic proteins from the intermembrane space to the cytosol. The first group consists of cytochrome c (cyt c), SMAC/DIABLO and the serine protease HtrA2/Omi. These proteins activate the caspase-depedent mitochondrial pathway. Cytochrome C binds and activates Apaf-1. In the presence of ATP, Apaf-1 recruits pro-caspase-9, forming a complex called “apoptosome”, involved in the activation of caspase-9. SMAC/DIABLO and HtrA2/Omi promote apoptosis by inhibiting IAP (inhibitors of apoptosis proteins) activity. IAPs inhibit activated caspases. The second group of pro-apoptotic proteins consists of AIF, endonuclease G and CAD, which are released from the mitochondria only in the late phase of apoptosis. AIF translocates to the nucleus and causes DNA fragmentation and chromatin condensation. Also endonuclease G translocates to the nucleus where it cleaves nuclear chromatin to produce oligonucleosomal DNA fragments. CAD is released from the mitochondria and translocates to the nucleus where, after cleavage by caspase-3, leads to oligonucleosomal DNA fragmentation and chromatin condensation (Elmore, 2007).

The control and regulation of these apoptotic mitochondrial events occur through members of the Bcl-2 family of proteins. The name of this family comes from the first member identified, Bcl-2, a gene involved in the B cell lymphoma. Bcl-2 family proteins are conserved throughout metazoan evolution, with homologues found in mammalian, avian, fish and amphibian species, as well as in invertebrates. The Bcl-2 family proteins governs mitochondrial membrane permeability and can be either pro-apoptotic or anti-apoptotic. To date, a total of 25 genes have been identified in the Bcl-2 family. Some of the anti-apoptotic proteins include Bcl-2, Bcl-x, Bcl-X_L, Bcl-X_S, Bcl-w, Mcl-1, BAG and some of the pro-apoptotic proteins include Bcl-10, Bax, Bak, Bid, Bad, Bim, Bik and Blk. These proteins a have special significance since they can determine if the cell commits to apoptosis or abort the process. Some of the Bcl-2 family genes produce two or more

proteins through alternative mRNA splicing, sometimes exerting opposing effects on cell death regulation (e.g. Bcl-X_L versus Bcl-X_S). Also, some of these proteins may display anti-apoptotic activity in some cellular backgrounds and have pro-apoptotic functions in other cellular contexts (e.g. Bcl-2, Bax). Gene ablation studies in mice suggest that each of the Bcl-2 family members plays unique roles in controlling cell survival *in vivo*, reflecting their tissue-specific patterns of expression or cell-context-dependent requirements for these proteins. Many Bcl-2 family proteins are constitutively localized to the membranes of mitochondria, whereas others are induced to target these organelles in response to specific stimuli. For example, Bcl-2 and Bcl-X_L and many others members of the Bcl-2 family have a hydrophobic stretch of amino acids near their C-termini (transmembrane domains) that anchors them in outer mitochondrial membrane. Some of these proteins also insert into endoplasmic reticulum and nuclear envelope, though their effects on cell death regulation in these compartments are poorly understood compared to those of mitochondria. Bid, Bim and BAD lack C-terminal transmembrane domains and are normally found in the cytosol but can be induced to target mitochondria (Hengartner, 2000).

The main function of this family is to regulate the mitochondrial homeostasis; the proteins of this family can act via different mechanisms, which can be summarized in:

- Formation of a pore, through which cytochrome C and other proteins can escape.
- Heterodimerization between pro- and anti- apoptotic family members.
- Direct regulation of caspases via adaptor molecules
- Interaction with other mitochondrial proteins, such as VDAC and the adenosine nucleotide transporter (ANT), either to generate a pore for cytochrome C exit, or to modulate mitochondrial homeostasis (for example, opening of the PTP)
- Oligomerization to form weakly selective ion channel.

The two pathways can interact between each other. One example of this cross-talk is Bid, that after the cleavage by caspase-8, migrates in the mitochondria and leads to release of cytochrome C. Serine phosphorylation of Bad is associated with 14-3-3, a member of a family of multifunctional phosphoserine binding molecules. When Bad is phosphorylated, it is trapped by 14-3-3 and sequestered in the cytosol; however, once Bad is unphosphorylated, it will translocate to the mitochondria to release cytochrome C. Bad can also heterodimerize with Bcl-X_L or Bcl-2, neutralizing their protective effect and promoting cell death. Puma and Noxa are two other members of the Bcl-2 family that are involved in pro-apoptosis. Puma plays an important role in p53-mediated apoptosis. *In vitro* overexpression of Puma is accompanied by increased BAX expression, BAX conformational change, translocation to the mitochondria, cytocrome C release and reduction in the mitochondrial membrane potential (Liu *et al.*, 2003). Noxa is also a

candidate mediator of p53-induced apoptosis. This protein can localize to the mitochondria and interact with antiapoptotic Bcl-2 family members, resulting in the activation of caspase-9 (Oda *et al.*, 2000). Since both Puma and Noxa are induced by p53, they might mediate the apoptosis that is elicited by geno-toxic damage or oncogene activation.

The extrinsic and intrinsic pathway both end at the point of execution phase, considered the final pathway of apoptosis. It is the activation of execution caspases that begins this phase of apoptosis. Caspase-3 is considered to be the most important of the executioner caspases and is activated by any of the initiator caspases (caspase-8, -9 and -10) (Elmore, 2007).

1.9 T regulatory cells

Homeostasis in the immune system depends on a balance between the responses that control infection and tumour growth and the reciprocal responses that prevent inflammation and autoimmune diseases. Regulatory T cells have a crucial role in suppressing immune response to self-antigens and in preventing autoimmune diseases. Evidence is also emerging that regulatory T cells control the immune system response to bacteria, virus, parasites and fungi. Many pathogens have evolved mechanisms to subvert the protective immune responses of the host to their advantage, generating conditions that ensure their survival for a longer time.
These strategies include:

- evasion of humoral and cellular immunity by antigenic variation
- interference with antigen processing or presentation
- subversion of phagocytosis or killing by cells of the innate immune system
- induction of regulatory response (normally associated with the termination of effector immune responses of the host).

At the beginning of 70's, Gershon was the first researcher to suggest the existence of lymphocytes with immunosuppressive activity, and these cells were called T suppressor lymphocytes. Because of several difficulties to characterize the cells and to understand the mechanisms of their immunoregulation, this theory has not been considered for twenty years. In the 90's several studies, which were carried out on animals with autoimmune diseases, demonstrated that a subset of T lymphocytes have a crucial role in the prevention of autoimmunity. Natural $CD4^+CD25^+$ T reg cells were first defined in 1995 by Sakaguchi and colleagues (Sakaguchi *et al.*, 1995). They described that a subset of peripheral $CD4^+$ T cells first appeared in the periphery 3 days after birth

in normal healthy mice and increased rapidly to 5-10% of the total peripheral $CD4^+$ T cell population within 2 weeks. Subsequently, an analogous subset of lymphocytes has been discovered also in humans, they represent 2-5 % of $CD4^+$ T cells in human PBMC (Bacchetta *et al.*, 2005).

Until now, several types of regulatory T cell have been described on the basis of their origin, generation and mechanism of action, with two main subsets identified. All of them are characterised by their ability to inhibit cytokine release and to suppress proliferation of various cell types, including $CD4^+$ and $CD8^+$ T cells, B lymphocytes, NK (natural killer) cells, NKT and DC (dendritic cells). Two main subsets of T regulatory cells have been indentified: natural and inducible.

The formers mainly develop in the thymus and can be detected in the foetus at an early stage of gestation (Cupedo *et al.*, 2005). Phenotipically they are defined by their constitutive expression of the high affinity IL-2 receptor α chain CD25 and the transcription factor, Foxp3 (Sakaguchi *et al.*, 1995; Shevach, 2002). Vice versa, inducible T regulatory cells develop in the periphery, expressing Foxp3 only when activated by certain stimulatory conditions. Inducible regulatory T-cell populations include T regulatory 1 (T_R1) cells, which secrete IL-10 and T helper 3 (T_H3) cells, which secrete TGF-β and converted $Foxp3^+$ regulatory cells. In addition, adaptive T reg can be induced from $CD4^+$T cells lacking the CD25 activation marker. Other cell-surface markers associated with regulatory properties have been described including glucocorticoid-induced TNF receptor family-relates gene (GITR), cytotoxic T-lymphocyte-associted protein (CTLA-4), integrin $\alpha E\beta 7$ (CD103), CD45RO and CD62L (Read *et al.*, 2000; Shimizu *et al.*, 2002).

These molecules are predominantly expressed at an elevated level on $CD4^+CD25^+$ T reg cells, which allows for their discrimination and subsequent isolation for functional studies. However, at present there is no single specific surface marker exclusively expressed by $CD4^+CD25^+$ T regs; thus, it remains a major challenge to identify a more specific and reliable functional marker for these cells.

Materials and Methods

2.1 Reagents

Phosphate-buffered saline (PBS), RPMI 1640 and Foetal calf serum (FCS) were purchased from Euroclone (Siziano, IT). Gentamicin and Hepes were purchased from Gibco (Scotland ,UK). Nutridoma-SP was purchased from Roche Applied Science (Basel, CH). Methanol, Red Ponceau, Ac-YVAD-CMK, ARL, DEPC, PMA, brefeldin A, Tetramethylbenzidine (TMB), Tween-20 and oATP were obtained from Sigma-Aldrich (St Louis, MO). Antibodies anti-IL1-β, GM-CSF and IL-4 were purchased from R&D Systems (Wiesbaden, DE). Antibodies anti-actin, horseradish peroxidise-conjugated anti-human IgG and ionomycin were obtained from Calbiochem (La Jolla, CA). Ficoll-Paque solution, Percoll, X-ray film and ECL (enhanced chemiluminescence system) were purchased from GE Healthcare (Buckinghamshire, UK). BCA protein assay reagent was purchased from Pierce (Rockford, IL). ATPlite kit was purchased from PerkinElmer Life Sciences (Boston, MA). TRIzol solution, 4-12% SDS-PAGE gels, SuperScript II, RNAse Out, LDS 4X sample buffer, NuPAGE antioxidant, NuPAGE MES 20X Running Buffer, NuPAGE 20x Transfer Buffer were obtained from Invitrogen (San Diego, CA). Antibody anti-CD4-PECY7 was obtained from Beckman Coulter (Fullerton, CA). Antibodies anti-CD25-PECY5, -CD14, -CD86, -CD80, -HLA-DR, -CD28, -CD49d were purchased from BD Biosciences (San Diego, CA) and antibody anti-Foxp3-FITC was purchased from e-Bioscience, (San Diego, CA). Antibody anti-TGF-β-PE was from IQProducts (Groningen, NL). IL-2 was provided by Novartis (Emeryville, CA). Polyclonal anti-PARP, anti-Caspase-3 and anti-Bcl-X_L antibodies were purchased from Cell Signaling (Beverly, MA). Annexin V FITC assay was from Bender Medsystem (Vienna, Austria).

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* gene

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: 5 min at 94°C, 30 cycles of 1 min at 94°C, 1 min at 50°C, and 45 sec at 72°C, and a final

extension cycle of 10 min at 72°C. Primers used were forward: 5'-ccggaattcacgatgaacatgtgtaca-3' and reverse: 5'-cccaagcttctaggctttcagggtagc-3', containing 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 µl 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 µg/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 µg/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 µl of buffer P1 (Qiagen), then were added 250 µl of buffer P2 (Qiagen) and the suspension was gently inverted 2-3 times; 350 µl 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 µl of buffer PE (Qiagen) and centrifuged 1 minute at 13,000 x g. The purified plasmid DNA was eluted from the column with 50 µl 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 µl of electroporation buffer to a final OD of 1.9. The bacteria were aliquoted in 70 µl. *Bacillus subtilis* (70µl) was electroporated using 1.5 µg of DNA; one pulse of 200 Ω, 2.3 κV, 2.5 µF. Electroporated cells were incubated 10 min at 25°C in 500 µl 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 µg/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 µg/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 Monocytes isolation from Buffy coat

Human monocytes were obtained from Buffy coat, from the Transfusional Centre of the Universitary Hospital of Padova, derived from 500 ml of blood of healthy donors. The buffy-coat was diluted 1:4 with sterile phosphate buffer (PBS) without Ca^{2+} and Mg^{2+} and then dextran 5% was added diluted 1:5, and erythrocytes sedimented for 30 min. The supernatant was recovered and washed with sterile PBS for 15 min at 50 x g. The cellular pellet was resuspended in 15 ml of sterile PBS, stratified on Ficoll-Paque and centrifuged 30 min at 400 x g without brake and accelerator. Lympho-monocytes were recovered and washed 15 min at 311 x g, and then stratified on Percoll gradient (15.76 ml RPMI 1640, 10% FCS (v/v), HEPES 4 mM, 50 μg/ml gentamycin, 285 mOsm; 15.54 ml 10% Percoll in PBS 10X sterile, 285 mOsm), and centrifuged 30 min at 400 x g without brake and accelerator. Monocytes were recovered and centrifugated for 15 min at 311 x g. The cells were harvested and resuspended in RPMI 1640, 2% FBS, 50 μg/ml gentamicin and HEPES 4 mM and further separated from contaminating lymphocytes by adherence (1h at 37°C) to plastic well. Adherent monocytes were extensively washed with medium to remove residual nonadherent cells.
Then, cells were treated for different time points with the appropriate stimuli. At the end of the culture period, cell supernatants were collected for the proteins level quantification. The monocytes were lysed for the mRNA extraction using TRIzol solution or using lysis buffer for protein extraction.

2.9 RNA extraction

For RNA isolation, 2 x 10^6 monocytes were seeded in 24 wells plate and incubated in 300 μl (for 1 and 5 h) and 500 μl (for 12 and 24 h) of RPMI 1640, 10% FCS, 50 μg/ml gentamicin and HEPES 4 mM in absence or presence of TpF1 1 μM. Adherent monocytes were lysed directly in the culture dish by adding 150 μl of TRIzol and passing the cell lysate several times through a pipette. The homogenized samples were incubated for 5 min at room temperature to permit the complete dissociation of nucleoprotein complexes. 30 μl of chloroform were added to cell lysate and the tubes were shaken vigorously for 15 seconds and incubated on ice for 15 min. The samples were centrifugated at 12,000 x g for 15 min at 4°C. After centrifugation, the mixture is separated in three phases: a lower phenol-chloroform phase with DNA, an interphase and a colourless upper aqueous phase which contained RNA. The latter was transferred into a new tube and RNA was precipitated by the addition of 70 μl of isopropyl alcohol at -80°C for at least 20 min. Then, the samples were centrifugated at 12,000 x g for 15 min at 4°C. The supernatants were removed, while the RNA pellets were washed twice with 500

µl of 70% ethanol RNase free at 7,500 x g for 5 min at 4°C. The RNA precipitates were vacuum-dried for 10-15 min at room temperature. The pellets were dissolved in 10 µl of RNase-free 0.1% v/v DEPC (diethylpyrocarbonate)-treated water.

2.10 cDNA synthesis

10 µl of total RNA was reverse transcribed with SuperScript II in the following reaction mix:

DTT 0.1 M	2.5 µl
Buffer 5X	5.0 µl
dNTPs 5 mM	2.5 µl
Random primers 50µM	1.0 µl
Total	11.0 µl

The samples were mixed by vortexing and denaturated by incubation for 5 min at 72° C and then placed on ice.

Samples were retrotranscribed to cDNA adding the following mix in a final volume of 25 µl:

H_2O	2.6 µl
Retrotranscriptase *Superscript* II (140U)	0.7 µl
RNAse out (98 U)	0.7 µl
Total	4.0 µl

The reaction program was:

- step1: 42°C for 50 min
- step2: 95°C for 5 min
- step3: 4°C

cDNAs were then precipitated with 0.1 Vol Sodium Acetate 3 M and 2.5 Vol of ethylic alcohol at -80°C for 1 h. cDNAs were centrifuged at 12,000 x g for 30 min at 4°C, supernatants were discharged and cDNA pellets were dissolved in 20 µl of sterile water.

2.11 Real time PCR analysis

1µl of diluted cDNAs were amplified in a final volume of 10 µl PCR reaction in 7900 HT FAST Real Time PCR System (Applied BioSystem) in 96-wells plates (Micro Amp Optical, Applied Biosystems).

Sample mix was prepared as follows:

Power SYBR Green PCR Master Mix	5.0 µl
Primer Forward 50 μM	0.18 µl
Primer Reverse 50 μM	0.18 µl
H_2O apyrogen	3.64 µl

The PCR cycle for the Real-Time PCR was:

- Initial denaturation: 95°C for 15 min
- Denaturation: 95°C for 15 seconds
- Annealing: 58°/60°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		Primers Sequence (5' - 3')	AT (C°)
GAPDH	F	AGCAACAGGGTGGTGGAC	58
	R	GTGTGGTGGGGGACTGAG	
IL-1β	F	CTGTCCTGCGTGTTGAAAGA	60
	R	TTGGGTAATTTTTGGGATCTACA	
IL-6	F	AACCTGAACCTTCCAAAGATGG	58
	R	TCTGGCTTGTTCCTCACTACT	
IL-10	F	AACCAAGACCCAGACATCAA	58
	R	CATTCTTCACCTGCTCCAC	
TGF-β	F	AGTGGTTGAGCCGTGGAG	58
	R	CCATGAGAAGCAGGAAAGG	
TNF-α	F	ATGAGCACTGAAAGCATGATCC	60
	R	GAGGCCTGATTAGAGAGAGGTC	

Tab I Primers sequences.

The primers were designed using Roche Universal Probe Library and were purchased from Invitrogen. (F: forward; R: reverse; AT: annealing temperature).

After the amplification, data analysis was performed using the second derivative method algorithm. For each sample, the amount of messenger RNA (mRNA) of each cytokine was expressed as the n-fold of the normalized amount of mRNA in untreated cells [1 arbitrary unit (AU) = cytokine mRNA concentration/GAPDH mRNA concentration (both in fmoles/μl)].

2.12 Protein Extraction

For protein extraction 6 x 10^6 were seeded on 12-well plate and stimulated with appropriate stimuli in RPMI 1649, 1% Nutridoma-SP, 50 μg/ml gentamicin and 4mM HEPES at different time points. The cells were collected, washed in ice-cold PBS and lysed with ice-cold lysis buffer (Tris 20 mM pH 7.15, NaCl 150 mM, NP40 0.25 %, supplemented with protease inhibitors PMSF 1 mM, leupeptin 1 μg/ml, and aprotinin 1 μg/ml) for 30 min on ice. Lysates were centrifuged at 12,000 x g for 20 min at 4°C, and the supernatants were collected and quantified on 96 well-plate using BCA Assay according to manufacturer's instructions. The plate was placed into a plate reader (Packard Fusion) and the optical density at 562 nm determined for each well.

2.13 Supernatant proteins preparation for IL-1β evaluation

For supernatant proteins extraction 6 x 10^6 monocytes were seeded on 12-well plate and stimulated with appropriate stimuli in RPMI 1640, 1% Nutridoma-SP, 50 μg/ml gentamycin and 4 mM HEPES at different time points. The supernatants were collected (500 μl total for each sample); 50 μl were used for IL-1β quantification by ELISA assay, whereas the rest was precipitated with Trichloroacetic acid (TCA) 10% for 1h at room temperature. The samples precipitated were centrifuged at 12,000 x g for 10 min. The supernatants were discharged while the pellets were resuspended in LDS.

2.14 SDS-PAGE (PolyAcrilamide Gel Electrophoresis)

5 μg of cell extracts and all supernatants precipitated in TCA 10% were diluted in Loading buffer which was prepared as follows:

- 1X NuPAGE® LDS Sample Buffer
- DTT 50 mM

The volume of each sample was brought to 15 μl. The samples were denaturated at 99 °C for 10 min. Samples were loaded on SDS 4-12% precast polyacrylamide gels. The electrophoresis was run in 1X MES Running buffer containing the antioxidant at 110 mA

and 200 V constant for 40 min.

2.15 Western Blot

After electrophoretic run, proteins were transferred from gels to nitrocellulose membranes. The gel and the membrane were equilibrated in Transfer Buffer. The Transfer Buffer was prepared as follows:

- 20X NuPAGE® Transfer buffer
- 10X NuPAGE® Antioxidant
- 10% Methanol

The volume was brought to 1 l with distilled water.
The transfer was obtained by applying a current of 170 mA and 30 V constant for 1 h. To evaluate the efficiency of the transfer, proteins were stained with Red Ponceau 1x. The staining was easily reversed by washing with distilled water.
Once the proteins were transferred on nitrocellulose membranes, the membranes were saturated with Blocking Buffer (2% no fat milk powder solubilizated in PBS with 0.2% TWEEN-20) for 1 h at room temperature, and then incubated overnight with the primary antibody of interest at 4°C. The membranes were then washed 3 times with PBS with 0.2% TWEEN-20 at room temperature and incubated with secondary antibody-HRP Conjugate, for 1 h at room temperature. Immunoreaction was revealed by ECL advance and followed by exposure to X- ray film.

2.16 Assay of TpF1-specific antibodies in sera of patients

Serum samples examined for the presence of TpF1-specific antibodies were obtained from 30 patients with secondary syphilis who were living in Latvia. Sera from 37 healthy control subjects were used as control. For the ELISA assay, the antigen (10 ng/μl) was incubated overnight at room temperature in a 96-well plate (100 μl/well). Then the plate was washed two times with 200 μl/well of PBS 1X, and incubated with blocking buffer (PBS, 1% bovine serum albumin, BSA) for 45 min at room temperature. After incubation, the plate was washed twice with 200 μl/well of PBS and serum samples (diluted 1:100 in PBS) were added and incubated for 1 h at room temperature. The plate was washed four times with PBS, 1% BSA, 0.05% Tween-20. Horseradish peroxidase–conjugated anti-human IgG antibody (1:5,000 in PBS, 1% BSA) was added to each well; after the addition of TMB (Tetramethylbenzidine) the plate was placed into a plate reader (Packard Fusion) and the optical density at 370 nm determined for each well.

2.17 Detection of IL-1β, IL-6, IL-10, TNF-α and TGF-β in culture supernatant

Culture supernatants of monocytes harvested for quantification of mRNAs, were collected and stored at -80°C for subsequent quantification of cytokine content by ELISA assay: specific kits for IL-6, IL-10 and TNF-α (Millipore, Billerica, MA), IL-1β (Biolegend, San Diego, CA) and TGF-β (Bender MedSystems Diagnostics GmbH, Vienna, AT) were used following manufacturer's instructions.

2.18 Detection of apoptosis

Phosphatidylserine (PS) is predominantly located in membrane leaflets, which face the cytosol. PS is exposed at the cell surface during the execution of apoptosis. Annexin V will bind to the PS-exposing apoptotic cell.

2 x 10^6 monocytes were cultured on 24-well plate in presence of appropriate stimuli in RPMI 1640, 10% FCS, 50 μg/ml gentamicin and 4 mM HEPES for different time points. The supernatants were collected and the adherent monocytes were incubated in Sodium EDTA 5 mM in PBS for 5-10 min at 37°C to detach them from the plate.

The cells were collected in FACS tubes and centrifugated at 226 x g for 10 min at 4°C with FACS buffer (PBS, 1% BSA). The supernatants were discharged and the pellets were resuspended in 195 μl of Binding buffer 1X. 5 μl of Annexin V-FITC were added to each tube and incubated for 20 min at 4°C in the dark. The tubes were filled up with FACS buffer and centrifugated at 226 x g at 4°C. The supernatants were discharged and the cells were resuspended in 190 μl of Binding buffer 1X. Before the measurement, 10 μl of propidium iodide (PI) were added and the viability was measured by flow cytometry (FACSCalibur, BD Biosciences, San Jose, CA) as the percentage of Annexin V/PI-negative events in the gated population.

2.19 Measurement of ATP

ATPLite™ is an Adenosine Triphosphate (ATP) monitoring system based on firefly (Photinus pyralis) luciferase. The reaction of ATP with added luciferase and D-luciferin is illustrated in the following scheme:

$$\text{ATP} + \text{D-luciferin} + \text{O2} \xrightarrow[\text{Luciferase}]{\text{Mg}^{2+}} \text{Oxyluciferin} + \text{AMP} + \text{PPi} + \text{CO2} + \text{light}$$

Extracellular ATP concentration was determined according to manufacturer's instructions. Briefly, 3 x 10^5 monocytes were seeded on 96-well microplate and were cultured with appropiate stimuli in 110 μl of RPMI 1640, 10% FCS, 50 μg/ml gentamicin and 4mM HEPES for different time points. After treatment, the supernatants were collected and centrifugated at 226 x g for 5 min to avoid the cell in suspension. 100 μl of each sample were transferred on a 96-well black microplate. 50 μl of mammalian cell lysis solution were added per well and the plate was shacked for 5 min in an orbital shaker at 700 rpm to lysate the cells and stabilize the ATP. 50 μl of substrate solution were added to the wells and the plate was shaked again for 5 min. The plate was incubated in the dark for 10 min. The plate was placed into a plate reader (Packard Fusion) and the luminescence was measured for each well. A standard curve was set up in the same microplate from a concentration of 1 x 10^{-5} M down to blank.

2.20 Determination of O_2^- production

To analyze the respiratory burst kinetics, the amount of O_2^- was assayed by detection of reduced cytochrome C produced by freshly isolated monocytes. Monocytes were resuspended in HBSS buffer supplemented with 10% FBS, 0.5 mM Ca^{2+} and 1 mg/ml glucose and plated at 1.5 x 10^5/100 μl in 96-wells plate. After 15 min of incubation at 37°C, the medium of each well was replaced with 200 μl of HBSS pH 7.4 containing 80 μM cytochrome C (Sigma) in presence or absence of TpF1 (1 and 10 μM) or β-glucan (50 μg/ml), as positive control. The plate was then placed in an automated plate reader ELX 808 (Bio-Tec instruments) pre-warmed at 37°C. Absorbance was measured at 550 during 2 h in 10-min intervals. O_2^- production in nmol was calculated using an extinction coefficient of 24.5 mM. Each condition was performed in triplicate.

2.21 Patients and flow cytometry analysis

Peripheral blood mononuclear cells were obtained after informed consent from 10 patients with secondary syphilis infection (six males and four females, median age 37 years, range 29-52) and 10 controls by four-colour flow cytometry. Controls were sex- and age matched healthy volunteers. Sample size was kept small according to the guidelines of the ethical committee. All clinical investigations were conducted according to the principles expressed in the Declaration of Helsinki.

PBMCs were isolated from fresh EDTA-treated blood by density gradient centrifugation using Ficoll-Paque solution. Regulatory T cells were defined as $CD4^+$ T cells expressing Foxp3. The phenotype of this cell subset was examined *ex vivo* in PBMC and after

antigen specific stimulation. Cells were stained for surface and intracellular markers with the following antibodies for flow-cytometry: anti-CD4-PECY7, anti-CD25-PECY5, anti-Foxp3-FITC, anti-TGF-β-PE. To evaluate the intracytoplasmatic concentration of TGF-β, according to commercial instructions, samples were stimulated with 25 ng/ml PMA plus 1 mg/ml ionomycin in the presence of brefeldin A (1 mg/ml). 1 x 10^6 cells were washed with 2 ml of PBS and stained for surface markers by incubation with the appropriate antibody for 30 min at 4°C. Cells were then washed with 2 ml of PBS and resuspended in 250 μl of Cytofix/Cytoperm solution (BD Biosciences) for 20 min at 4°C. Permeabilized cells were washed with 2 ml of Perm/Wash Buffer (BD Biosciences) and stained for intracellular Foxp3 and TGF-β at 4°C for 30 min. After intracellular staining, cells were washed with 2 ml of Perm/Wash Buffer and resuspended in 250 μl PBS. For each sample, a minimum of 50,000 $CD4^+$ events were acquired. Data acquisition was performed in a FACScan flow cytometer using the Diva software program (Becton Dickinson).

2.22 Isolation of $CD14^+$ and of $CD4^+CD25^{+high}$ cells and generation of DCs

$CD14^+$ and $CD3^+CD4^+CD25^+$ T cells were selected from PBMCs by immunomagnetic cell sorting (Miltenyi Biotec, Bisley, Germany).
Dendritic cells (DC) were generated by culturing purified $CD14^+$ cells in medium supplemented with 5% FCS, 50 ng/ml GM-CSF, and 20 ng/ml IL-4. On day 7, cells were analyzed by flow cytometry for CD1a, CD14, CD86, CD80, and HLA-DR expression.

2.23 Antigen-specific T-cell lines

$CD4^+CD25^{+high}$ cells, isolated from peripheral blood of patients with secondary syphilis and from healthy donors, were stimulated with autologous DC (1:1 ratio) loaded or not with TpF1 (150 nM) in the presence of recombinant IL-2 (100 U/ml). After 7 days they were assessed for the expression of Foxp3 and TGF-β after stimulation with TpF1 (150 nM) plus anti-human CD28 (1 μg/ml) and anti-human CD49d (1 mg/ml) for 36 h, being the last two mAbs added in the presence of brefeldin A (5 μg/ml).

2.24 Statistical analysis

Data were reported as mean values ± SD. Statistical significance was calculated by unpaired Student's t-test. A probability (p) of less than 0.05 was considered significant

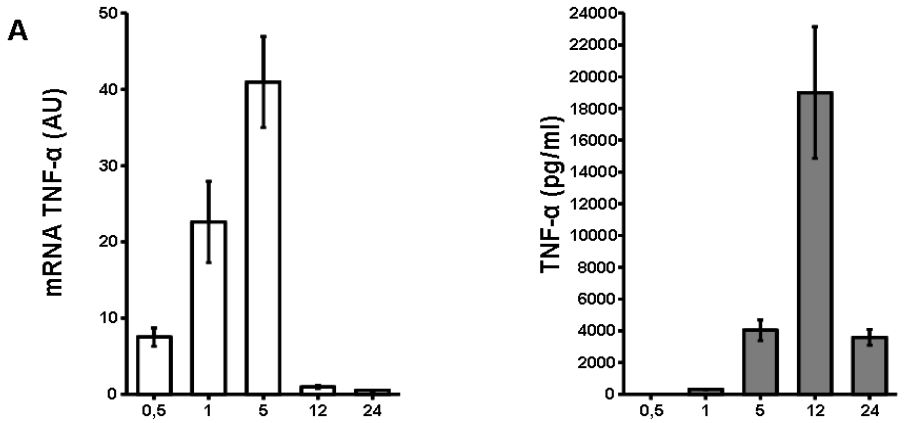
and was indicated with *, a $p < 0.01$ was indicated with ** and a $p < 0.001$ was indicated with ***.

Results

3.1 TpF1 activates monocytes to release pro-inflammatory cytokines

The tissue destruction characteristic of syphilis infection is probably caused by inflammation due to *T. pallidum* and the ensuing immune responses to the pathogen. *T. pallidum* membrane proteins are thought to be potent inducers of inflammation during the early stages of infection (Sellati *et al.*, 1999). However, either the specific membrane proteins or other secreted products crucial in triggering inflammation, are not yet identified. The protein TpF1, also called 4D antigen, TpN19 or TP1038, has been described as a periplasmic antigen (Radolf *et al.*, 1986); however, considering that it induces an important seroconversion particularly in primary syphilis patients (Coates *et al.*, 1986), it is conceivable that a quote of TpF1 is somehow released by the bacterium. Therefore, we wondered if TpF1 could contribute in causing the inflammation.

Firstly, monocytes isolated from buffy-coat of healthy donors were exposed to TpF1 1 μM at different time points; PBS (vehicle of TpF1) was used as negative control. The concentration of TpF1 adopted in our study was selected based on a dose response experiment (data not shown). Total RNA was extracted from monocytes, retrotranscribed and amplified by real-time PCR in the presence of primers specific for TNF-α, IL-6 and IL-1β. All these three cytokines play a key role in mediating acute and chronic inflammatory reactions (Feghali & Wright, 1997). The amount of mRNA for these three cytokines was markedly increased (Fig. 5 A-C, left panel) and, though at different extent, their expression showed a peak at 5 h before rapidly decreasing. In terms of temporal dynamics, the accumulation of cytokines in culture supernatants perfectly matched the kinetics of cytokines mRNA levels (Fig. 5 A-C, right panel).



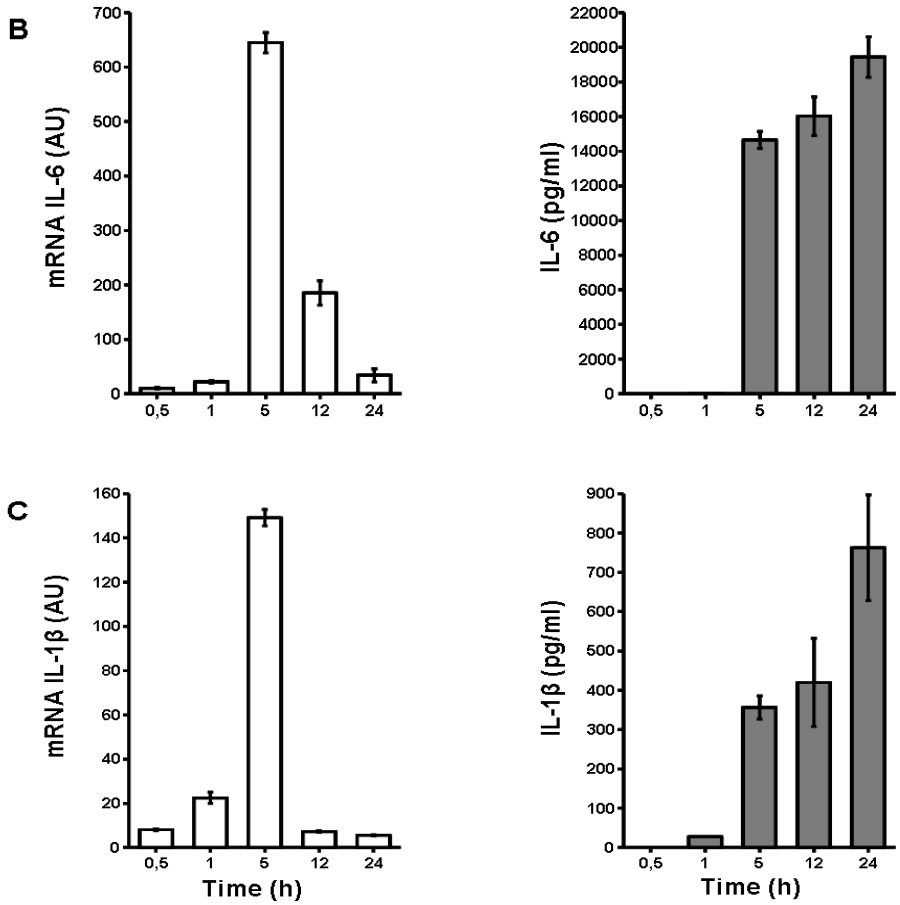


Fig. 5 Kinetics of TNF-α, IL-6 and IL-1β synthesis and production in monocytes stimulated with TpF1. TNF-α, IL-6 and IL-1β mRNAs were determined by quantitative Real time PCR at the indicated time points after TpF1 administration (1 μM) (A-C, left panels). TNF-α, IL-6 and IL-1β protein levels were measured in the culture supernatants of the same monocytes (A-C, right panels) harvested for mRNA evaluation. The amount of cytokine mRNA was expressed as n fold of the normalized amount of mRNA from untreated cells (1 AU = mRNA cytokine concentration [fmol/μl]/mRNA GAPDH [fmol/μl]). Figure shows the mean values ± SD of duplicate assays for each time point, obtained from three experiments conducted with different cell preparations, under the same conditions.

Based on these premises, we were interested in elucidating the signalling triggered by TpF1 ending with the secretion of IL-1β. IL-1β is produced as inactive form of 31 kDa (pro-IL-1β) and subsequently is processed in a mature form of 17 kDa (IL-1β), which is then released by an unconventional pathway, probably mediated by vesicles (Andrei *et al.*, 1999). Human monocytes were treated with TpF1, PBS as negative control and LPS, as positive control. LPS, the bacterial endotoxin, is the best characterized TLR-ligand and its ability to induce IL-1β production is well documented (Dinarello, 1998). After 3 and 18 h of stimulation, cells were collected and lysed. Lysates were quantified by means of BCA assay and 5 μg of each cell extract were loaded on a 4-12% SDS-PAGE. The amounts of

the immature and the mature form of IL-1β were analyzed by immunoblotting using polyclonal antibody anti-human IL-1β.

We found that, already after 3 h of stimulation and even more after 18 h, TpF1 is able to induce the synthesis of pro-IL-1β at levels comparable to those of the positive control, LPS (Dinarello, 1998); as expected, in the cell lysed before stimulation (T0) and cells non-stimulated (PBS) we did not detect any form of IL-1β (Fig. 6). To exclude that the different level of expression of pro-IL-1β at 3 h with respect to 18 h was not due to different loading of total proteins, we analysed the expression of actin, a housekeeping gene that is expressed independently of treatment (Fig. 6).

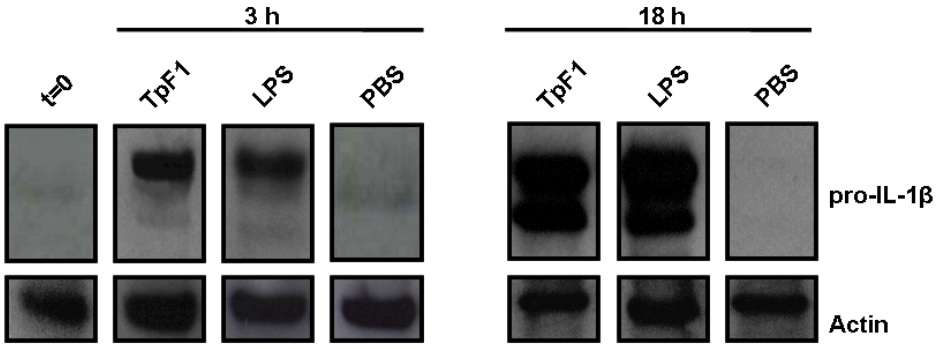


Fig. 6 Production of pro IL-1β in TpF1-stimulated monocytes.
Monocytes were cultured in absence (PBS) or presence of TpF1 (1μM) or LPS (1μg/ml). After 3 and 18 h, the monocytes were lysed; cell proteins were separated by SDS PAGE and analysed by immunoblotting. Specific antibodies were used to reveal pro-IL-1β and actin. T0 corresponds to cells harvested before the application of stimuli.

3.2 TpF1 induces IL-1β release

In addition to the cell extract (see Paragraph 3.1), we also evaluated the release of IL-1β into the supernatants of cells. Supernatants from stimulated and non-stimulated monocytes were collected (500 μl total for each sample); 50 μl were used for IL-1β quantification by a specific ELISA assay, whereas the rest was precipitated with 10% of trichloroacetic acid (TCA) and the total pellet was resuspended in LDS and loaded onto a 4-12% SDS-PAGE. We found that TpF1 induces the release of IL-1β (Fig. 7), like LPS does (Dinarello, 1998). IL-1β was undetectable in monocytes untreated (T0) or treated with PBS (Fig. 7A). The data were confirmed by means of ELISA assay (Fig. 7B).

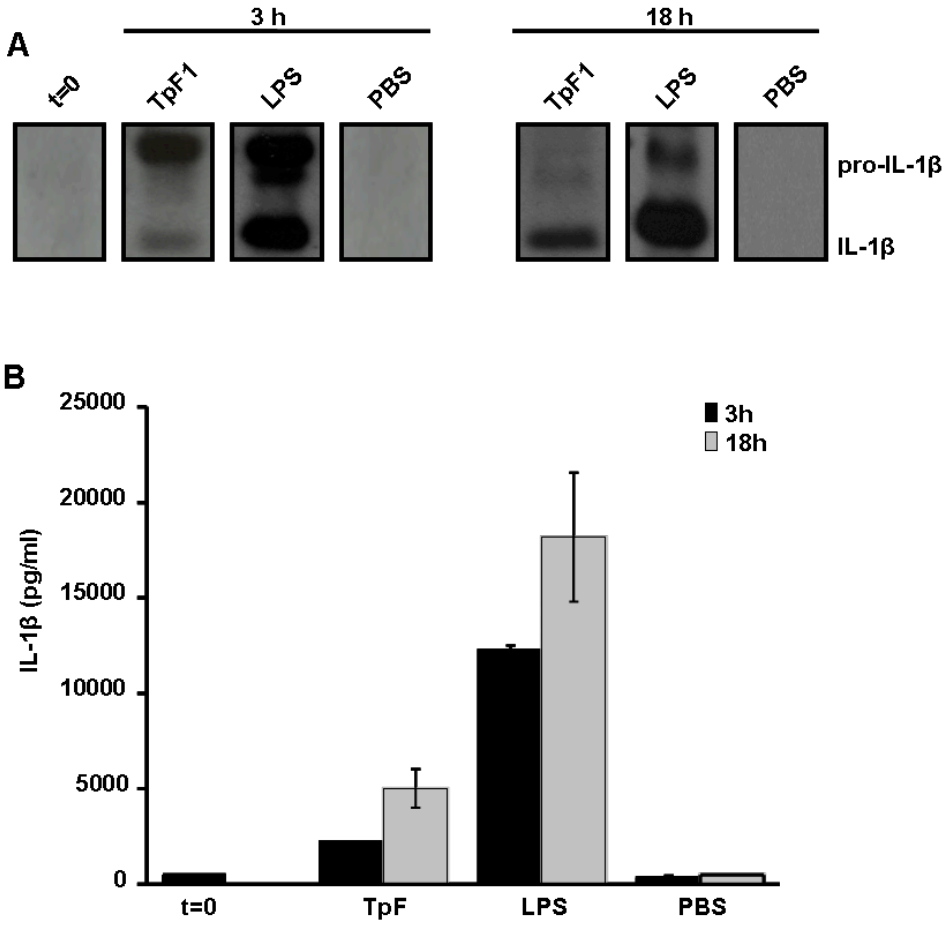


Fig. 7 Production of pro IL-1β in TpF1-stimulated monocytes.

Monocytes were cultured in absence (PBS) or presence of TpF1 (1 µM) or LPS (1 µg/ml). After 3 and 18 h, the supernatants were collected. IL-1β accumulation in the culture supernatant was assessed by western Blot (A) and ELISA assay (B).

3.3 TpF1-induced ATP secretion leads to the release of the pro-inflammatory cytokine IL-1β

On the basis of the ability of TpF1 to promote the secretion of IL-1β, we were interested to elucidate the signalling triggered by the bacterial protein leading to the secretion of the cytokine. Indeed, biologically active IL-1β can be released from its cytosolic precursor by the cystein protease caspase-1, which is, in turn, activated by the assembly of inflammasome. In order to confirm that TpF1 induces the release of IL-1β via this pathway, monocytes were exposed to TpF1 in the presence or absence of the caspase-1 inhibitor Ac-YVAD-CMK (Thornberry *et al.*, 1992) and the intracellular content of pro-IL-1β, as well as the secretion of the mature cytokine, were evaluated after 18 h by immunoblot and ELISA assay. As expected, the inhibitor of caspase-1 repressed the processing of IL-1β but not its secretion. Indeed, in the cell treated with TpF1 plus inhibitor the amount of pro-IL-1β was similar to that of TpF1-treated cells (Fig. 8A). On the

contrary, in the supernatants, the presence of inhibitor resulted in the accumulation of the immature form of the cytokine (Fig. 8B).

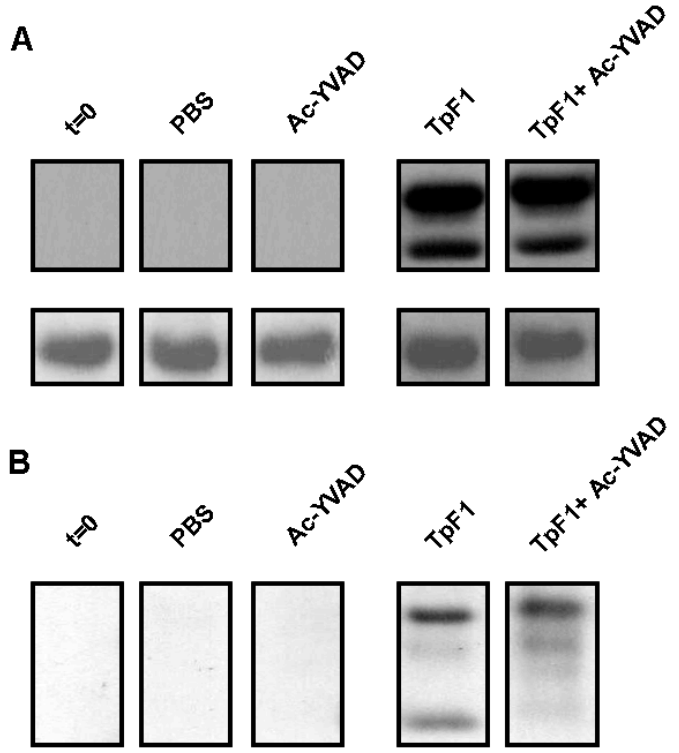


Fig. 8 IL-1β production after blocking of caspase-1.

Monocytes were stimulated 18 h with TpF1 (1 µM), in absence or presence of Ac-YVAD-CMK (50 µM). Cell lysates (A) and supernatants (B) were analyzed for the presence of pro-IL-1β and IL-1β by immunoblot. One experiment of three independent is shown.

It is now widely accepted that extracellular ATP is crucial for an efficient IL-1β conversion and secretion, in virtue of a series of events that follow its binding to the $P2X_7$ receptor ($P2X_7R$) (Ferrari *et al.*, 2006). For the activation of the inflammasome, human monocytes only require the stimulation with either TLR2 and TLR4 ligands, resulting in the release of endogenous ATP and processed IL-1β.

We wondered whether TpF1 was able to induce the release of the mature form of IL-1β via the secretion of endogenous ATP. To answer this question, we investigated whether TpF1 was able to induce the release of ATP from monocytes. We treated monocytes with TpF1 for different time points, collected the supernatants and measured the release of ATP by Luciferase assay. We found that TpF1-stimulation was rapidly followed by ATP release. Moreover, in the presence of an inhibitor of ectoATPase (ARL) (Karczewska *et al.*, 2007), the levels of ATP detected in supernatants at the different time points was increased, accordingly with the balance between the ATP release and the ATP hydrolysis by extracellular ectonucleotisades in extracellular medium (Fig. 9). The

amount of extracellular ATP was only partially reduced by the inhibitor at 2 h (as compared to 30 min and 6 hours); this time point probably represents the moment of the maximum nucleotide secretion by the cells.

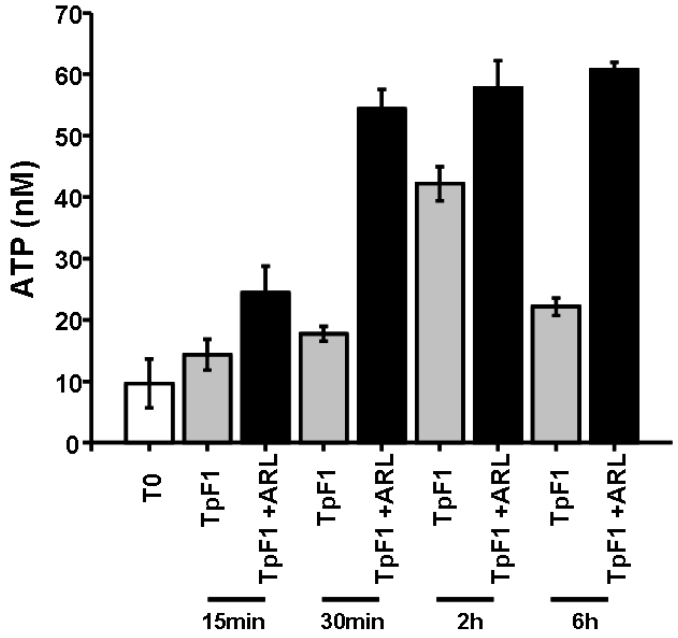


Fig. 9 Endogenous ATP release by TpF1-treated monocytes induced.

Human monocytes were cultured for different time points in the absence or presence of TpF1 (1 μM) and ARL (200 μM). ATP level in supernatants was determined with using ATP determination kit (ATPlite PerkinElmer) and is expressed as nM of ATP secreted per $3 \cdot 10^5$ cells. The values of untreated cells were subtracted from those of TpF1 stimulated cells.

To investigate the role of endogenous ATP in the TpF1-induced inflammasome activation, we treated monocytes for 18 h with TpF1 1 μM in presence or absence of oATP 300 μM, a $P2X_7$ receptor irreversible antagonist. In the presence of oATP, the mature form of IL-1β was absent in the extracellular medium (Fig. 10B and 11), according to the role of ATP in driving inflammasome assembly and ensuing caspase-1 activation. Differently from what we observed with caspase-1 inhibitor, the immature form was also absent in extracellular medium in the presence of oATP, suggesting a role of ATP also in the exocytosis of IL-1β-containing vesicles (Andrei *et al.*, 2004; Ferrari *et al.*, 2006). Notably, the reduction of both forms of IL-1β in the culture supernatant is not accompanied by an increased accumulation of pro-IL-1β in the cell cytoplasm; a reduced NF-κB activation, due to oATP (Di Virgilio, 2003), is probably responsible for a reduction of pro-IL-1β synthesis (Fig. 10A).

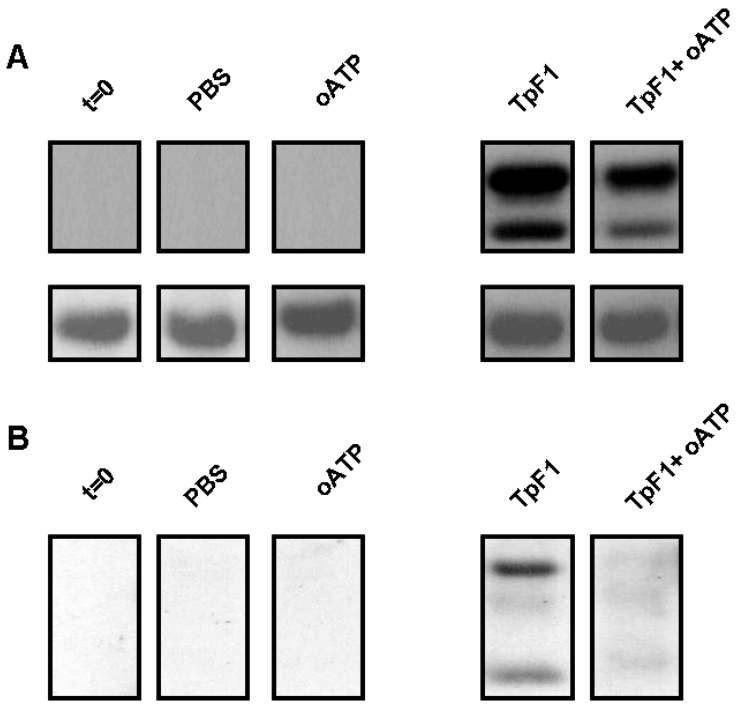


Fig. 10 IL-1 β production after blocking of P2X7R.

Monocytes were stimulated 18 h with TpF1 (1 µM), in absence or presence of oATP (300 µM). Cell lysates (A) and supernatants (B) were analyzed for the presence of pro-IL-1β and IL-1β by immunoblot. One experiment of three independent is shown.

Data on Fig. 8 and Fig. 10 were also confirmed by ELISA assay (Fig. 11).

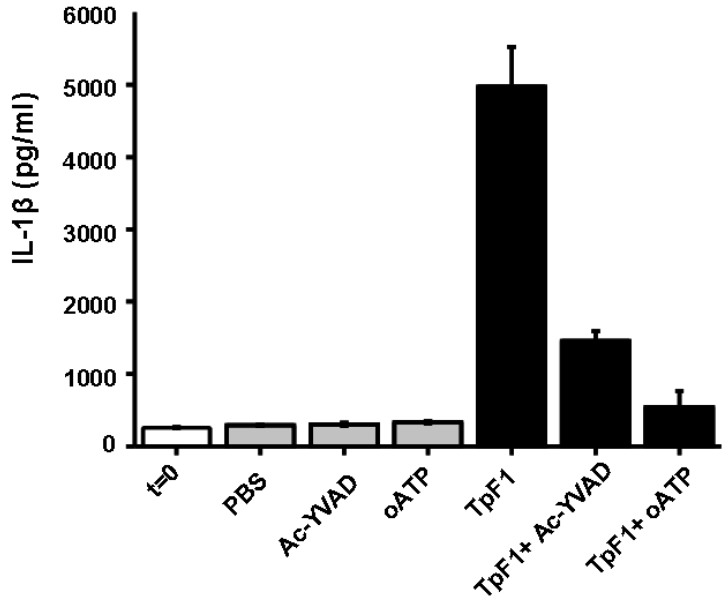


Fig. 11 IL-1β production after blocking of caspase-1 and P2X$_7$R.

Monocytes were stimulated 18 h with TpF1 (1 µM), in absence or presence of Ac-YVAD-CMK (50 µM) or oATP (300 µM). Supernatants were analyzed for the presence of pro-IL-1β and IL-1β by ELISA assay.

3.4 TpF1 does not induce generation of ROS

The role of reactive oxygen species (ROS) in the activation of inflammasome is controversial. It has been reported that ROS induce the NF-κB activation and different studies *in vitro* proposed that the inflammasome activation could be triggered by ROS (Stutz *et al.*, 2009; Zhou *et al.*, 2010). On the contrary, some investigation reported the anti-inflammatory effect of NADPH oxidase and ROS (Schappi *et al.*, 2008). Based on these observations and considering that there could be a crosstalk among the different mechanisms of inflammasome activation, we wondered whether TpF1 could be able to trigger the assembly of inflammasome also via ROS production.

In order to evaluate the ability of TpF1 to induce ROS production, we treated the monocytes at different time points with TpF1 at two different concentrations, 1 and 10 μM, and with β-glucan, as positive control. β-glucan, a lipopolysaccharide of yeast wall, is known to induce the respiratory burst. We measured the production of anion superoxide (O_2^-) by the cytocrome C reduction assay at different time points. We found that, as expected, upon β-glucan stimulation, the production of O_2^- was markedly increased compared to basal level, with a trend showing significant increases over time. Vice versa, the production of O_2^- induced by TpF1 did not change from basal level remaining comparable to that of the negative control. The same result was obtained even when the concentration of TpF1 applied was ten times higher (Fig. 12).

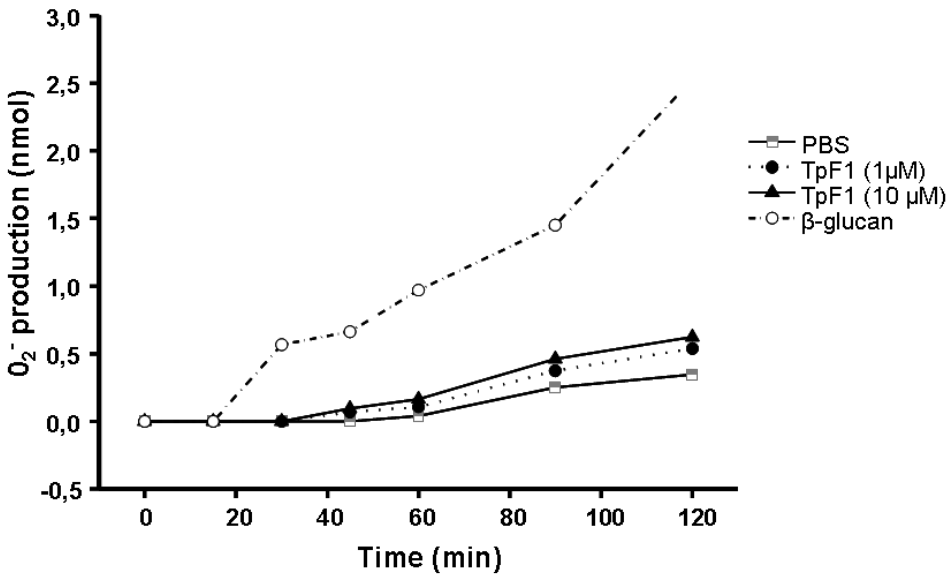


Fig. 12 Kineticof O_2- production in response to TpF1.

Monocytes were cultured in medium supplemented with cytochrome C (80 μM) and were stimulated in the absence or presence of TpF1 (1 and 10 μM) or β-glucan (50 μg/ml). Reduction of cytochrome C was measured every 10 minutes at 550 nm following addition of stimuli.

3.5 TpF1 prevents apoptosis in monocytes

In vitro human monocytes undergo spontaneous apoptosis, which could be prevented by proinflammatory cytokines, such as TNF-α, IL-1β and granulocyte-monocyte-colony-stimulating factor (GM-CSF) (Flad *et al.*, 1998). Considering that TpF1 induces the expression of TNF-α and IL-1β in monocytes (see Paragraph 4.1) and that monocytes exposed to TpF1 survived longer that untreated monocytes (our observation), we hypothesized a role for TpF1 in monocytes survival. To test this hypothesis, we monitored the percentage of apoptotic cells in presence or absence of TpF1 at different time points: 24, 48, 72 h, 5 and 7 days.

At the indicated time points cells were collected and labelled with Annexin V- FITC and propidium iodide. Annexin V staining clearly revealed that, while untreated monocytes progressively underwent apoptosis after 24 h and were all died after 5 days, the administration of TpF1 to cells significantly protected them from apoptosis (Fig. 13).

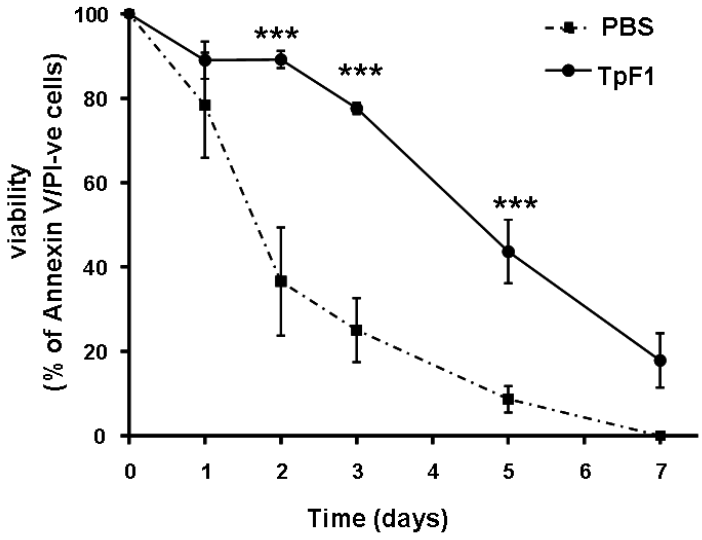


Fig. 13 Monocytes viability after TpF1 treatment.

Human monocytes were treated with TpF1 (1 μM) or PBS (vehicle). At the indicated time points cells were collected and labelled with Annexin V-FITC and propidium iodide. Viability was determined cytofluorimetrically and expressed as the percentage of Annexin V/PI-negative cells. Data represent mean ± SD of three independent experiments. Significance was determined by Student's t-test for paired data of TpF1-treated cells versus untreated cells; ***$P < 0.001$.

Subsequently, we investigated whether TpF1 promotes the survival of monocytes via induction of anti-apoptotic genes. After 24, 48 and 72 h of TpF1 stimulation cells were collected and lysed, and cells extracts were analysed by immunoblot (Fig. 14). We evaluated the activation of caspase-3 and poly-ADP-ribose polymerase (PARP), two well known markers of apoptosis activation. Caspase-3 is a crucial executioner caspase for the apoptotic process, originating from the cleavage of pro-caspase-3. We evaluated the level of both forms upon TpF1 stimulation. Among the caspase-3 targets there is PARP, the cleavage of which causes the irreversible nuclear alteration. To evaluate the activation of caspase-3 and PARP we used polyclonal antibodies able to recognize both the inactive and active forms. As expected, upon TpF1 treatment pro-caspase-3 and PARP remained uncleaved, suggesting that TpF1 prevents the apoptotic program. Vice versa, PBS treatment resulted in cleavage of both proteins (Fig. 14) as early as after 24 h.

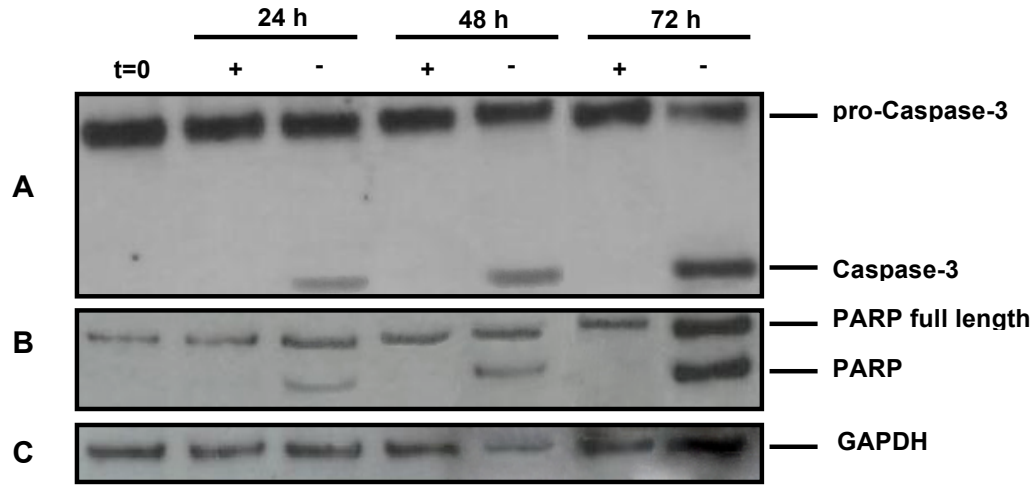


Fig. 14 TpF1 prevents caspase-3 and PARP activation.

Monocytes were cultured in absence (-) or presence of TpF1 (+) 1 μM and were lysed at indicated times. Equal amounts of cell extracts were separated by SDS PAGE and immunoblotted. Caspase-3 and PARP were revealed with specific polyclonal antibodies; an anti-GAPDH monoclonal antibody was used as control for equal loading. Cell lysate at time 0 (t=0) was considered as untreated baseline condition.

To corroborate the fact that TpF1 prevents the activation of caspase-3 and PARP and therefore slows the naturally occurring apoptotic process, we analysed by immunoblot the expression of two anti-apoptotic proteins: Mcl-2 and Bcl-X_L.

Both proteins regulate mitochondrial homeostasis blocking the release of cytocrome c and caspase-9 activation. The latter, as caspase-8, activates the effector caspases, which, in turn, have an effect on their targets, such as caspase-3. The expression of Mcl-2 and Bcl-X_L progressively decreased in untreated cells; this decrease

reflected the increase in number of apoptotic cells. On the contrary, the expression of Mcl-2 and Bcl-X_L remained unchanged in the presence of TpF1 (Fig. 15).

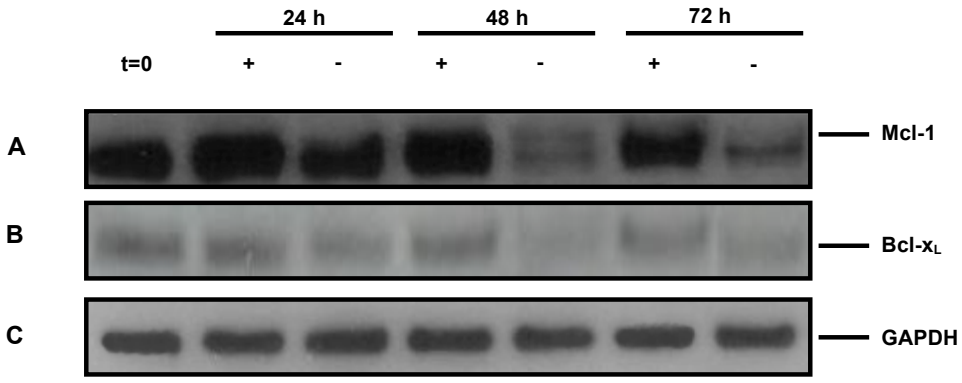


Fig. 15 TpF1 induces the expression of anti-apoptotic genes.

Monocytes were cultured in absence (-) or presence of TpF1 (+) 1 µM and were lysed at indicated times. Equal amounts of cell extracts were separated by SDS PAGE and immunoblotted. Mcl-1 (A) and Bcl-xL (B) expressions were revealed by specific antibodies; anti-GAPDH monoclonal antibody was used as control for equal loading (C). Cell lysate at time 0 (t=0) was considered as untreated baseline condition.

3.6 TpF1 antibody response in patients with syphilis

The importance of TpF1 in the antibody response to syphilis was already suggested several years ago by Coates and colleagues, who focused their evaluation on sera from primary syphilis patients, and demonstrated that 55% of sera reacted with TpF1 (Coates *et al.*, 1986). We wanted to expand this observation and verify whether TpF1 might be an immunodominant antigen also during the progression of the disease. For this purpose, we tested by ELISA whether serum samples from *T. pallidum* infected patients with secondary syphilis contained specific antibodies against this antigen. Among 30 patients, 20 of them (66%) showed circulating antibodies specific for TpF1, whereas in 37 healthy control subjects a minimal or no reactivity with this antigen was detected (Fig. 16).

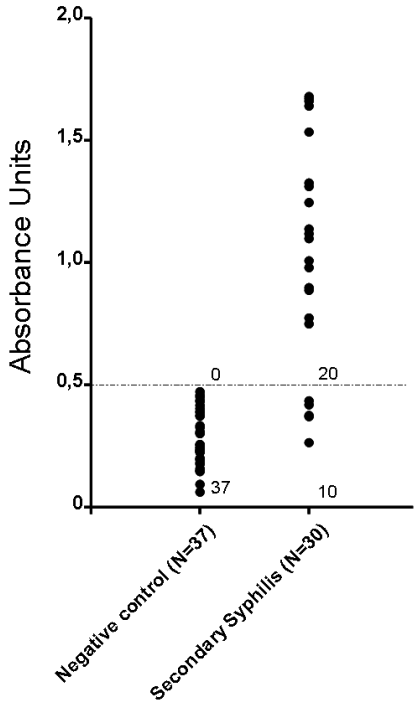


Fig. 16 TpF1 is immunogenic in patients with secondary syphilis.

The presence of TpF1-specific antibodies were determined with a specific ELISA assay in 37 patients with secondary syphilis and 30 healthy controls. TpF1-specific antibodies were detected in serum samples from 66% of the secondary syphilis patients. Healthy control subjects were seronegative for TpF1 and 0.5 absorbance units (horizontal line) was considered as threshold.

3.7 TpF1 activation of monocytes leads to release of cytokines essential for the differentiation of T regulatory cells

Several studies suggest that, during *Treponema* infection, lymphocytes may act to suppress the effect of macrophages (Tabor *et al.*, 1984; Tabor *et al.*, 1987). This strategy has been indicated as a possible mechanism for *Treponema* to escape the elimination by the immune system. This subset of lymphocytes, initially called T suppressor cells, are now known as T regulatory cells (T reg). The fully-differentiated T cell subtypes influence the immune system partially through the secretion of specific cytokines. Progenitor T cells (Th0) differentiate into functionally distinct T cell subsets, Th1, Th2, Th17 or T reg under the influence of specific cytokines. In the first stage of the disease, an increased expression of the Th1 cytokines IL-2, IFN-γ and TNF-α occurs. In secondary early syphilis the ability of Th1 lymphocytes to secrete cytokines slightly decreases, while the ability of cells to produce IL-6 and IL-10 increases. Finally, the late syphilis stage is characterized by high level of Th2 cytokines (IL-6 and IL-10) and low level of Th1 cytokines (Podwinska *et al.*, 2000). IL-10 and transforming growth factor-β (TGF-β) are two cytokines involved in the differentiation of T reg cells.

We wanted to evaluate the capacity of TpF1 to favour a cytokine milieu pushing towards the T regulatory phenotype. To this purpose, we treated the monocytes isolated from healthy donors with TpF1 1 μM for different time points; PBS was used as negative control. Total RNA was extracted from monocytes, retrotranscribed and amplified by real-time PCR in the presence of primers specific for IL-10 and TGF-β. Figure 17 A shows that TpF1 was able to induce the expression of IL-10 in monocytes and the kinetic of this cytokine expression was consistent with the accumulation of the protein in culture supernatant. In addition, being TGF-β crucial for the conversion of $CD4^+CD25^-$ T cells into $CD4^+CD25^+$ T suppressor cells (Chen *et al.*, 2003; Kretschmer *et al.*, 2005), we also assessed whether monocytes stimulated with TpF1 released TGF-β (Fig. 17 B).

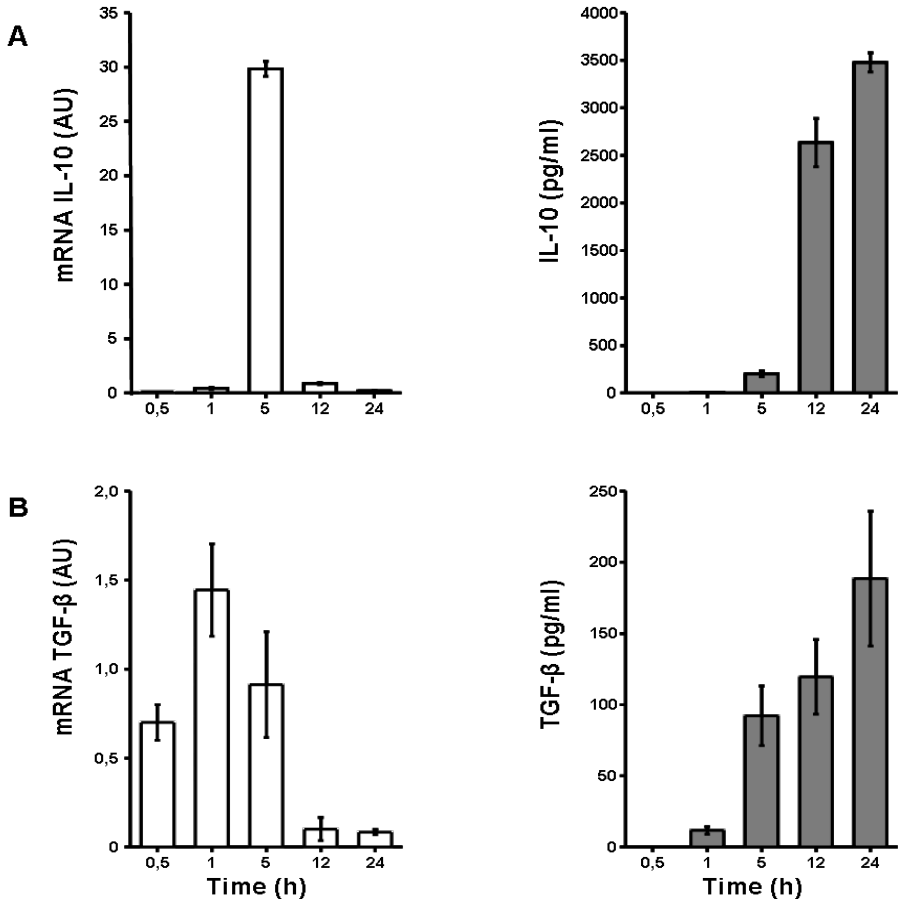


Fig. 17 Kinetics of IL-10 and TGF-β synthesis and production in monocytes stimulated with TpF1.
IL-10 and TGF-β mRNAs were determined by quantitative Real time PCR at the indicated time points after TpF1 administration (A-B, left panels). IL-10 and TGF-β protein levels were measured in the culture supernatants of the same monocytes (A-B right panels) harvested for messengers evaluation. The amount of cytokine mRNA was expressed as n fold of the normalized amount of mRNA from untreated cells (1 AU = mRNA cytokine concentration [fmol/μl]/mRNA GAPDH [fmol/μl]). Figure show the mean values ± SD of duplicate assays for each time point, obtained from three experiments conducted with different cell preparations, under the same conditions.

Figure 17 B shows that, although TpF1 weakly induced TGF-β mRNA expression, according to the post-transcriptional mechanism that governs its secretion (Grotendorst *et al*., 1989), accumulation of this cytokine in cell supernatant was clearly observed.

In conclusion, we have demonstrated that, upon TpF1 stimulation, peripheral blood monocytes produce IL-10 and TGF-β; these cytokines are expected to promote a T reg immune response.

3.8 Evaluation of $CD4^{+}CD25^{+high}$ cells in the peripheral blood of patients with secondary syphilis

We evaluated whether a portion of circulating T cells from patients with secondary syphilis were T regulatory cells. For this purpose, $CD3^{+}$ $CD4^{+}CD25^{+}$ T cells were purified by immunomagnetic cell sorting from peripheral blood mononuclear cells (PBMCs) of 10 patients (six males and four females, median age 37 years, range 29-52) and 10 healthy donors (sex and age matched healthy volunteers). Isolated cells were stimulated with PMA and ionomycin and evaluated for the internal expression of forkhead box P3 (Foxp3), a transcription factor known to be crucial for T reg cells development and function (Sakaguchi, 2005) and for TGF-β production (Fig. 18 A and B).

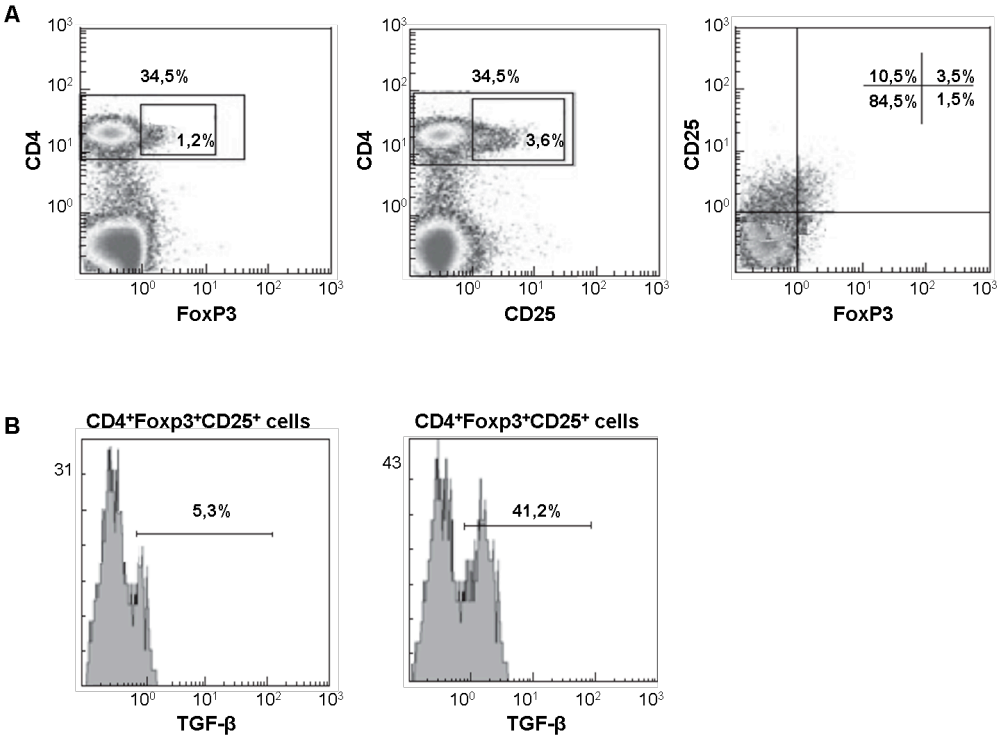


Fig. 18 Evaluation of CD4+CD25+high cells and TGF-β production in peripheral blood of patients with secondary syphilis.

(A) Dot plots showing expression of forkhead box P3 (Foxp3) (left), CD25 (middle) and co-expression of both CD25 and Foxp3 (right) on $CD4^{+}$ T cells in a representative *T. pallidum*-infected patient. (B) Histograms showing

the expression of TGF-β on T regulatory (Treg) cells, isolated from healthy donor (left) and *T. pallidum*-infected patient (right) and stimulated with PMA and Ionomycin.

As showed in Table 1, each *T. pallidum*-patient (P) showed a greater number of $CD4^{+}CD25^{+high}$ $Foxp3^{+}$ circulating cells than healthy donors (HD). The mean percentage of T reg cells in *T. pallidum*-infected patients was significantly higher than that recorded for healthy donors (3.66% vs 1.32% $p < 0.0001$). In addition, the percentage of $CD4^{+}CD25^{+high}$ $Foxp3^{+}$ cells freshly isolated from peripheral blood and producing TGF-β, after mitogen stimulation, was greater in P than in HD (40.97% vs 5.08% $p < 0.0001$) (Table II).

Subject	% of $CD4^{+}$ $CD25^{+high}$ $FoxP3^{+}$ cells		% of $CD4^{+}$ $CD25^{+high}$ $FoxP3^{+}$ $TGFβ^{+}$ cells	
	P	HD	P	HD
1	3.70 ± 0.14	1.00 ± 0.14	42.50 ± 0.99	5.10 ± 0.28
2	4.20 ± 0.14	1.20 ± 0.14	43.40 ± 0.99	5.40 ± 0.14
3	2.70 ± 0.14	1.50 ± 0.14	40.90 ± 1.98	5.70 ± 0.14
4	3.45 ± 0.07	1.00 ± 0.14	40.85 ± 0.49	4.80 ± 0.42
5	4.05 ± 0.21	1.35 ± 0.07	38.20 ± 0.99	5.20 ± 0.42
6	3.00 ± 0.28	1.40 ± 0.28	39.45 ± 1.20	5.00 ± 0.28
7	4.40 ± 0.28	1.40 ± 0.14	37.50 ± 0.14	5.45 ± 0.21
8	3.90 ± 0.28	1.20 ± 0.14	42.90 ± 0.42	4.10 ± 0.28
9	3.70 ± 0.28	1.95 ± 0.21	41.85 ± 0.64	5.10 ± 0.28
10	3.45 ± 0.21	1.20 ± 0.14	42.10 ± 0.99	4.90 ± 0.28
Mean	**3.66 ± 0.53**	**1.32 ± 0.28**	**40.97 ± 2.00**	**5.08 ± 0.44**

Table II

Percentage of fresh T reg cells producing TGF-β in peripheral blood of patients with secondary syphilis (P) and healthy donors (HD). Values represent the mean ± SD obtained from two independent experiments.

3.9 TpF1 promotes T reg response and TGF-β production

To evaluate whether TpF1 was able to increase TGF-β production, the $CD4^{+}CD25^{+high}$ cells isolated from peripheral blood of P and HD were stimulated with autologous dendritic cells (1:1 ratio) loaded or not with TpF1 (150 nM) in the presence of IL-2 (100 U/ml). After 7 days $CD4^{+}CD25^{+high}$ cells were assessed by cytofluorimetric analysis for the intra-cytoplasmatic expression of Foxp3 and TGF- β after mitogen stimulation.

Data summarized in Figure 19 clearly show that in none of the HD it was possible to identify T reg cells following TpF1 stimulation. In contrast, in all P the percentage of T reg cells, obtained after the induction of TpF1-specific T cell lines, was increased

compared to that of cells from fresh peripheral blood (9.1 vs 3.7 $p < 0.0001$). Furthermore, it is worthy of note that in TpF1-induced cell lines the percentage of T reg cells producing TGF-β, after stimulation with PMA and ionomycin, was significantly increased (76.4 vs 41 $p < 0.0001$). Thus, we can conclude that TpF1 not only promotes the proliferation of T reg cells but it also increases the production of TGF-β.

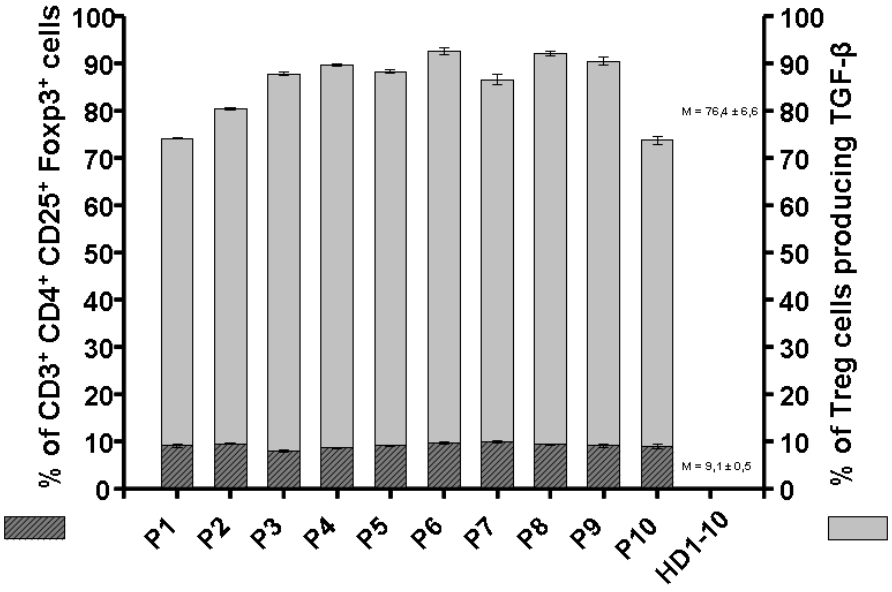


Fig. 19 TpF1 promotes T reg ($CD3^+CD4^+CD25^+Foxp3^+$) response and TGF-β production in patients with secondary syphilis.

Percentage of T reg cells in TpF1-induced T cell line (dashed grey); (M) mean: 9.1%. TGF-β production in patients with secondary syphilis (P) and healthy donors (HD). TGF-β production of T reg cells induced by TpF1, following mitogen activation (light grey); (M) mean: 76.4.

Discussion

Syphilis is a chronic disease caused by *Treponema pallidum*. This disease still constitutes an important global health problem and it remains a major sexually transmitted disease.

Up to now, a vaccine to prevent the syphilis has not been developed yet. One of the main reasons for this lack is the difficulty to determine the virulence factors of *T. pallidum*, because of its peculiar characteristics, such as a striking lack of metabolic capabilities, sensitivity to oxygen and temperature (Lafond & Lukehart, 2006). Our study was focused on TpF1, an oligomeric protein of 190 KDa localised in the periplasm of *T. pallidum*. Although TpF1 was identified and purified as early as in 1984 (Fehniger *et al.*, 1984), its function remained unknown. We have selected TpF1 as a potential candidate to have a role in syphilis for different reasons. In the literature, it has been reported that the *intra vena* administration of TpF1 in subsequently treponema-challenged rabbits reduced the mean incubation periods for lesion development (Borenstein *et al.*, 1988), suggesting a role for this protein in inducing an immune response. Moreover, TpF1 belongs to the Dps-like protein family and it is homolog to two proteins of this family, HP-NAP from *Helicobacter pylori* and NAPA from *Borrelia Burgdorferi.* These proteins have been studied in our lab and their pro-inflammatory and immune-modulatory properties have been demonstrated to be crucial in sustaining chronic diseases (Amedei *et al.*, 2006; Codolo *et al.*, 2008). Therefore, the aim of our study was to verify whether TpF1 has a role in triggering inflammation and, eventually, in down-modulating the adaptive immune response, thus permitting the survival of the bacterium in the host.

Firstly, we examined the pro-inflammatory potential of TpF1 and we demonstrated that the antigen activates monocytes to express and release the pro-inflammatory cytokines IL-1β, IL-6 and TNF-α, suggesting that TpF1 may contribute to inflammation. Considering that TpF1 induces the production of IL-1β, we investigated the molecular mechanisms by which TpF1 promotes this effect.

IL-1β is one of the crucial mediators of inflammation and a defective control of its release may cause serious diseases. This cytokine is produced as inactive precursor and it is subsequently processed into its mature form by caspase-1. The latter is activated following the assembling of the multiprotein complex, called inflammasome. The process of IL-1β secretion requires two signals. Firstly, the gene expression and the synthesis of the IL-1β precursor (pro-IL-1β) are induced by inflammatory signals, such as PAMPs (Dinarello, 1998). Then the second signal, a danger signal, such as ATP exogenous or ROS, induces inflammasome activation: this event allows the activation of caspase-1 and subsequent conversion of pro-IL-1β into its mature form. ATP release from monocytes is followed by autocrine stimulation of the purinergic receptor $P2X_7$ ($P2X_7R$), which activates

the pannexin-1 hemichannel, resulting in K^+ efflux and increase in the concentration of cytosolic Ca^{2+}. Potassium efflux is considered as the crucial signal for inflammasome activation and IL-1β release (Andrei *et al.*, 2004).

We were interested to elucidate the signalling triggered by TpF1 leading to IL-1β secretion. We found that caspase-1 inhibitor prevented IL-1β maturation, but not the release of pro-IL-1β release, in agreement to the fact that caspase-1 is not involved in secretion. We then measured the ATP release by Luciferase assay and we observed that TpF1-stimulation is immediately followed by ATP release; moreover, in the presence of the inhibitor, the level of ATP detected in supernatants was increased, accordingly to the balance between the ATP release and the ATP hydrolysis by extracellular ectonucleotisades. Subsequently, we blocked the activation of $P2X_7R$ with oATP, an antagonist of the receptor and we found that in the presence of oATP both the mature and immature form were absent. This confirms that extracellular ATP is crucial not only for the conversion of IL-1β via the activation of inflammasome, but also for the secretion of the cytokine.

Based on the observation that there could be a crosstalk among the different mechanisms of inflammasome activation, we investigated whether TpF1 could trigger the assembly of inflammasome also via ROS production (Stutz *et al.*, 2009). While the production of O_2^- upon β-glucan (positive control) stimulation was markedly increased compared to basal level, the production of O_2^- induced by TpF1 did not change as compared to the basal level, suggesting that TpF1 does not induce ROS generation.
In this first part of our work, we demonstrated that TpF1 induces endogenous ATP release in monocytes. The engagement of $P2X_7$ receptor by ATP results in the activation of inflammasome, leading, as a final effect, to the release of IL-1β.

One of the main factors that could end the exacerbation of the inflammation is the natural apoptotic death of innate immune cells. Considering the capacity of TpF1 to induce the production of pro-inflammatory cytokines in monocytes, such as TNF-α and IL-1β, which are known to prevent the spontaneous apoptosis (Flad *et al.*, 1998), we evaluated whether TpF1 contributes to inflammation increasing the lifespan of monocytes. We observed that TpF1 delayed apoptosis of monocytes with respect to untreated cells. In addition, we found that TpF1-exposed monocytes did not undergo activation of caspase-3 and poly-ADP-ribose polymerase (PARP), an executioner caspase and one of its substrates, respectively. In parallel, $Bcl\text{-}X_L$ and Mcl-1, two anti-apoptotic proteins, progressively decreased in untreated cells, while remained unchanged in the presence of TpF1. Considering that we observed that TpF1 induces the inflammasome activation and prevents the apoptosis, we can conclude that TpF1 could be crucial in causing early inflammation.

Despite the fact that a strong specific immune response is evoked after

establishment of bacterial infection, in absence of antibiotic treatment *T. pallidum* can survive in the host and the syphilis evolves into chronic disease. Many mechanisms have been suggested to ensure the continued existence of the microorganism in the host. Several years ago Tabor and colleagues suggested that *T. pallidum* infection induces the activation of “suppressor T cells”, which suppress the macrophages’ activity (Tabor *et al.*, 1984; Tabor *et al.*, 1987). This strategy has been indicated as a possible mechanism for *T. pallidum* to escape the elimination by the immune system and explains the fact the disease becomes chronic with a possible latent stage. This subset of lymphocytes, initially called T suppressor cells, are now known as T regulatory cells (T reg). We have demonstrated that, upon TpF1 stimulation, peripheral blood monocytes produce IL-10 and TGF-β; these cytokines are, in turn, able to promote a T reg phenotype in cells. This result is of particular interest in view of the fact that secondary syphilis is a systemic disease and that the presence of T regulatory cells has been suggested in syphilis patients. Until now, the involvement of these cells in syphilis remained an unexplored issue. In this work, we reported for the first time that $CD4^{+}CD25^{+high}$ T cells are increased in the peripheral blood of patients with secondary syphilis, compared to healthy donors. Furthermore, these cells are Foxp3 positive and are able to produce TGF-β. Moreover, we demonstrated that TpF1 is able to promote T reg response and TGF-β production from T cells obtained from patients with secondary syphilis.

In conclusion, in this work, for the first time, the function of TpF1 from *Treponema pallidum* has been described. These functional elucidations are important, especially if we consider the low knowledge of virulence factors of this dangerous pathogen, *Treponema pallidum*, that causes 12 million new cases of syphilis each year.

We have demonstrated that, in one hand TpF1 promotes and probably sustains the inflammation, whereas, on the other side, TpF1 counteracts the immune response via induction of T regulatory cells. We can explain these two opposite effects on the immune response taking into account the different stages of the disease. We hypothesize that TpF1 could exert its pro-inflammatory activity at the onset of the disease, when the infection is confined in the specific region. Subsequently, when the disease progresses becoming systemic, TpF1 helps to overcame the immune response activity promoting T reg phenotype; indeed, we have demonstrated the presence of T reg lymphocytes in peripheral blood of secondary syphilis patients.

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