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1 RIASSUNTO DELL'ATTIVITA' SVOLTA

Borrelia burgdorferi è l'agente eziologico della malattia di Lyme. La malattia di Lyme può essere causata da tre specie di *Borrelia* correlate tra loro: *B. burgdorferi*, *B. afzelii* and *B. garinii*. In Europa la malattia è causata da tutte e tre le specie, mentre, in America, *Borrelia burgdorferi* è l'unica causa.

La malattia di Lyme è una patologia multisistemica e può essere suddivisa in tre stadi: il primo è caratterizzato da un'infezione cutanea localizzata a livello del sito del morso della zecca; nella fase successiva, le spirochete disseminano in diversi tessuti, e l'ultimo stadio è caratterizzato dalla cronicizzazione dell'infezione. La malattia di Lyme ha inizio con una caratteristica lesione cutanea che, nelle ore e giorni successivi al morso, si espande, motivo per cui questo stadio della malattia viene chiamato *Eritema migrans*. Nelle settimane successive le spirochete disseminano in diversi siti. Dopo mesi, soprattutto nei pazienti che non vengono trattati con terapia antibiotica, l'infezione può cronicizzare causando artrite che colpisce le articolazioni, in particolare le ginocchia (artritie di Lyme).

Scopo di questo progetto è quello di contribuire alla comprensione dei meccansimi molecolari e cellulari coinvolti nello sviluppo dell'artrite. E' oramai stabilito che la risposta immunitaria dell'ospite nei confronti di *Borrelia burgdorferi* influenza quella che sarà la manifestazione clinica dell'infezione. In particolare, fino a qualche anno fa si riteneva che i linfociti T di tipo Th1, secernenti IFN-γ, fossero il tipo cellulare principalmente coinvolto nell'artrite di Lyme. Recentemente , utilizzando un modello murino, è stato dimostrato che i linfociti Th1 non erano essenziali nell'indurre l'artrite di Lyme, suggerendo quindi il coinvolgimento di altri tipi cellulari e di mediatoridiversi da IFN-γ e IL-12 prodotti dalle cellule Th1. L'attenzione si è focalizzata su un nuovo sottotipo di linfociti T helper: i linfociti Th17. E' riportato che queste cellule giocano un ruolo importante nell'induzione di danno tissutale in malattie autoimmuni attraverso la secrezione di interleuchina (IL)-17. La IL-17 induce il rilascio di varie citochine e chemochine ad attività pro-infiammatoria da parte di cellule stromali, sinoviociti, condrociti, fibroblasti e macrofagi, inoltre, sono in grado di reclutare neutrofili. Un'importante osservazione è costituita dal fatto che la IL-17 si trova a livelli elevati nei liquidi sinoviali di pazienti con artrite di Lyme, dove è in grado di

promuovere la formazione di osteoclasti e diverse osservazioni sperimentali suggeriscono che IL-17 possa essere coinvolta nella patogenesi dell'artrite di Lyme.

Non era ancora noto, tuttavia, quali fossero i fattori batterici coinvolti nell'induzione di una risposta immunitaria Th17 mediata.

In precedenza, nel nostro laboratorio, era stata caratterizzata una proteina prodotta da *Helicobacter pylori*, batterio che nell'uomo provoca infezione cronica, così come è in grado di fare *Borrelia burgdorferi*, chiamata HP-NAP (*Helicobacter pylori* Neutrophil Activating Protein). Si tratta di una proteina fortemente immunogena dotata di specificihe proprietà immunomodulanti. Anche Borrelia possiede una proteina simile ad HP-NAP la cui unica caratterizzazione funzionale aveva portato alla dimostrazione che fosse essenziale per la persistenza della spirocheta nella zecca.

Sulla base dell'omologia con HP-NAP abbiamo voluto verificare se anche il prodotto del gene bb0690 di Borrelia e codificante per la proteina NapA potesse giocare un ruolo nello sviluppo dell'artite di Lyme attraverso una specifica modulazione del sistema immunitario.

In una prima fase dello studio abbiamo verificato la presenza di anticorpi specifici contro NapA nei sieri di pazienti affetti da artrite di Lyme, mentre, nei pazienti con *eritema migrans* o paralisi facciale, manifestazioni cliniche che caratterizzano gli stadi precoci della malattia, raramente sono stati trovati anticorpi contro NapA.

Successivamente, considerando l'emergente idea che i linfociti Th17 avessero un ruolo cruciale nello sviluppo dell'artrite di Lyme, abbiamo deciso di valutareo il profilo funzionale di linfociti T isolati da pazienti con artrite di Lyme e la eventuale loro specificità verso la proteina NapA. Abbiamo così dimostrato che nella cavità sinoviale di pazienti affetti da artrite di lyme ci sono linfociti Th17 specifici per NapA; inoltre, l'aggiunta della proteina ai suddetti linfociti opportunamente stimolati in vitro, si traduceva nella produzione di IL-17. Sulla base di questi risultati siamo passati a valutare se la capacità di NapA di polarizzare i linfociti T helper verso il profilo Th17, fosse il risultato dell'induzione di un particolare mielieu ricco in IL-6, IL-1β, TGF-β e IL-23, note citochine che insieme contribuiscono al differenziamento e alla proliferazione dei linfociti Th17. Utilizzando neutrofili e monociti umani come modello

sperimentale, in quanto abbondantemente rappresentati nella cavità sinoviale in corso di infiammazione, abbiamo dimostrato che NapA è capace di indurre in queste cellule una maggiore sintesi e rilascio di IL-6, IL-1β, TGF-β e IL-23.
Una forte risposta infiammatoria a *Borrelia* è una caratteristica di tutti gli stadi della malattia di Lyme. A dare inizio a questa risposta infiammatoria è l'attivazione dell'espressione di geni dovuta al legame delle componenti batteriche ai *Toll-like receptors* (TLRs). Per tale ragione, al fine di indagare il meccanismo di attivazione dei monociti da parte di NapA, abbiamo considerato la possibilità che questa proteina attivasse un *signaling* mediato da un TLR.
Abbiamo dimostrato che NapA è un ligando del TLR2, e che questo recettore è coinvolto nell'attivazione dei monociti da parte di NapA; questo dato è stato supportato dall'osservazione che l'espressione e produzione di IL-23 veniva abrogata in presenza di un anticorpo specifico bloccante il TLR2.
Complessivamente i dati da noi ottenuti hanno dimostrato che NapA è un fattore di virulenza di *Borrelia burgdorferi* coinvolto nella patogenesi dell'infiammazione Th17 mediata dell'artrite di Lyme (Codolo G. et al., 2008).

Sebbene le sequenze di NapA e HP-NAP abbiano il 28% di identità, esse hanno diverse attività immunomodulanti; la prima induce una risposta di tipo Th17, la seconda invece promuove risposte di tipo Th1.
L'allineamento tra NapA e HP-NAP ha mostrato che NapA è 13 aminoacidi più lunga all'estremità N-terminale e 20 aminoacidi più lunga all'estremità C-terminale. Sulla base di tali differenze, abbiamo voluto valutare se le proprietà pro-Th17 di NapA risiedessero in queste due porzioni della proteina. Sono state prodotte due proteine mutanti, una priva dei 13 residui all'N-terminale e una senza 20 aminoacidi al C-terminale, e abbiamo valutato le loro proprietà pro-Th17. A tale scopo è stata analizzata l'espressione di IL-6, IL-1β, TGF-β e IL-23 in monociti trattati con NapA a diversi tempi. Inaspettatamente, le due proteine delete hanno mostrato avere una capacità addirittura maggiore, rispetto a NapA intera, di indurre la sintesi delle citochine prese in esame.
Inoltre, siamo andati a vedere se le due proteine mutate fossero ancora in grado di legare il TLR2; per fare questo abbiamo analizzato l'induzione di IL-23 in presenza di uno specifico anticorpo bloccante il TLR2. Abbiamo osservato che la

presenza dell'anticorpo bloccante riduce in modo significativo la capacità di NapA e delle due proteine delete la capacità di promuovere la sintesi e il rilascio di IL-23, suggerendo che la porzione di proteina responsabile del legame con il TLR2 e dotata di attività immunomodulante non sia localizzata alle estremità (Codolo G. et al. Submitted).

L'artrite di Lyme è caratterizzata dal progressivo danneggiamento dell'articolazione, che è mediato da diversi meccanismi. L'erosione della cartilagine e dell'osso è associata alla formazione del panno sinoviale proliferante. Il processo infiammatorio è caratterizzato dall'infiltrazione di cellule infiammatorie nell'articolazione, che portano alla proliferazione dei sinoviociti simil-fibroblasti (*fibroblast-like synoviocytes*, FLS), e alla distruzione della cartilagine e dell'osso.
L'extravasazione dei neutrofili nell'articolazione è uno dei primi step chiave nello sviluppo dell'infiammazione delle articolazioni, come dimostrato utilizzando un modello murino.
La migrazione dei leucociti nella sinovia è un processo multistep finemente regolato che coinvolge l'interazione tra leucociti e cellule endoteliali e le molecole di adesione di superficie, e anche tra leucociti e chemochine e recettori per le chemochine. Il tessuto sinoviale e il liquido sinoviale di pazienti affetti da artrite di Lyme, così come in altre patologie legate ad artriti croniche, contengono elevate concentrazioni di chemochine. Il sistema delle chemochine si ritiene essere implicato nella patogenesi dell'artrite di Lyme attraverso il reclutamento di neutrofili, monociti e linfociti T nelle articolazioni.
L'ultima parte del lavoro che abbiamo svolto aveva lo scopo di capire se NapA fosse in grado di reclutare cellule infiammatorie ed immunitarie. Innanzitutto abbiamo dimostrato, utilizzando un modello di artrite indotta in ratto, che NapA è in grado di reclutare neutrofili e linfociti T capaci di produrre IFN-γ. Inoltre, abbiamo osservato che la proteina è in grado di indurre aumento dell'espressione di chemochine, quali CCL2, CCL20 e CXCL1, coinvolte nel reclutamento di neutrofili e linfociti T. Tali cellule rappresentano il maggior infiltrato di cellule infiammatorie richiamate a livello delle articolazioni di pazienti affetti da artrite di Lyme.

Sulla base dei risultati ottenuti da questi esperimenti, abbiamo voluto capire quale fosse il ruolo di NapA nel reclutamento di neutrofili e linfociti T. Da esperimenti preliminari, abbiamo osservato che la proteina NapA non è in grado, da sola, di reclutare tali cellule. Queste osservazioni suggeriscono che probabilmente NapA non possiede un'attività chemotattica diretta, perciò è possibile ipotizzare che NapA agisca in modo indiretto inducendo la produzione di fattori in grado di richiamare le cellule al sito di infezione.

In particolare, sulla base delle chemochine accumulate nella cavità sinoviale dei ratti trattati con NapA, abbiamo ritenuto fosse interessante valutare quali tipi cellulari, attivati da NapA, fossero coinvolti nella produzione di queste chemochine.

Abbiamo condotto degli esperimanti preliminari mirati a valutare la produzione di chemochine da parte di macrofagi e neutrofili; sono state scelte queste cellule come modello sperimentale perché i macrofagi sono cellule normalmente residenti in sinovia e quindi in grado, una volta attivati, di produrre molecole pro-infiammatorie coinvolte nel reclutamento di altre cellule al sito di infezione; per quanto riguarda i neutrofili, invece, è noto che essi sono le prime cellule ad essere richiamate in gran numero nella cavità sinoviale di pazienti affetti da artrite di Lyme.

Dai primi dati ottenuti, sembra che entrambi i tipi cellulari siano in grado di produrre chemochine responsabili in particolare del reclutamento di neutrofili e linfociti T. Si tratta di un aspetto molto interessante che dovrà essere indagato più in dettaglio. Sembrerebbe che questi dati siano in accordo con quanto riportato da Pellettier e colleghi, che hanno recentemente dimostrato che neutrofili attivati in presenza di IFN-γ e LPS producono chemochine, quali CCL2 e CCL20, in grado di reclutare linfociti Th17. Inoltre, hanno dimostrato che neutrofili attivati sono in grado di reclutare linfociti Th1 attraverso il rilascio di CXCL10 e CCL2. Un altro interessante aspetto messo in evidenza in questo lavoro, è che i linfociti Th17 possono, a loro volta, richiamare neutrofili attraverso il rilascio di CXCL8.

Dunque, come proposto da Pellettier et al., l'interazione tra neutrofili e Th17 potrebbe creare un loop pro-infiammatorio che aumenta il locale accumulo di neutrofili e linfociti T helper in un contesto di malattie infiammatorie. E' possibile ipotizzare che tale loop si possa verificare anche nella risposta

infiammatoria che caratterizza la patogenesi dell'artrite di Lyme. Al fine di verificare questa ipotesi, stiamo facendo esperimenti finalizzati alla valutazione del fenotipo dei linfociti T helper reclutati attraverso l'endotelio, sotto lo stimolo di supernatanti di coltura che derivano da neutrofili o macrofagi stimolati con NapA.

2 SUMMARY

Borrelia burgdorferi is a spirochetal bacterium responsible for the human Lyme disease, a multisistemic illness transmitted by ticks. This pathology was described for the first time at the beginning of nineteens. Lyme borreliosis is due to three related species *B. burgdorferi, B. afzelii and B. garinii*; in Europe the disease is caused by the three species whereas in the U.S.A. *B. burgdorferi* is the sole cause. Lyme disease is a multisistemic illness and can be divided in three stages: the first one is characterized by a localized infection of the skin in the site of tick's bite, the second one is the dissemination of the spirochetes and the last stage consists in a persistent infection. Lyme disease usually begins with a characteristic expanding skin lesion named erythema migrans. After days or weeks, the spirochetae may spread to different sites of the host involving heart, muscles and nervous system causing different clinical manifestation. Months later, in untreated patients, the infection could chronicize causing arthritis, affecting joints, in particular knee.

The aim of this project is to contribute in understanding which are the molecular and cellular mechanisms involved in the development of arthritis. Now it is established that the host immune response to *B. burgdorferi* influences the clinical outcome of the infection. In particular, until a few years ago, T lymphocytes, particularly IFN-γ secreting Th1 cells, were proposed to play a central role in Lyme arthritis. Recently, it was demonstrated, using a mouse model, that Th1 cells were not essential in inducing Lyme arthritis, suggesting the involvement of mediators different from IFN-γ and IL-12; consequentially a different T cell subsets is supposed to be involved in the pathogenesis of the desease. The attention was focused on a recent subset of T helper lymphocytes: Th17 cells. Th17 cells play a crucial role in the induction of autoimmune tissue injury via IL-17 release. IL-17 induces the release of several pro-inflammatory cytokines and chemokines by stromal cells, synoviocytes, chondrocytes, fibroblasts and macrophages, and recruits and activates neutrophils. Moreover, IL-17 is elevated in synovial fluids of patients with rheumatoid arthritis, where it promotes formation of osteoclasts and several experimental observations suggest its involvement in the pathogenesis of murine Lyme arthritis.

A major unanswered question remained that of the chemical nature of the bacterial factor(s) responsible for the induction of Th17 cells. Previously, in our laboratory, we have characterized a protein produced by *Helicobacter pylori*, a bacterium that causes chronic infections in humans, as *B. burgdorferi* does, termed HP-NAP (*H. pylori* Neutrophil Activating Protein). This protein is a strong immunogen and is endowed with immunomodulatory properties. Borrelia burgdorferi produces a protein which is homologue to HP-NAP, and the sole functional characterization had shown to be a protein essential for the persistence of spirochetae within ticks.

On the basis of this homology with HP-NAP, we decided to verify whether the product of the gene *bb0690* of *Borelia* encoding for the protein NapA could play a role in the development of Lyme arthritis through a specific modulation of immune system.

In a first set of experiments we verify the presence of specific anti-NapA antobodies in serum samples from patients with Lyme arthritis, whereas, in samples from patients with erythema migrans and facial palsy, caracteristic of the early phase of the disease, anti-NapA specific antobodies were rarely detected.

Successively, considering the important role of Th17 cells in the development of Lyme arthritis, we decided to examine the functional profile of synovial T cells from patients with Lyme arthritis, and their specificity toward NapA. We have demonstrated that in synovial cavity of patients with Lyme arthritis there are Th17 lymphocytes NapA specific; moreover, when these cells, appropriately stimulated, were exposed to NapA in vitro, they were able to produce IL-17. On the basis of these observations, we evaluated whether the ability of NapA to polarize T helper lymphocytes toward a Th17 phenotype, could be the result of a cytokine environment rich in IL-6, TGF-β, IL-1β and IL-23, tipically involved in the differentiation and proliferation of Th17 cells.

To this aim we used neutrophils and monocytes as experimental model, cells which are recruited in the synovial cavity during inflammation, and we have demonstrated that NapA is able to induce upregulation of synthesis of IL-6, TGF-β, IL-1β and IL-23 in these cells.

A robust inflammatory response to *B. burgdorferi* is the hallmark of both early-stage (erythema migrans) and middle and late-stage (arthritis)

manifestations of Lyme disease. These inflammatory responses are likely initiated by the expression of genes activated by the binding of borrelial components to Toll-like receptors (TLRs). To investigate the mechanism of the NapA activation of monocytes, we evaluated the possibility that NapA activates a TLR-mediated signalling. We demonstrate that NapA is a TLR2 agonist able to activate NF-κB in HEK TLR2-transfected cells, and the involvement of TLR2 receptor in the activation of monocytes by NapA is also supported by the abrogation of IL-23 expression by a specific anti-TLR2 blocking antibody.

Altogether these results suggest that NapA is a *B. burgdorferi* virulence factor involved in the pathogenesis of Th17 inflammation in human Lyme arthritis (Codolo G. et al., 2008 Arthr andRheum).

Although NapA and HP-NAP sequences are identical to 28%, they are endowed with different immunological properties, being the former a pro-Th17 agonist and the latter an inducer of Th1-oriented responses.

The allignment between the protein NapA and HP-NAP from *H.pylori*, shows that NapA is 13 amino acids longer at the N-terminus and 20 amino acids longer at the C-terminus than HP-NAP. On the basis of these differences, we were concerned to investigate whether in these two portions of the molecule could reside the peculiar Th17-orienting property of NapA. To elucidate whether the immuno-modulating activity of NapA resides either in its N-terminal or in its C-terminal tail, we produced two NapA mutants lacking 13 residues at the N-terminus and 20 at the C-terminus, respectively, and assayed them for their Th17-orienting property. To this aim we analized the expression of IL-6, IL-1β, TGF-β and IL-23 in monocytes treated with NapA for different time points; surprisingly the exposure to both the deleted forms of NapA resulted in a even more pronounced induction of synthesis of these cytokines.

Finally, to further investigate whether NapA mutants retain the ability to engage the TLR2, which is crucial for the immuno-modulatory properties of the wild type protein, we evaluated the induction of IL-23 in the presence of a blocking anti-TLR2 antibody. The presence of the antibody significantly reduced the ability of NapA, both wt and mutant, to promote the synthesis and release of IL-23, suggesting that the portion of the protein responsible for the binding to the

receptor does not localize within the two N- and C-terminal tails (Codolo G. et al., submitted).

Lyme arthritis is characterized by progressive joint damage that is mediated by several mechanisms. Early erosion of cartilage and bone is associated with the formation of a proliferating pannus.

The inflammatory process is characterized by infiltration of inflammatory cells into the joints, leading to the proliferation of fibroblast-like synoviocytes (FLS) and the destruction of cartilage and bone.

Neutrophils extravasation into the infected joint is a key initial step in the development of joint inflammation, as reported in mouse model studies.

Migration of leukocytes into the synovium is a regulated multistep process involving interactions between leukocytes and endothelial cells and cellular adhesion molecules, as well as between leukocytes and chemokines and chemokine receptors. Synovial tissue and synovial fluid from Lyme arthritis patients, such as other chronic inflammatory arthritides, contain increased concentrations of several chemokines. The chemokine system is considered to be implicated in LA pathogenesis via the recruitment and retention of neutrophils, monocytes and T lymphocytes into the joints.

The last part of our work was aimed to understand whether NapA could be able to recruit immune cells involved in the inflammatory process. We first demonstrate, using a rat arthritis model, that NapA is able to recruit neutrophils and T lymphocytes able to produce IFN-γ. Moreover, analysis of cytokines and chemokines in synovial cavity of animals, reveals that NapA induces an increased production of chemokines such as CCL2, CCL20 and CXCL1, involved in the recruitment of neutrophils and T lymphocytes. These cells represent the major inflammatory cells recruited in joint of patients with Lyme arthritis..

On the basis of the results described above, we investigated the role of NapA in neutrophils and T lymphocytes recruitment. In a first set of experiments we observed that NapA, *per se*, is not able to recruit neutrophils and T lymphocytes. These observations suggest that, probably, NapA is not directly a chemotactict factor for immune cells. Therefore, it could be hypothesized that

NapA acts indirectly inducing the production of factors able to attract neutrophils and T lymphocytes to the site of infection.

Particularly, on the basis of CCL2, CCL20 and CXCL1 accumulation in synovial fluids of rats treated with NapA, we wondered which cells could be the source of these mediators.

We performed preliminar experiments aimed to evaluate the production of chemokines in macrophages and neutrophils, after NapA stimulation. We focused our attention on these cells because macrophages are resident cells in synovia, and for this reason, once activated, macrophages are able to produce pro-inflammatory molecules involved in the recruitment of other cells to the site of infection. Neutrophils, instead, are the first cells recruited in synovial cavity of Lyme arthritis-affected patients.

From our preliminary observations it seems that both cell types might be able to produce chemokines involved in neutrophils and T lymphocytes recruitment. This is an interesting observation that we will deeply investigate. These data could correlate with those reported by Pellettier and colleagues who have recently demonstrated that IFN-γ plus LPS-activated neutrophils release chemokines, such as CCL2 and CCL20 which, in turn, are responsible for the recruitment of Th17 cells. Moreover, they showed that activated neutrophils, *via* the release of CXCL10 and CCL2, may also trigger the chemotaxis of Th1 cells. Finally, they demonstrated that, upon activation, human Th17 cells can directly chemoattract neutrophils through the release of CXCL8.

Therefore, according to Pellettier *et al.*, neutrophils/Th cells interaction might create a pathogenic proinflammatory loop that ultimately amplifies the local accumulation of neutrophils and Th cells during inflammatory disease. We can speculate that such a loop could also occur during the inflammatory events in the pathogenesis of Lyme arthritis. In order to verify this hypothesis, we are currently performing experiments aimed to evaluate the phenotype of T lymphocytes recruited across a HUVEC-derived endothelium, under stimulation with supernatants harvested from either neutrophils or macrophages activated with NapA.

3 INTRODUCTION

In the late 20th century, Lyme disease, or Lyme borreliosis, was recognized as an important emerging infection (Steere, 2001). It is now the most commonly reported arthropod-borne illness in the US and Europe and was also found in Asia.

Lyme disease was recognized as a separate entity in 1976 because of geographic clustering of children in the Lyme, Connecticut, area who were thought to have jouvenile rheumatoid arthritis (Steere, 1989). It then became apparent that Lyme arthritis was a late manifestation of an apparently tick-transmitted, multisystemic disease, of which some manifestations had been recognized previously in Europe and America.

In 1981, Burgdorfer and collegue discovered previously unidentified spirochetal bacterium, called *Borrelia burgdorferi*, in a nymphal Ixodes scapularis tick (Burgdorfer *et al.*, 1982). This spirochaete was then cultured from patients with early Lyme disease, and patients' immune responses were linked conclusively with that organism, proving the spirochetal etiology of the infection (Steere *et al.*, 1983, Benach *et al.*, 1983).

Based on genotyping of bacteria isolated from ticks, animals and humans, the formerely designated *B. burgdorferi* has been suddivided into multiple *Borrelia* species, including three that cause human infection: *Borrelia burgdorferi sl*, *Borrelia afzelii* and *Borrelia garinii*.

3.1 Biology of *Borrelia burgdorferi*

The agents of Lyme borreliosis belong to the phylum of Spirochetes, unicellular spiral microorganisms without a rigid cellular wall; the length can range from 10 to 30 μm and the width of helices from 0,2 to 0,3 μm (Hayes *et al.*, 1983). About 7-14 flagella at each end of the bacterium are responsible for *Borrelia* mobility, they are localized in the periplasmic space (Figure 1).

The composition of the cell envelope is similar to Gram negative bacteria with some expressive differences, e.g. absence of lipopolysaccaride and an abundance of lipoproteins in the outer cell membrane. These outer surface

proteins include OspA, OspB and OspC; although the role of these Osps during infection is not known; different Osp genes are expressed at different time during transmission from the tick vector to the mammalian host (de Silva & Fikrig, 1997). Other important antigens of *B. burgdorferi* are the 60-kDa antigen, which has been termed "common antigen" and belongs to the heat-shock protein family, and the flagellin proteins – FlaA (38 kDa) and FlaB (41 kDa) – which constitute the flagella (Ge *et al.*, 1998).

Figure1: A scanning electron microscopy of B. burgdorferi spirochetes in the midgut of a nymphal I. scapularis tick (from (Steere *et al.*, 2004).

The *B. burgdorferi* (strain B31) genome has been completely sequenced (Fraser *et al.*, 1997); it has a linear chromosome in addition to twelve linear and nine circular plasmids, that constitute 40% of its DNA (Casjens *et al.*, 2000). A unique feature of *Borrelia spp*. is that the genes for the immunodominant outer-surface proteins OspA, OspB and OspC are located on plasmids. The flagellin protein and the 60-kDa antigen are not species-specific (Shapiro & Gerber, 2000).

3.2 Borrelia burgdorferi: epidemiology

Lyme disease is the most common vector-borne disease in the United States, where approximately 15.000 cases are reported every year. In the U.S., most of the cases of Lyme disease occur in southern New England, the Eastern part of the middle Atlantic states and the upper Midwest.
In Europe, most cases occur in the Scandinavian countries and in central Europe, especially Germany, Austria and Switzerland.
In endemic areas, the reported annual incidence is highest among children aged 5-10 years (Shapiro & Gerber, 2000).
In Italy, the first case of Lyme disease was reported in 1983. Lyme borreliosis has been detected particularly in northern Italy, mainly in the northeastern part (Friuli-Venezia Giulia region), where the number of cases of human Lyme borreliosis has increased annually. On the contrary, it seems to occur sporadically in central Italy (Cinco *et al.*, 1998).

3.3 Borrelia burgdorferi life cycle

Lyme disease is considered a zoonosis because the causative agent is maintained in a natural infectious cycle that does not include humans, who only inadvertently become infected. Ticks both acquire and transmit *B. burgdorferi* sensu lato (s.l.) by feeding on a variety of small mammals that act as reservoir hosts (Magnarelli & Anderson, 1988). *Borrelia burgdorferi* s.l. is transmitted by *Ixodes* ticks, mainly *Ixodes scapularis* and *Ixodes pacificus* in the United States, and *Ixodes ricinus* and *Ixodes persulcatus* in Europe and Asia (Lane *et al.*, 1991).

Ixodes ticks feed once at each of the three active stages – larval, nymphal and adult. Therefore, uninfected larval ticks generally acquire *B. burgdorferi* s.l. by feeding on infectious vertebrates, and uninfected mammals acquire spirochetes when fed on by infected nymphal ticks.

After the ingestion with the bloodmeal, spirochetes multiply in the midgut of the larval tick, where they persist through the moult to the nymphal stage. With few exceptions, the spirochaetes are restricted to the midgut and do not infect other tissues or organs in the ticks. When the nymphal tick feeds, spirochetes

again replicate and some leave the midgut and migrate to the salivary glands, from where they are transmitted by saliva. Only small numbers of spirochetes are transiently present in the salivary glands. *Ixodes* ticks feed slowly and take 3-7 days to complete a bloodmeal. Efficient transmission of *B. burgdorferi* from infected nymphs occurs after approximately 48 hours from ticks attachment (Piesman *et al.*, 1987).

The dynamics of *Borrelia* infection in the mammalian host have not been clearly defined because very low numbers of organisms are present in immunocompetent hosts and therefore the diffusion of the bacteria in the body is difficult to detect. However, it has been established in a mouse model of Lyme disease that *B. burgdorferi* initially causes a localized infection in the skin at the site of the tick bite, transiently disseminates via the bloodstream and subsequently establishes persistent infection in various tissues, including skin, joints, heart and bladder (Barthold *et al.*, 1991).

3.4 Genes expression and regulation during the *Borrelia burgdorferi* life cycle

B. burgdorferi has evolved remarkable abilities to survive in a wide range of organisms such as arthropods and vertebrates like birds and mammals. In contrast to other pathogenic bacteria, *B. burgdorferi* has devoted a large portion of its genome towards producing lipoproteins. It has been reported the ability of the bacterium to alter its surface structure by differential lipoprotein gene expression at various stages of its life cycle in mammal and ticks; this is likely a strategy aimed to host adaptation and immune evasion (Pal & Fikrig, 2003).

A few host molecules were also identified that are involved in interactions with spirochete ligands and thus aid in bacterial survivability in different host environments. In addition to the transcriptional activation of selective genes, events such as variable recombination also contribute to the alteration of spirochete structure. For example, the *B. burgdorferi vlsE* locus consists of an active telomeric expression site flanked by a number of upstream silent *vls* cassettes. Recombination events between the *vlsE* gene and *vls* cassettes occur in

mammals and most likely in feeding ticks, producing a genetically diverse population of spirochetes (Pal & Fikrig, 2003).

A series of in vitro studies have identified a number of environmental signals that are thought to regulate gene expression in spirochetes: temperature, pH, cell density and host factors. Interestingly, many of these regulated genes are differentially expressed in vivo, and include *hsp, mlp, dbpA, bbk32, erp, ospA and ospC*.

3.4.1 *Adaptation in ticks*

The tick environment is exposed to changes related to temperature and blood meal. Among the proteins expressed by *B. burgdorferi* in the tick, OspA is one of the best examples of an antigen with a definitive spatial pattern of expression (Anguita *et al.*, 2003).

OspA first appears on the surface of *Borrelia* during larval acquisition and subsequent colonization of the tick gut (Schwan & Piesman, 2000). The spirochaetes continue to produce abundand OspA during its persistence within the gut of the tick until the next blood meal and subsequent transmission back to a new host. OspA serves as an anchor for the spirochete that allows to bind and colonize the tick gut (Pal *et al.*, 2001).

When a tick starts feeding, many spirochetes downregulate *ospA* and upregulate *ospC*; while downregulation of *ospA* favors *Borrelia* exit from the gut, *ospC* is needed for their efficient migration from the gut via salivary gland and transmission to the new host (Schwan & Piesman, 2000).

3.4.2 *Adaptation in the host*

An important function that *Borrelia burgdorferi* needs to accomplish during infection of the mammalian host is the evasion from the immune responses.

Besides downregulation of antibody-targeted surface proteins, two other potential mechanisms allow the spirochetes to survive in their host in the presence of immune responses. These include a recombination system that allows the

spirochete to change antigenic determinants of a surface protein, VlsE, and the inhibition of complement-based phagocytosis (Anguita *et al*., 2003).

VlsE is a protein produced by *Borrelia* that is required for persistent mammalian infection. Although its function is unknown, this lipoprotein has an elaborate system for variation (Tilly *et al.*, 2008).

The complement cascade is an important part of the innate immune system as a barrier method to prevent the invasion of microorganism. The pathway activates with an initial deposition of the C3 protein to the pathogen surface, followed by several amplification loops, ultimately resulting in the formation of the membrane attack complex, which kills the pathogen.

Some of the Erp proteins, which belong to a family of surface-exposed lipoproteins, have been reported to be synthetized by *B. burgdorferi* during early mammalian infection. Studies have revealed that Erps contribute to the ability of Borrelia to infect mammals by blocking host complement-mediated killing. The mechanism of resistance to complement is mediated by the binding of two host-derived complement control proteins: factor H and factor H-like protein 1 (FHLP-1), with subsequent promotion of factor I-mediated degradation of C3 (Pal & Fikrig, 2003).

3.5 Clinical features of Lyme disease

After transmission of the spirochaetes, human Lyme disease generally occurs in stages, with remissions and exacerbations and different clinical manifestations at each stage. Early infection consistes of stage 1, characterized by localized infection of the skin, followed within days or weeks by stage 2. During this phase the spirochaetes disseminate to different site of the host, affecting muscle tissue, heart and nervous system. The third stage of the disease can occur months to years later, and it is characterized by a persistent infection. However, the infection is variable; some patients have only localized infection of the skin, while others have only later manifestations of the illness, such as arthritis. Moreover, there are regional variations, primarily between the illness found in America and that found in Europe and Asia (Steere *et al*., 2004).

3.5.1 *Erythema migrans*

Erythema migrans is the most important and frequent clinical sign of the early stage of Lyme borreliosis, affecting people of all ages and both sexes. Days to weeks after the infectious tick bite, a small red macula appears on the skin, usually at the site of the bite. As the red patch slowly enlarges, central clearing usually begins, resulting in a ring-like lesion. Untreated lesions persist and expand over days to several months. About half of adult European patients report local symptoms at the site of erythema migrans - usually mild itching, burning, or pain – whereas a smaller proportion has systemic symptoms such as fatigue and malaise, headache and muscle or joint pain, which are intermittent and vary in intensity and location. The proportion of patients with systemic symptoms is higher in the USA than in Europe. Solitary erythema migrans with systemic symptoms might indicate dissemination of the spirochaetes. Systemic dissemination is proven by appearance of additional, secondary skin lesions similar to primary erythema migrans, but cannot be excluded even in the absence of systemic symptoms (Stanek & Strle, 2003).

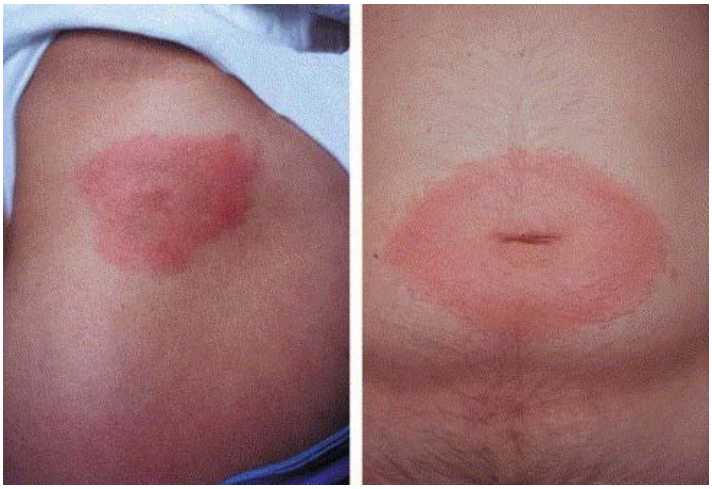

Figure2: Skin manifestations of Lyme borreliosis. Erythema migrans on the upper leg, about 2 weeks after tick bite and a week after onset of the lesion (left panel); erythema migrans expanding around the umbilicus; note central clearing (from (Stanek & Strle, 2003).

3.5.2 *Borrelial lymphocytoma*

Borrelial lymphocytoma, another manifestation of the early phase of the disease, is a solitary bluish-red swelling with a diameter of up to a few centimetres, consisting of a dense lymphocytic infiltration of cutis. The infiltration is polyclonal with a predominance of B lymphocytes, and often shows germinal centres. The lesion is most frequently located on the earlobe in children, and in the region of the mammary areola in adults. This rare manifestation arises later and is of longer duration than erythema migrans, but also resolves spontaneously. Most borrelial lymphocytomas arise in the vicinity of a previous or concurrent erythema migrans lesion (Strle *et al.*, 1992).

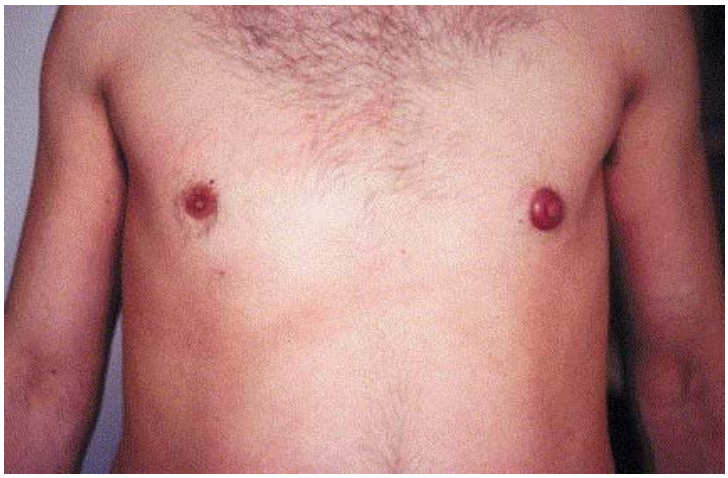

Figure3: Borrelial lymphocytoma on the left nipple; borders of erythema migrans can be seen on the thoracic skin above the nipples. from(Stanek & Strle, 2003).

3.5.3 *Acrodermatitis chronica atrophicans*

Acrodermatitis chronica atrophicans is a relatively frequent chronic skin manifestation of Lyme borreliosis that does not resolve spontaneously. It is most often located on the extensor sites of the hands and feet. Initially the lesion is usually unilateral; later on it may become more or less symmetrical. Previous other signs of Lyme borreliosis are rarely reported by patients with acrodermatitis chronica atrophicans. The onset is hardly noticeable; a slightly bluish-red discolouration and oedema appear on the dorsal part of a hand, foot or knee. The lesion enlarges very slowly over months to years, after which the oedema slowly

vanishes and atrophy becomes gradually prominent. The skin then becomes thin and wrinkled and the discolouration become violet. Veins become prominent and healing of skin damage is impaired. Pathohistological findings usually show a pronounced lymphocytes and plasma-cell infiltration of dermis, and sometimes subcutis, with or without atrophy (Stanek & Strle, 2003).

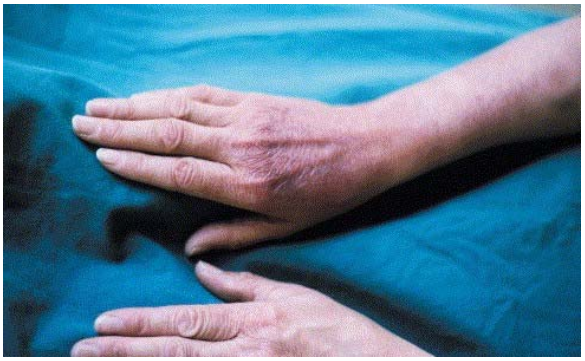

Figure4: Acrodermatitis chronica atrophicans on the dorsal side of the hands (from (Stanek & Strle, 2003).

3.5.4 *Involvement of nervous system*

Neuroborreliosis can arise at any time during the course of Lyme borreliosis. Usually, the most pronounced clinical symptom is pain as a result of radiculoneuritis. Patients have severe pain, usually in the thoracic or abdominal region. Motor nerves can be affected with paresis that is usually non-symmetrical.
Any cranial nerve can be affected in early neuroborreliosis, but facial nerves are most frequently involved, resulting in unilateral or bilateral peripheral facial palsy. Patients with this symptom often have lymphocytic pleocytosis, even when there is no sign or symptom of meningitis. Prognosis of borrelial peripheral facial palsy is good, even in patients who are not treated with appropriate antibiotics.
Up to 10% European patients develop features of disseminated encephalomyelitis that can resemble those seen in multiple sclerosis. Peripheral neuritis, with or without acrodermatitis chronica atrophicans, arises in several patients. Subtle encephalopathy has been reported predominantly in patients with American Lyme borreliosis (Logigian *et al.*, 1990).

3.5.5 *Lyme carditis*

Cardiac disease due to *Borrelia burgdorferi* tipically develops weeks to months later after infection and is usually manifested by a fluctuating degree of atrioventricular block which may cause the patient to complain of dizziness, palpitations, dyspnoea, chest pain or syncope. Pericarditis with effusion is less common. Carditis was reported in 8% of US patients before the widespread use of antibiotics for erythema migrans. In more recent series the incidence has been lower, in both the USA (<1%) and in Europe (<4%). Heart block is typically at or above the atrioventricular node, but can affect the entire cardiac conduction system (Nadelman & Wormser, 1998).

3.5.6 *Lyme arthritis*

In the United States, arthritis is the most common manifestation of late Lyme disease (LD). Arthritis is observed in about 60% of untreated or incompletely treated patients a few months after the onset of disease (Steere, 2001). Patients begin to have intermittent attacks of joint swelling and pain, primarily in large joints, especially the knee (Steere, 1989). Synovial tissue from affected patients shows synovial hypertrophy, vascular proliferation, and a marked infiltration of mononuclear cells, sometimes with pseudolymphoid follicles that are reminiscent of a peripheral lymph node (Steere *et al.*, 1988). During attacks of arthritis, innate immune responses to *B. burgdorferi* lipoproteins are found, and γ/δ T cells in joint fluid may aid in this response. In addition, there are marked adaptive immune responses to many spirochetal proteins (Chen *et al.*, 1999). A *Borrelia*-specific, inflammatory Th1 response is concentrated in joint fluid, but antiinflammatory (Th2) cytokines are also present. Furthermore, patients with Lyme arthritis usually have higher *Borrelia*-specific antibody titers than patients with anyn other manifestation of the illness, including late neuroborreliosis (Gross *et al.*, 1998b).

Recurrent inflammation may continue for weeks to months. If left untreated, many of these patients eventually develop an acute or subacute arthritis of one or both knee joints resembling, in an adult, septic arthritis, crystalline-

induced arthritis, or reactive arthritis. The affected joint may have a large effusion and the synovial fluid (SF) is inflammatory but nonpurulent. Virtually all untreated patients with LA have serum immunoglobulin G (IgG) antibodies to *Borrelia burgdorferi* by Western blotting. Culture of synovial fluid does not reveal *B. burgdorferi*, but *Borrelia* DNA can be demonstrated by polymerase chain reaction (PCR) in a majority of untreated patients with LA. Both oral and parenteral regimens have been used successfully to treat LA.

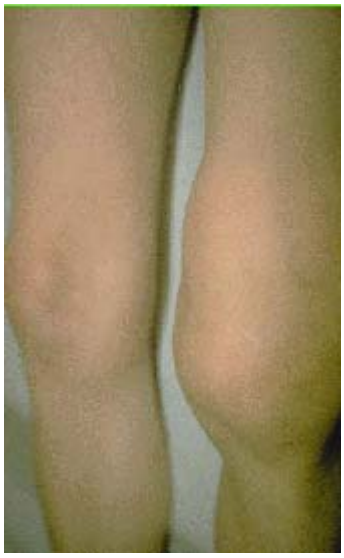

Figure 5: Lyme arthritis. This is an example of Lyme arthritis in the knee. Joints may become red, swollen, and painful.

3.5.7 *Post-Lyme sindrom*

A number of patients have persistent chronic complaints that last more than 6 months after previous treatment of Lyme borreliosis (Stanek & Strle, 2003).

These include musculoskeletal or radicular pain, dysaesthesia, and neurocognitive symptoms that are often associated with fatigue. This condition of unspecific persistent symptoms in association with a positive serology is termed ‘Post Lyme Disease (PLD) syndrome’. The PLD syndrome is reported to be much less common in Europe than in America. Clear diagnostic criteria for this condition are lacking (Steiner, 2003). Its pathogenesis is not known. A persistent (ongoing) infection or post-infectious immune-mediated mechanisms have been implicated. Others have questioned if the PLD syndrome is really an organic condition or

primarily a psychiatric disorder. Controlled trials investigating whether prolonged antibiotic treatment is effective for patients with the PLD syndrome showed that additional antibiotic therapy was not more beneficial than a placebo (Klempner *et al.*, 2001). However, due to the absence of accepted diagnostic criteria or biological markers, the interpretation of these data is limited by the heterogeneity of the patient group studied.

3.6 Immune response to *Borrelia burgdorferi* infection

The ability of *B. burgdorferi* to avoid clearance by the immune system may be attributed in part to an inadequate innate immune response upon infection. However, innate cells such as monocytes, macrophages, and dendritic cells display a vigorous response against borrelial infection and play a major role in the activation of the adaptive immune response against the spirochete. Despite these efforts, though, arthritis still develops in a significant number of patients infected with *B. burgdorferi*. It appears that the robust response elicited by monocytes, macrophages, and dendritic cells against the spirochete may be viewed as insufficient in early infection but excessive in the later stages. Paradoxically, in mounting such a strong defense against the organism, these cells may inadvertently contribute to the induction of Lyme arthritis. Unlike neutrophils, large populations of monocytes, activated macrophages, and mature dendritic cells are found in erythema migrans lesions, the sites of initial *B. burgdorferi* infection. The presence of TLR2, which binds borrelial lipoprotein, on these cell types may induce the expression of inflammatory cytokines, chemokines, and mediators in an attempt to eradicate the spirochaetes within the lesion. In addition, interaction of macrophages with *Borrelia* antigens induces the production of proinflammatory mediators such as nitric oxide, IL-1, TNF-α, IL-6, and IL-12, as well as mediators of tissue destruction such as matrix metalloproteinase 9 (MMP-9) (Nardelli *et al.*, 2008). Moreover, the spread of *B. burgdorferi* is hindered by the ability of macrophages and dendritic cells to readily bind and phagocytize *B. burgdorferi* by a variety of means. It is demonstrated that the presence of *B. burgdorferi* in the skin upon initial infection establishes a rapid, robust innate response by monocytes, macrophages, and dendritic cells in an attempt to stem the

dissemination of spirochetes. However, despite this strong initial response, it is not sufficient to prevent chronic disease in all infected patients.

An early investigation demonstrated that, upon stimulation with *Borrelia* antigens, synovial fluid cells from patients with chronic Lyme arthritis produced IL-2, TNF-α and IFN-γ but not IL-3, IL-4, or IL-5. Following this finding, various studies implicated IFN-γ as a key modulator of arthritic development. As a result, the inflammation characterizing Lyme arthritis has traditionally been assigned as a Th1-mediated response. To support the hypothesis it was demonstrated that administration of anti-IFN-γ antibodies to *Borrelia*-infected mice reduced the degree of paw swelling and reduced the spirochete load in the joints, while administration of anti-IL-4 antibodies to these mice increased both the severity of paw swelling and the number of spirochetes found in the joints (Matyniak & Reiner, 1995).

However, recent findings have questioned the absolute requirement for IFN-γ in the development of Lyme arthritis (Glickstein *et al.*, 2001).

For example, Brown and Reiner (Brown & Reiner, 1998); (Brown & Reiner, 1999) showed that IFN-γ is not required for the induction of Lyme arthritis. They demonstrated that depletion of IFN-γ-producing NK cells in arthritis-susceptible C3H mice did not affect the development of arthritis in challenged mice.

It was also shown that *Borrelia*-infected IFN-γ-deficient mice developed arthritis to the same degree as wild-type mice. In addition, infected mice deficient in the IFN-γ receptor displayed pathology to a similar extent as the wild-type parental strain (Brown & Reiner, 1999).

Furthermore, vaccinated and challenged IFN-γ-deficient C57BL/6 mice developed a chronic, severe, destructive osteoarthropathy characterized by destruction of cartilage and erosion of bone. These results suggest that Lyme arthritis is induced by cytokines other than IFN-γ (Christopherson *et al.*, 2003).

The findings that arthritis can develop in the absence of IFN-γ indicate that the currently accepted model of Lyme arthritis as a Th1 cytokine-driven inflammatory response is incomplete. That the prototypical Th1 cytokine is not

absolutely required for the induction of arthritis following *B. burgdorferi* infection warrants a modification of the paradigm to include additional proinflammatory cytokines, chemokines, or immune modulators.

A recently discovered subset of helper T cells, Th17 cells, which are distinct from Th1 and Th2 cells (Park *et al.*, 2005) and are characterized by the production of the inflammatory cytokine IL-17, has been shown to play a role in the development of arthritis (Hirota *et al.*, 2007) and in bone erosion(Sato K et al., 2006). In addition, high levels of IL-17 are present in the synovial fluid of patients with rheumatoid arthritis. IL-17 induces the production of proinflammatory cytokines from stromal cells, synoviocytes, chondrocytes, and macrophages and shows synergy with other cytokines for the induction of bone resorption and stimulation of osteoclast differentiation (Kotake *et al.*, 1999). Furthermore, neutralization of IL-17 causes substantial reduction of collagenase activity, osteoclast formation, and production of proinflammatory cytokines (Chabaud *et al.*, 2000). Moreover, *B. burgdorferi* or its lipoproteins have been shown to induce the production of IL-17. These properties make the Th17 cell subset a strong candidate for an additional route by which Lyme arthritis may be induced.

There are evidence that the administration of antibodies to IL-17 or to the IL-17 receptor to IFN-γ-deficient *Borrelia*- infected mice prevent the development of the destructive arthritis observed in untreated control mice. The induction of destructive arthritis has also been observed in wild-type C57BL/6 *Borrelia*-vaccinated and -challenged mice and has been prevented by administration of anti-IL-17 antibodies. These findings suggest that IL-17, and presumably the subset of activated T cells that produce it, play a major role in the development of *Borrelia*-induced arthritis, even in the presence of an intact Th1 cytokine response (Burchill *et al.*, 2003). These and other findings suggest that the IL-17 responsible for the development of *Borrelia*-induced arthritis may be derived from a subset of helper T cells distinct from Th1 cells, implying the necessity to modify the current paradigm of Lyme arthritis as solely a Th1-mediated response.

3.7 Autoimmunity induced by *Borrelia burgdorferi*

Borrelia burgdorferi infection triggers autoimmunity in the joint because of T-cell epitope mimicry between a spirochetal and a host protein or because of bystander activation of a T-cell response to a self-epitope that is not related to a spirochetal protein. Either way, the T-cell response or a linked antibody response to the self-protein might stimulate persistent synovial inflammation.

Because of the large phenotypic variation in MHC alleles in the population, only some MHC molecules bind a particular autoantigen, accounting for the HLA associations with most autoimmune diseases. The first indication that antibiotic-treatment-resistant Lyme arthritis might have an autoimmune pathogenesis was a study of HLA alleles among patients with Lyme arthritis (Steere *et al.*, 1990). Compared with patients with Lyme arthritis of brief or moderate duration, those with arthritis of prolonged duration had an increased frequency of HLA-DR4, as determined by serological typing methods.

In addition, this HLA-DR specificity was associated with a lack of response to antibiotic therapy. More recently, most patients with treatment-resistant Lyme arthritis have been shown to have the HLA-DRB1*0401 or HLA-DRB1*0101 alleles, or to a lesser degree, the HLA-DRB1*0404 allele. Of great interest, these three alleles, which have a similar shared sequence in the third hypervariable region of the HLA-DRB1 chain, are associated with severity of adult rheumatoid arthritis (Steere *et al.*, 2003).

3.8 Diagnosis of Lyme disease

The culture of *Borrelia burgdorferi* from specimens in Barbour-Stoenner-Kelly (BSK) medium permits a definitive diagnosis. However, except in the case of few patients with acrodermatitis, positive cultures have been obtained only early in the illness, primarily from biopsy samples of erythema migrans lesions, less often from plasma samples and only occasionally from cerebrospinal fluid samples in patients with meningitis.

Later in the infection, PCR testing is greatly superior to culture in the detection of *Borrelia* in joint fluid; *B. burgdorferi* DNA has been detected in

cerebrospinal fluid samples in only a small number of such patients. The Lyme urine antigen test, which has given grossly unreliable results, should not be used to support the diagnosis of Lyme disease (Steere, 2001).

Borrelia burgdorferi has been identified with silver stains and with immunohistochemical stains with monoclonal or polyclonal antibodies; however, spirochaetes are difficult to be discriminated from elastic tissue fibres or procollagen fibres and other artifacts.

A two-step serological approach has been proposed to increase specificity. A positive or equivocal first test (usually an enzyme-linked immunosorbent assay, ELISA) is followed on the same serum sample by an immunoblot test which can detect IgM and IgG antibodies to individual *Borrelia* antigens. If immunoblot is negative the ELISA will probably have been a false-positive (Nadelman & Wormser, 1998).

Both the accuracy and the reproducibility of currently available serological tests are poor. Use of commercial diagnostic test kits for Lyme disease results in high rate of mis-diagnosis.

3.9 Lyme disease treatment

Although most manifestations of Lyme borreliosis resolve spontaneously without treatment, antibiotics may hasten resolution and prevent disease progression.

Most person treated for Lyme borreliosis have an excellent prognosis.

For the early localized disease and early disseminated disease, either doxycycline or amoxicillin is the drug of choice for patients aged > 8 years. The reccomended treatment is a 14- to 21-day course of therapy for early localized Lyme disease, although it is likely that shorter courses are effective. Treatment of erythema migrans almost always prevents development of the later stages of Lyme disease.

For patients with objective evidence of neurologic symptoms, a 2- to 4-weekcourse of intravenous ceftriaxone is most commonly given. The signs and symptoms of acute neuroborreliosis usually resolve within weeks, but those of chronic neuroborreliosis improve slowly over a period of months.

After appropriately treated Lyme disease, a small percentage of patients continue to have subjective symptoms that may last for years.

3.10 The protein NapA produced by *Borrelia burgdorferi*

The *B. burgdorferi* chromosomal gene *bb0690* encodes a Dps homologue, which is also known as NapA (Fraser et al., 1997).

The Dps protein was first identified in *Escherichia coli* as a non-specific DNA-binding protein from starved bacteria, which protects DNA against damage during starvation and oxidative stress (Almiron *et al.*, 1992, Martinez & Kolter, 1997). Dps homologues have been found in many different bacteria (Kim *et al.*, 2004). The involvement of Dps proteins in the oxidative stress response has been demonstrated in a growing list of bacteria. Crystallography studies have shown that Dps proteins are ferritin-like molecules, which form dodecameric complexes capable of sequestering iron (Grant *et al.*, 1998, Papinutto *et al.*, 2002, Zanotti *et al.*, 2002, Ceci *et al.*, 2003, Ren *et al.*, 2003, Roy *et al.*, 2004). Both the iron- and the DNA-binding properties of the Dps protein contribute to its ability to protect DNA. In response to nutritional or oxidative stress, Dps proteins in *E. coli* and *Staphylococcus aureus* pack the nucleoid tightly into cocrystals, physically sequestering DNA to prevent damage (Wolf *et al.*, 1999, Frenkiel-Krispin *et al.*, 2004, Morikawa *et al.*, 2006). Dps protein also protects DNA from oxidative damage by sequestering iron to prevent the Fenton reaction, which generates reactive oxygen species (Zhao *et al.*, 2002). In our laboratory it was demonstrated that not all Dps proteins bind to DNA *in vitro* (unpublished data), and NapA belongs to this category.

3.10.1 *NapA structure*

The gene encoding NapA was cloned and expressed in *Bacillus subtilis* in our laboratory in collaboration with the group of Professor Zanotti in Padova. The final product was checked for purity in a Comassie brilliant blue-stained gel and analyzed by western blot with a specific polyclonal antibody (Figure 6 A). The dimensions of the oligomers formed by ferritins and proteins of the Dps family

are such that they can be studied at low resolution by electron microscopy. Electron micrograph of negatively stained samples of NapA (Figure 6 B) clearly shows that it forms oligomers with a central cavity, a structure reminiscent of those of other Dps-like proteins (Grant et al., 1998).

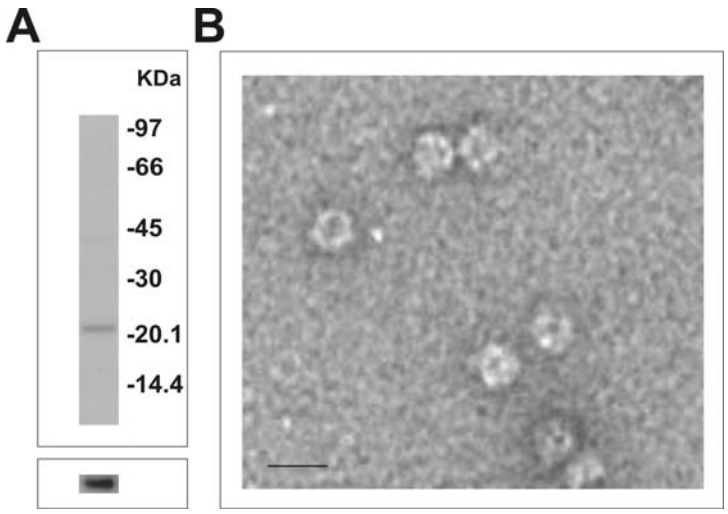

Figure 6:Purification of recombinant NapA from B. subtilis. (A) Comassie blue-stained SDS-PAGE of purified NapA reveals the purity of the protein. The identity of NapA was confirmed by using a specific polyclonal antibody in a Western blot analysis (lower box). (B) Electron microscopy analysis of negatively stained NapA.

To gain more detailed information about the structural organization of NapA, the recombinant protein was crystallized and its structure determined by x-ray diffraction at 3 Å resolution. The NapA monomer (Figure 7 A) was found to adopt the typical folding of the Dps-like family members (Grant et al., 1998, Papinutto et al., 2002, Zanotti et al., 2002, Ceci et al., 2003) characterized by a four-helix bundle. Helices A (residues from 15 to 40) and B (from 46 to 53) are connected through a long strand to helices C (residues from 113 to 127) and D (residues from 133 to 153). Another short piece of helix, quite distorted in our model (residues 82 - 88), is present in the center of the long strand that connects the first two to the last two helices. Superposition of the Cα chain trace of NapA monomer with that of other bacterial miniferritins gives root mean square values ranging from 1.4 to 1.8 Å. The major significant difference is represented by a turn (residues from 102 to 107) present at the beginning of α-helix C, since the presence of a Pro residue in position 104 prevents the formation of the α-helix.

Other differences can be observed at the N- and C-terminus. In particular, NapA has a longer N-terminal (about 10 residues) and C-terminal tail (about 19 residues) with respect to most of the other members of the family. Both the N- and C-terminus extensions cannot be seen in the electron density map, indicating that they are flexible and/or disordered. The C-terminus is rich in cysteine residues (6 Cys in 19 residues), but the electron density map for our model stops at residue 161, and only Cys 160 is visible.

The quaternary organization of NapA is that of a dodecamer with 23 symmetry. The macromolecular complex has a nearly spherical shape that tends to become a tetrahedron (Figure 7 B). The diameter of the assembly is more than 90 Å, with an internal cavity of about 50 Å. The dodecamer possesses four three-fold axes, each of them passing through the shell in two different 3-folds environments. One of the two three-fold pores corresponds to the iron entry channel postulated for Flp (Ilari *et al.*, 2000). This pore has a definitely hydrophilic, negatively charged environment. From the outside to the inside, nine potentially negatively charged residues are conserved in all the proteins of this family. In NapA they are Asp 128, 140 and 141 and their symmetry mates. The other pore is only a virtual one, since it is closed by hydrophobic side chains, in particular Val 48.

In the NapA dodecamer twelve cations binding sites are present, two per dimer. The relatively low resolution of our crystal does not allow a detailed description of this site. The electron density map is clear enough to allow us to state that two ions are present in each site, as in the case of *B. brevis* Dps structure (Ren et al., 2003). Two Fe ions were refined in each site, along with two water molecules. One of the two irons presents a nearly tetrahedral coordination, given by Glu 169 and Asp 65 of one subunit, His 38 from another and a solvent molecule, whilst the second is coordinated to the same water molecule (that consequently bridges the two ions), His 50 and a second water molecule. The latter is kept in place by Gln 54 (Figure 7 C).

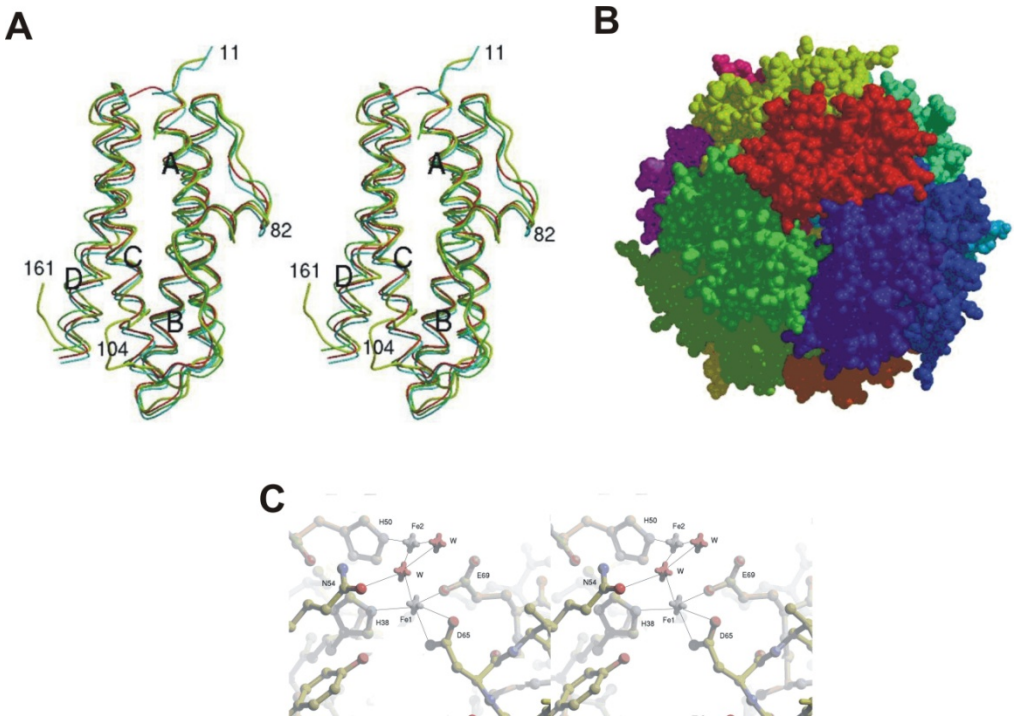

Figure 7:Three-dimensional model of the NapA monomer and oligomer. (A) Superposition of Cα chain trace of monomer of NapA (yellow) superimposed to that of other proteins of the family: Dps from Bacillus brevis (blue, PDB code 1N1Q) (Ren et al., 2003), HP-NAP from Helicobacter pylori (green, PDB code 1JI4) (Zanotti et al., 2002), Dlp-2 from Bacillus anthracis (red, PDB code 1JIG) (Papinutto et al., 2002). Residues are numbered according to (Li *et al.*, 2007). (B) van der Waals representation of the NapA dodecamer. Each monomer is coloured differently. One three-fold 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. (C) The Fe binding site. One of the iron binding sites inside NapA cavity. Two Fe ions (grey) and two water molecules (red) are coordinated by protein side chain residues. Black lines represent coordination distances.

3.10.2 *NapA functional analysis*

It has been shown that the Dps level in *B. burgdorferi* is reduced under oxygen-limiting conditions (Seshu *et al.*, 2004). *In vitro* studies on the *B. burgdorferi* Fur homologue, also known as BosR for *Borrelia* oxidative stress regulator, have identified the promoter of *bb0690* as a target of the transcription regulator (Boylan *et al.*, 2003, Katona *et al.*, 2004). These results suggest that the *B. burgdorferi* Dps homologue may play a role in the oxidative stress response. On the other hand, it has been demonstrated that *Borrelia* does not require or assimilate iron during growth in vitro (Posey & Gherardini, 2000), which raises

the question as to whether Dps supposed to be a ferritin-like molecule, has a similar function in *B. burgdorferi* as described in other bacteria.

The study conducted by Li *et al.* demonstrated that the Lyme disease spirochaete requires Dps for persistence within ticks. The *dps*-deficient spirochaetes are not defective in colonizing the tick midgut but rather fail to survive prolonged period in unfed ticks.

Dps expression was high in spirochaetes residing in unfed ticks; immediately after tick attachment and during tick feeding, the *dps* expression dropped to a level comparable to that in spirochaetes grown *in vitro*. After tick feeding, the *dps* expression in spirochaetes showed a significant trend of increase (Li et al., 2007).

As already mentioned the host immune response to *B. burgdorferi* influences the clinical outcome of the infection. In the last few years, a new subset of T helper lymphocytes was considered important in induction of Lyme arthritis.

A major unanswered question remained that of the chemical nature of the bacterial factor(s) responsible for the induction of Th17 cells. Although a role for the outer membrane lipoproteins has been suggested (Infante-Duarte *et al.*, 2000) bacterial products are known to possess immunomodulatory properties and to drive different types of innate and adaptive responses.

Previously, we have characterized a protein produced by *Helicobacter pylori*, a bacterium that gives chronic infections in humans as *B. burgdorferi* does, termed HP-NAP (*H. pylori* Neutrophil Activating Protein). This protein is a strong immunogen and is endowed with immunomodulatory properties (D'Elios *et al.*, 2007, Tonello *et al.*, 1999, Montecucco & de Bernard, 2003), (Satin *et al.*, 2000); the protein NapA (Neutrophil Activating Protein A) is homologous to HP-NAP (Fraser et al., 1997, Tonello et al., 1999). Therefore, we have hypotesized that NapA could be one of the borrelial factor involved in the pathogenesis of Th17 inflammation in human Lyme arthritis.

4 MATHERIALS AND METHODS

4.1 REAGENTS

Human recombinant IL-2, TT (tetanus toxoid) and PPD (purified protein derivative) were provided by Chiron Corp. Policlonal antibody anti NapA was raised in rabbit using the purified recombinant protein as antigen. Anti-CD3 IgG from OKT3 hybridoma supernatants were affinity purified on Mabtrap (GE Healthcare). The monoclonal blocking antibody against TLR2 was from Genentech.

4.2 METHODS

4.2.1 Bacterial strains

Escherichia coli Top10 strain were used for cloning *napA* wt and deleted genes. *Bacillus subtilis* SMS118 strain was used for expression and purification of NapA wt and N- and C-terminal mutants proteins.

4.2.2 DNA digestion

Digestion of DNA was performed with restriction endonucleases which recognize unique DNA palindromic sequences at which they hydrolyze the phosphoester linkage.
An analytical restriction enzyme reaction contained:

1 µg DNA
2 µl 10X Buffer (depending on the restriction enzyme)
1 µl restriction enzyme 5 U
Sterile H_2O up to a final volume of 20 µl

The reaction mix was incubated for 2 hours on a water bath at 37°C.

4.2.3 Agarose gel electrophoresis

The DNA samples were loaded on 1% agarose (Invitrogen) gels together with 1X Loading Buffer (0.04% bromophenol blue, 0.04% xylene cyanol, 5% glycerol in H_2O) and ran at 80 V for approximately 30 min.
The size of the separated DNA strands was determined by comparison of their relative position to that of the DNA strands of the 1000bp DNA Ladder (MBI Fermentas).

4.2.4 DNA fragments purification from agarose gels

Following agarose gel electrophoresis, the gel slice containing the desired DNA band was excised with a sharp scalpel and purified using the QIAquick Gel Extraction kit (Qiagen) following the manifacturer's instruction.

Briefly, 3 volumes of buffer QG (Qiagen) was added to DNA fragment (100 mg of gel weigh correspond to a volume of 100 µl); sample was incubated 10 minutes at 50°C. Then, one gel volume of isopropyl alcohol was added and the mix was applied to a QIAquick column and centrifuged 1 minute at 13000 g. The column was washed two times by adding 750 µl of buffer PE (Qiagen) and centrifuged 1 minute at 13000 g.
The purified DNA was eluted from the column with 50 µl of sterile water.

4.2.5 DNA ligation

DNA ligation was performed by incubating the DNA fragments with the appropriately linearized cloning vector in the presence of 2-4 units of T4 DNA ligase (3 units/µL), 10X T4 ligase buffer (50 mM TrisHCl pH 7.5, 10 mM MgCl2, 10 mM DTT, 25 µg/ml BSA, 1 mM ATP) and sterile water to a final volume of 10 µl . A vector: insert molar ratio of 1:3 was usually used.
The reaction mix was incubated overnight at 16°C.

4.2.6 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 minutes. The cells were heat-shocked for 30-40 seconds at 42°C and then incubated for 30 minutes at 37°C in 500 µl of Luria-Bertani (LB) broth (10g/L Bacto Tryptone, 5g/L Bacto yeast extract, 10g/L NaCl). The mix was plated out on LB agar which contained the antibiotic chloramphenicol that selects for transformants.

4.2.7 PCR analysis of recombinant plasmid DNA

For each sample, the following 50 µl PCR reaction was set up in a 0.2 mlmicrocentrifuge tube:

Recombinant plasmid DNA (100 ng)	1 µl
10X PCR Buffer (Celbio)	5 µl
50 mM MgCl2 (Celbio)	1.5 µl
10 mM dNTP Mix	1 µl
PCR Primers 50 µM	1 µl
EuroTaq DNA polymerase (Celbio)	2,5 U
Sterile Water	until 50 µl

The PCR was amplified using the following cycling parameters:

Initial Denaturation 15 min 95°C

Denaturation 1 min 94°C
Annealing 1 min 55°C X 35 cycles
Extension 45 sec 72°C

Final extension 5 min 72°C

5-10 µl from the reaction was removed and analyzed by agarose gel electrophoresis.

4.2.8 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 13000 g (microcentrifuge Biofuge, Haeraeus) for 3 minutes and the plasmid DNA was isolated using the QIAprep Spin miniprep kit(Qiagen) following the manifacturer'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 minutes at 13000 g.
Supernatants were applied in the Qiaprep spin column and centrifuged 1 minute at 13000 g; the column was washed two times by adding 750 µl of buffer PE (Qiagen) and centrifuged 1 minute at 13000 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 OD260-280

4.2.9 DNA sequencing

DNA sequencing was performed by the BMR Genomics Center of the University of Padova.

4.2.10 *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 hours at 150 rpm at 37°C in 50 ml of LB broth (to an OD of 0,6).

Bacteria were centrifuged 10 minutes at 1000 g and washed two times with Hepes 1 mM 10 minutes at 1000 g.

Cells were then treated two times with electroporation buffer (Hepes 1 mM pH 7, PEG 8000 25% v/v, mannitol 0,1 M.

Bacteria were risuspended in 250 μl of electroporation buffer to a final OD of 1,9.

Bacillus subtilis was electroporated using 1,5 μg of DNA; one pulse of 200 Ω, 2,3 κV, 2,5 μF.

Electroporated cells were incubated 10 minutes at 25°C in 500 μl of LB and then incubated 2 hours and 30 minutes at 37°C at 120-140 rpm. At the end of this time bacteria were plated in LB-agar in the presence of 20 μg/ml of chloramphenicol.

4.2.11 Construction of the plasmid pSM214G-*napA* wt and mutant genes

NapA was cloned and expressed in *Bacillus subtilis.* The *napA* gene was amplified by PCR from *B. burgdorferi* strain B31, using standard methods, with the following primers: 5'-CCCGAGCTCATAAAGGAGATAGTTATG-3' and 5'-CCCAAGCTTCTATTTTGCATCACACTC-3'. The N-terminal and C-terminal deletion mutants of NapA (ΔN-ter and ΔC-ter) were obtained amplifying by PCR the *napA* gene with the following couple of primers: 5'-CCCGAGCTCTATAAAAAAGGATGATTTAATG-3' and 5'-CCCAAGCTTCTATTTTGCATCACACTC-3' for the ΔN-ter mutant; and 5'-CCCGAGCTCATAAAGGAGATAGTTATG-3' and 5'-CCCAAGCTTTTATTCAAGCAATGCCTTATGC-3' for the ΔC-ter mutant.

These primers contain a *Sac*I or *Hind*III restriction enzymes sites (underlined). Bacterial samples were lised 5 minutes at 95°C.

For each sample, the following 50 µl PCR reaction was set up in a 0.2 mlmicrocentrifuge tube:

Template	2 µl
10X PCR Buffer (Celbio)	5 µl
50 mM MgCl2 (Celbio)	1.5 µl
10 mM dNTP Mix	1 µl
PCR Primers 50 µM	1 µl
EuroTaq DNA polymerase	2,5 U
Sterile H_2O	until 50 µl

PCR reaction was carried out using standard methods and the thermal cycling parameters were as follows:

Initial Denaturation 15 min 95°C

Denaturation 1 min 94°C
Annealing 1 min 55°C X 35 cycles
Extension 45 sec 72°C

Final extension 5 min 72°C

PCR products were digested 4 hours at 37°C with *Sac*I and *Hind*III.

At the same time pSM214G plasmid was digested with the same restriction enzymes 4 hours at 37°C, and ligated with the DNA fragment.

Escherichia coli cells were heat shock transformed with DNA plasmids, and bacterial colonies were PCR analyzed. The DNA plasmid was purified and sequenced. The plasmid with the correct sequence was used to transform *Bacillus subtilis*. NapA wt and deleted expression was assessed by SDS-PAGE and the best clones were used for proteins purification.

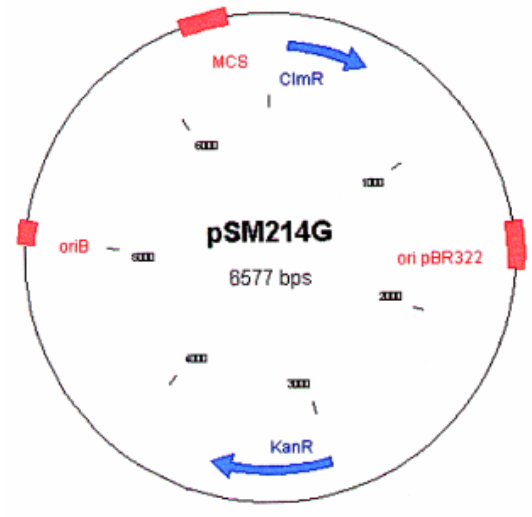

Figure 8:Map of pSM216G plasmid. oriA and oriB are the replication origin of *Bacillus subtilis* and *Escherichia coli*, respectively. MCS is the polyclonal site; ClmR is the chloramphenicol resistance and KanR is the kanamicyn resistance.

4.2.12 RNA extraction

Total RNA extraction from neutrophils and monocytes was performed using TRIzol reagent (Invitrogen), a monophasic solution of phenol and guanidine isothiocyanate, according to the manufacturer instruction. Briefly, cells were lysed directly in the culture dish by adding 150 µl of TRIzol and passing the lysate several times through a pipette. The homogenized samples were incubated for 5 minutes at room temperature to permit the complete dissociation of nucleoprotein complexes. Then, 30 µl of chloroform were added, tubes were shaken vigorously for 15 seconds and incubated on ice for 15 minutes. Samples were centrifuged at 12,000 × g for 15 minutes at 4°C. Following centrifugation, the mixture separated into a lower red, phenol-chloroform phase, an interphase, and a colorless upper aqueous phase which contained RNA. Aqueous phase was transferred into a new tube and RNA was precipitated by the addition of 70 µl of isopropyl alcohol. Following 60 minutes incubation at -20°C, samples were centrifuged again at 12,000 × g for 15 minutes at 4°C and supernatant was discarded. RNA precipitate was washed two times with 500 µl of 70% ethanol RNase free. The RNA was air dried and dissolved in 10 µl RNase-free DEPC (diethylpyrocarbonate) (Sigma)-treated water.

RNA extraction from HUVEC cells was performed using RNeasy RNA extraction kit (Promega) according to the manufacturer's instructions. Highquality RNA was then eluted in 50 µl of DEPC treated water.

An aliquot was diluted in water and the absorbance of the solution was measured spectrophotometrically at 260 and 280 nm to determine the purity and the concentration of isolated RNA. Preparations with the ratio A260/A280 higher than 1.8 were considered for further analysis.

4.2.13 cDNA synthesis

Reverse transcription reactions were performed using 1 to 5 µg of total RNA.
Before the RNA was denatured in:

DTT	10 mM
Buffer	1X
dNTPs	0,5 mM
Random pdNG primer	2 µM

RNA was denatured at 72°C for 5 minutes and then placed on ice.
Samples were retrotranscribed in cDNA using the following mix:

Superscript II (Invitrogen)	140 U
RNasi out (Invitrogen)	98U
DEPC water	

The retrotranscription was performed in a final volume of 25 µl usimg following conditions:

42°C 50 minutes
95°C 5 minutes
4°C

cDNA was then precipitated with 0,1 Vol Sodium Acetate 3 M and 2,5 Vol of ethylic alcohol at -80°C for 1 hour. cDNA was centrifuged at 12000 x g for 30 minutes at 4°C, supernatant was discarded and cDNA was dissolved in 20 µl of sterile water.

4.2.14 Real-time PCR

As the name suggests, real time PCR is a technique used to monitor the progress of a PCR reaction in real time. At the same time, a relatively small amount of PCR product (DNA, cDNA or RNA) can be quantified. Real Time PCR is based on the detection of the fluorescence produced by a reporter molecule which increases, as the reaction proceeds. This occurs due to the accumulation of the PCR product with each cycle of amplification. These fluorescent reporter molecules include dyes that bind to the double-stranded DNA (i.e. SYBR® Green) or sequence specific probes (i.e. Molecular Beacons or TaqMan® Probes). Real time PCR facilitates the monitoring of the reaction as it progresses. It is possible to start with minimal amounts of nucleic acid and quantify the end product accurately.

In real time PCR, DNA binding dyes are used as fluorescent reporters to monitor the real time PCR reaction. The fluorescence of the reporter dye increases as the product accumulates with each successive cycle of amplification. By recording the amount of fluorescence emission at each cycle, it is possible to monitor the PCR reaction during exponential phase.

The initial amount of template DNA is inversely proportional to a parameter measured for each reaction, the threshold cycle (Ct).

SYBR Green specifically binds double-stranded DNA by intercalating between base pairs, and fluoresces only when bound to DNA. Detection of the fluorescent signal occurs during the PCR cycle at the end of either the annealing or the extension step when the greatest amount of double-stranded DNA product is present. However, SYBR Green detects any double-stranded DNA non-specifically. Therefore, the reaction must contain a combination of primers and master mix that only generates a single gene-specific amplicon without producing any non-specific secondary products.

SYBR Green detection also uniquely allows to check the specificity of the PCR using melting (also known as dissociation) curves.

At low temperature, the PCR DNA product is double stranded, and it binds SYBR Green, which fluoresces. With increasing temperature, the DNA product melts or dissociates becoming single stranded, releasing SYBR Green and decreasing the fluorescent signal.

The inflection point in the melting curve then becomes a peak.

Single peaks indicate a single product while, multiple peaks usually indicate multiple products. These other products can have many sources including primer dimers, and genomic DNA contamination, but also they arise from unreported (un-annotated) splice variants of the gene of interest.

To compare gene expression between biological samples, first calculate the ΔΔCt. A ΔCt value is calculated for each sample as the difference between the Ct values for the gene of interest and the housekeeping gene in each sample. The ΔΔCt value is the difference between the ΔCt values of an experimental sample and the control sample. The fold-change in gene expression is equal to $2^{-\Delta\Delta Ct}$ if the PCR replication efficiency for all genes is 100 percent. That is, the amount of the amplicon product perfectly doubles with each cycle. The PCR amplification efficiency can be determined from the slope of the calibration curve. A slope (m) equal to -3.3 indicates 100 percent efficiency. If the PCR efficiency is less than 1 (that is, if the slope of the calibration curves is greater than –3.3), then the fold-change in gene expression is equal to $10^{\Delta\Delta Ct/m}$, where m is the average slope of the calibration curves for the gene of interest and the housekeeping gene.

For each sample, was set up the following 12 µl PCR mix:

cDNA	2 µl
Primers	0,75 µM
SYBR Green mix (Qiagen)	6,27 µl
DNase free H_2O	3,45 µl

The PCR was amplified using the following cycling parameters:

Initial denaturation	15 minutes 95°C	
Denaturation	15 seconds 95°C	
Annealing	20 seconds 60°C	X 50 cycles
Extension	10 seconds 72°C	

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

GAPDH	5'-AGCAACAGGGTGGTGGAC-3'
	5'-GTGTGGTGGGGGACTGAG-3'
IL-23p19	5'-TCCACCAGGGTCTGATTTTT-3'
	5'-TTGAAGCGGAGAAGGAGACG-3'
IL-12p40	5'-ACAAAGGAGGCGAGGTTCTAA-3'
	5'-CCCTTGGGGGTCAGAAGAG-3'
IL-6	5'-AACCTGAACCTTCCAAAGATGG-3'
	5’-TCTGGCTTGTTCCTCACTACT-3'
TGF-β	5'-AGTGGTTGAGCCGTGGAG-3'
	5'-CCATGAGAAGCAGGAAAGG-3'
IL-1β	5’-CTGTCCTGCGTGTTGAAAGA-3’
	5’-TTGGGTAATTTTTGGGATCTACA-3’
CCL2	5’-AGTCTCTGCCGCCCTTCT-3’
	5’-GTGACTGGGGCATTGATTG-3’
CCL20	5’-GCGCAAATCCAAAACAGACT-3’
	5’-CAAGTCCAGTGAGGCACAAA-3’

4.2.15 NapA purification

B. subtilis strain SMS 118 containing the plasmid pSM214G-NapA (either wt or mutant) was grown for 15 hours in YT medium (15 g/l yeast extract, 16 g/l bactotryptone, 5 g/l NaCl) with 20 µg/ml chloramphenicol. Cells were pelleted by centrifugation at 4000 g and resuspended in 10 ml of 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 32000 g, ammonium sulphate (60% for wt protein and 40% for the mutants) was added to the supernatant and kept for 3 hours at 4°C with slow continuous stirring. At this percentage of ammonium sulphate most of the protein NapA remained in solution. The solution was centrifuged at 32000 g and the supernatant was dialyzed overnight in buffer A (Tris-HCl 30 mM, pH 7.8, NaCl 0.1 M) and then loaded onto an anion exchange pre-packed column (MonoQ FPLC, GE Healthcare) equilibrated with the same buffer. Using a linear gradient from 0.1 to 0.4 M NaCl in buffer A, NapA was eluted in a range from 0.41 to 0.51 M NaCl. The fractions containing the protein were poole. NapA was further purified by gel filtration chromatography (Superdex200 HR 10/30, GE Healthcare) with phosphate buffer, 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.

4.2.16 SDS-PAGE (PolyAcrilamide Gel Electrophoresis)

Protein samples were diluted in 1X NuPAGE® LDS Sample Buffer (Invitrogen) and 50 mM DTT and denatured at 99°C for 10 minutes.

Samples were loaded on SDS 4-12 % precast polyacrylamide gels (NuPAGE Novex Bis tris-gels Invitrogen). The electrophoresis was run in 1X MES Running buffer (Invitrogen) for 45 minutes minutes at 200V constant.

Gels were stained with Simply Blue Safestain (Invitrogen).

4.2.17 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 following:

20X NuPAGE® Transfer buffer(Invitogen)

10X NuPAGE® Antioxidant (Invitogen)

10% Methanol (Sigma-Aldrich)

The volume was brought to 1l with distilled water.

The transfer was obtained by applying a current of 300mA for 1 hour. To evaluate the efficiency of transfer, proteins were stained with Red Ponceau 1x (Sigma). 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 (5% no fat milk powder solubilizated in TBS 1X) for 1 hour at room temperature and were overnight incubated with primary antibody diluted in TBS 1X with 3% no fat milk powder at 4°C . Then membranes were washed 3 times with TBS-Tween-20 0,2% and incubated with a 1:10000 dilution of a secondary antibody-HRP Conjugate dissolved in TBS-Tween-20 0,2% 3% no-fat milk, for 1 hour at room temperature. Membranes were washed 3 times in TBS-Tween-20 0,2%. Immunoreaction was revealed by ECL (Millipore) and followed by exposure to X-ray film (KODAK Sigma-Aldrich).

4.2.18 ELISA assay (Enzyme-Linked ImmunoSorbent Assay)

This method allows to quantify the antibody titre in a sample.

The antigen (10 ng/µl in PBS) was incubated overnight at room temperature in a 96 well plate (100 µl/well).

The plate was then washed two times with 200 µl/well of PBS, and incubated with blocking buffer (PBS 1% BSA) 45 minutes at room temperature. The plate was washed two times and incubated with sera 1:100 1 hour at room temperature.

The plate was washed four times with PBS, 1% BSA, 0,05% Tween-20, followed by incubation with secondary antibody HRP conjugated 1:2000 for 1 hour at room

temperature. Then was added to the plate the substrate solution ABTS (2,2'-Azino-bis3-ethylbenzthiazoline-6-6sulfonic acid). Samples absorbance was read at 405 nm.

Culture supernatants of neutrophils and monocytes, harvested for mRNA quantification, were collected at the same time points, and the amount of IL-23 for both cell type and IL-6, TGF-β and IL-1β for monocytes was quantified by ELISA (BioSource International).

Samples from rat synovial fluid lavage were assessed for cytokines and chemokines levels using a rat multiplex assay (Millipore) according to the manifacturer's instructions.

4.2.19 Assay of NapA-specific antibodies and patients

Serum samples, which were obtained from 46 patients with Lyme disease in the north-eastern United States, were investigated for the presence of NapA-specific antibodies. Of the 46 patients, 27 had Lyme arthritis, 9 had erythema migrans, and 10 had facial palsy. All patients with Lyme disease met the Center for Disease Control case definition for diagnosis of that infection (Wharton *et al.*, 1990). For comparison, serum samples were tested from 30 healthy control subjects, 20 patients with rheumatoid arthritis, 20 patients with sepsis and 20 patients with pneumonia. The 9 patients with erythema migrans participated in a study of early Lyme disease; the serum samples were selected randomly from those with skin lesions from which *B. burgdorferi* was cultured (Vaz *et al.*, 2001). The 10 patients with facial palsy had positive IgM or IgG antibody responses to *B. burgdorferi*, and the 27 patients with Lyme arthritis had swelling of a knee accompanied by a positive IgG antibody response to the spirochete by the two-test approach of ELISA and Western blot, interpreted according to the criteria of the Centers for Disease Control and Prevention.

The serum samples from patients with facial palsy or Lyme arthritis were selected randomly from patients seen during a 25-year period (1977-2001). However, the group of patients with Lyme arthritis was enriched for those with an antibiotic-refractory course. Thus, 15 of the 27 patients had antibiotic-refractory arthritis, a

rare outcome of the infection, 10 had antibiotic-responsive arthritis, and 2 patients, who were seen in the late 1970s, were not treated with antibiotics. They participated in study called "Immunity in Lyme arthritis" (Kannian *et al.*, 2007). Clinical information about these patients was obtained by chart review.
For the ELISA, serum samples were added to a 96 well plate coated with purified recombinant NapA (1 μg/100 μl). Horseradish peroxidise (HRP)-conjugated anti-human IgG subclass antibody was added into each well and colour was developed with 2,2'-azino-bis-3- ethylbenzthiazoline-6-sulfonic acid. Samples absorbance was read at 450 nm.

4.2.20 Synovial fluid T cells of Lyme arthritis patients

Upon the approval of the local Ethical Committee, synovial fluid T cells were obtained, after informed consent, from five patients with Lyme arthritis (three males and two females, median age 51, range 42 – 58) seropositive for anti-NapA antibodies. Aspiration of synovial fluid was performed by biopsy of the knee joints for diagnostic or therapeutic reasons. Fresh synovial fluid-derived T cells (1×10^5) were stimulated by immobilized anti-CD3 (OKT3) monoclonal antibody (5 μg/ml) in round-bottom microwell plates (Boncristiano M. et al., 2003 J Exp Med 198:1887). After 72 h of stimulation supernatants were collected, and assayed for IL-17, and TNF-α by ELISA (R&D, and BioSource respectively). After removal of the supernatant, IL-2 was added to the synovial fluid-derived antiCD3-induced T-cell line every 3 days. At day 15, T cell blasts of each synovial fluid T-cell line and T cells from peripheral blood mononuclear cells (PBMCs) were stimulated with NapA, Tetanus toxoid (TT) or Purified protein derivative (PPD) in the presence of autologous antigen presenting cells (APCs) for 48 hours in ELISPOT microplates coated with anti-IL-17 antibody (eBioscience). Cells stimulated with medium alone served as negative controls. At the end of culture period the number of IL-17 spot-forming cells (SFCs) were counted.

4.2.21 TLR screening

Human embryonic kidney (HEK) 293 cells constitutively expressing TLR2/CD14 (HEK293-TLR2/CD14) or TLR4/CD14/MD2 (HEK293-TLR4/CD14/MD2) (Invivogen) were grown in low-glucose Dulbecco modified Eagle medium supplemented with 10% heat-inactivated foetal calf serum (FCS). Before the experiments, HEK293 cells were plated in 24-well tissue culture plates at a density of 5 x 10^5 cells/ml. Adherent cells were collected at various times after the addition of 100 ng/ml LPS (as positive control ligand of TLR4) or 1.3 μg/ml *Helicobacter pylori* HP-NAP (as positive control ligand of TLR2) (Amedei *et al.*, 2006) or 1.7 μg/ml NapA. Monolayers were washed with ice-cold PBS. Cells were lysed in lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100 and 1 μg/ml each of sodium ortovanadate, PMSF, leupeptin, pepstatin and aprotinin) by incubation on ice for 5 minutes.

Lysates were cleared by centrifugation and proteins were separated in SDS-PAGE and transferred to nitrocellulose membranes; after being blocked with TBS-T (150 mM NaCl, 50 mM Tris-HCl pH 7.4, 0.02% Tween-20) supplemented with 3% milk, membranes were blotted with anti phospho-IκBα antibody (Cell Signaling Technology). After stripping, blots were re-probed with an anti-α actin antibody (GE Healthcare) used as a control for equal loading.

4.2.22 Neutrophils isolation from Buffy coat

Human neutrophils were obtained from Buffy coat, from the Transfusional Centre of the Universitary Hospital of Padova, derived from healthy donors.
The blood was diluted 1:4 with sterile phosphate buffer (PBS) without Ca^{2+} and $Mg^{2+}$and then dextran 5% w/V was added diluted 1:5, and erytrocytes sedimented for 30 minutes. The supernatant was recovered and wash with sterile PBS for 15 minutes at 50g. The cellular pellet was resuspended in 15 ml of sterile PBS, stratified on Ficoll-Paque (GE-Healthcare) and centrifuged 30 minutes at 400g without brake and accelerator.
The cellular pellet was washed 15 minutes at 311g.

The remaining erytrocytes were lysed with a hypotonic NaCl solution; then the cells were risuspended in RPMI 1640 (Gibco), 10% FCS (Euroclone), 50 μg/ml gentamycin (Gibco), seeded $2x10^6$ cells in 24 wells plates and treated for different time points with the appropriate stimuli.
At the end of the culture period, cell supernatants were collected for the protein level quantification, moreover, cells were lysed for the mRNA extraction using TRIzol solution (Invitrogen) according to the manifacturer's instructions.

4.2.23 Monocytes isolation from Buffy coat

Human monocytes were obtained from Buffy coat, from the Transfusional Centre of the Universitary Hospital of Padova, derived from healthy donors.
The blood 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 erytrocytes sedimented for 30 minutes. The supernatant was recovered and wash with sterile PBS for 15 minutes at 50g. The cellular pellet was resuspended in 15 ml of sterile PBS, stratified on Ficoll-Paque and centrifuged 30 minutes at 400g without brake and accelerator.
Lympho-monocytes were recovered and washed 15 minutes at 311g, and then stratified on Percoll (GE-Healthcare) gradient (15,76 ml RPMI 1640, 10% FCS (v/v), HEPES 4 mM (Gibco), 50 μg/ml gentamycin, 285 mOsm; 15,54 ml 10% Percoll in 10x sterile PBS 285 mOsm), and centrifuged 30 minutes at 400g without brake and accelerator.
Monocytes were recovered and washed 15 minutes at 311 g, then the cells were risuspended in RPMI 1640, 2% FBS, 50 μg/ml gentamycin, seeded $2x10^6$ cells in 24 wells plates and separated from contaminating lymphocytes by adherence (1 hour at 37°C). 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 protein slevel quantification, moreover, cells were lysed for the mRNA extraction using TRIzol solution (Invitrogen) according to the manifacturer's instructions.

4.2.24 Induction of Arthritis

Arthritis was induced by intraarticular administration of NapA (10 μM/50μl of sterile saline) into the right knee, while saline was injected into the left knee, which served as a control. Animals were examined for the development of arthritis by measuring swelling of the right and left knees with a caliper. At different time points after arthritis induction (2 hours - 2 days), the animals were killed, and the periarticular tissue of the knee joints was gently removed to expose the intraarticular cavity for lavage with 2 ml of saline. A ZB1 Coulter Counter (Beckman Coulter, Luton, UK) was used to measure the total cell count in the synovial lavage fluid, and the number of polymorphonuclear neutrophils (PMNs) was assessed by measuring their myeloperoxidase content, as previously reported (Marzari *et al.*, 2002), and the number of lympho-monocytes was assessed using May Grunwald- Giemsa staining.

The same fluids were centrifuged to remove the cells and used to evaluate cytokines and chemokines levels .

Part of the anterior capsule of the joint was removed,embedded in OCT compound (Miles, Milan, Italy), snapfrozen in liquid nitrogen, and kept at 80°C until used for immunohistochemical analysis. The knee joints were fixed for 6 days in 10% buffered formalin, decalcified for 5 days in Decalcifier I (SurgiPath, Richmond IL [distributed by Bio- Optica, Milan, Italy]), and embedded in paraffin.

4.2.25 Immunohistochemical analysis

Tissue presence of lymphocyte CD3 and IFNγ was assessed on frozen/paraffined sections incubated with goat anti-rat CD3 IgG (Santa Cruz) for 60 minutes at room temperature and further exposed to alcaline phosphatise-labeled rabbit anti-goat IgG (Sigma) for an additional 60 minutes at room temperature and finally to alkaline phosphatase substrate (BCIP-NBT). A similar approach was followed to examine synovial tissue for the presence of IFNγ, using rabbit anti-rat IFNγ IgG (Hycult Biotech) dilution, followed by StreptABCcomplex/HRP (Dako, Milan, Italy).

4.2.30 Statistical analysis

Data were expressed 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 $p<0,005$ was indicated with ***.

5 RESULTS AND DISCUSSION

In 1986, Mosmann and Coffman (Mosmann & Coffman, 1989) introduced the concept of distinct types of helper T cells, which was based on the types of cytokines that T cells produce when they are stimulated to differentiate. They named these lymphocytes type 1 helper T cells (Th1 cells) and type 2 helper T cells (Th2 cells). Th1 cells produce large quantities of interferon(IFN)-γ, induce delayed hypersensitivity reactions, activate macrophages, and are essential for the defense against intracellular pathogens (Figure 9). Th2 cells produce mainly interleukin(IL)-4 and are important in inducing IgE production, recruiting eosinophils to sites of inflammation, and helping to clear parasitic infections (Figure 9). More recently, T cells were shown to produce cytokines that could not be classified according to the Th1–Th2 scheme. Interleukin-17 was among these cytokines, and the T cells that preferentially produce IL-17, but not IFN-γ or IL-4, were named Th17 cells. Since these T cells constitute a distinct lineage, we now have three types of effector helper T cells: Th1, Th2, and Th17 (Figure 9).

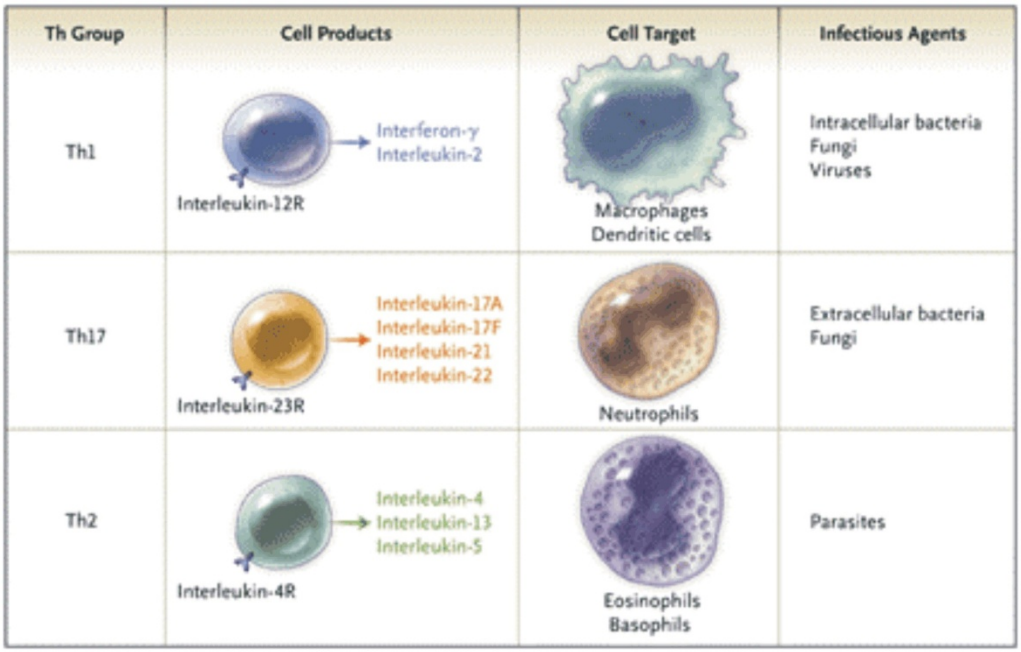

Figure 9: T helper lymphocytes subgroups and effector functions. The cytokine profile of Th1, Th17 and Th2 cells, their cell targets and infectious agents against they respond, are shown (from(Miossec *et al.*, 2009).

The differentiation of Th17 cells is induced by IL-1β, IL-6 and TGF-β. This finding was surprising, since TGF-β was classified as an immunosuppressive cytokine, not as an inducer of T-cell differentiation. Furthermore, naive T cells that are exposed to TGF-β alone express forkhead box P3 (Foxp3), the master transcription factor that induces regulatory T cells - T cells that suppress inflammation and inhibit autoimmunity (Li *et al.*, 2006). A relevant finding is that IL-6 is a potent inhibitor of TGF-β-driven induction of $Foxp3^+$ regulatory T cells. Interleukin-6 not only suppresses the generation of these cells, but together with TGF-β, it also forces naive T cells to express interleukin-17 and to become Th17 cells. Thus, Th17 cells and $Foxp3^+$ regulatory T cells are reciprocally related: TGF-β induces naïve T cells to develop into suppressor regulatory T cells, whereas IL-6 switches the transcriptional program initiated by TGF-β in a way that triggers the development of Th17 cells.

Th17 cells express a unique transcription factor, ROR-γt (Ivanov *et al.*, 2006), which induces transcription of the *il-17* gene in naive helper T cells and is required for the development of IL-17 producing cells in the presence of IL-6 and TGF-β. Activation of ROR-γt also causes expression of the receptor for IL-23, indicating that IL-23 acts on T cells that are already committed to the Th17 lineage. Exposure of developing Th17 cells to IL-23 not only enhances the expression of IL-17 but also induces IL-22 and suppresses IL-10 and IFN-γ, which are not normally associated with the Th17 phenotype (McGeachy *et al.*, 2007). Thus, IL-23 is essential for stabilizing the Th17 phenotype.

As with Th1 and Th2 cells, no single surface marker is specific for Th17 cells. However, coexpression of the chemokine receptors CCR4 and CCR6 or expression of CCR2 in the absence of CCR5 appear to define human Th17 cells (Acosta-Rodriguez *et al.*, 2007, Sato *et al.*, 2007).

Th17 cells can rapidly initiate an inflammatory response that is dominated by neutrophils (Figure 9); indeed, acute inflammation in which neutrophils are prominent is typical of Th17-driven inflammation.

The involvement of Th17 cells was suggest also in the pathogenesis of murine Lyme arthritis (Infante-Duarte et al., 2000, Burchill et al., 2003). However, a major unanswered question remains that of the chemical nature of the bacterial factor(s) responsible for the induction of Th17 cells. Although a role for the outer membrane lipoproteins has been suggested (Infante-Duarte et al., 2000) bacterial products are known to possess immunomodulatory properties and to drive different types of innate and adaptive responses. Previously, we have characterized a protein produced by *Helicobacter pylori*, a bacterium that gives chronic infections in humans as *B. burgdorferi* does, termed HP-NAP (*H. pylori* Neutrophil Activating Protein). This protein is a strong immunogen and is endowed with immunomodulatory properties (D'Elios et al., 2007, Tonello et al., 1999, Montecucco & de Bernard, 2003, Zanotti et al., 2002, Satin et al., 2000). The chromosomal gene *bb0690* of *B. Burgdorferi* encodes a protein termed NapA (Neutrophil Activating Protein A), homologous to HP-NAP (Tonello et al., 1999, Fraser et al., 1997), that was shown to be essential for the persistence of spirochetae within ticks (Li et al., 2007).

5.1 NapA of *Borrelia burgdorferi* drives Th17 inflammation in Lyme arthritis

5.1.1 *NapA antibody response in human Lyme arthritis*

The aim of our study is to understand whether NapA possesses immunomodulatory activity, and whether, in case, it could have an immunodominant role. In order to define this aspect, we evaluate the role of NapA in the *B. burgdorferi* associated human pathologies, we tested by ELISA whether serum samples from infected individuals, with different clinical outcomes of Lyme disease, contained specific antibodies to this protein. We considered for our study 27 patients with Lyme arthritis, a late manifestation of the disorder. Among these subjects, 13 (48%) had circulating antibodies specific for NapA (Figure 10). In contrast, 19 patients with earlier manifestations of the disorder, 9 with erythema migrans and 10 with facial palsy, had minimal or no reactivity with this antigen and no anti-NapA antibodies were present in the sera of 30 healthy

control patients. In order to define the relevance of NapA serological findings in Lyme arthritis-affected patients and to exclude potential cross reactivity, we tested for NapA reactivity 20 sera collected from patients with rheumatoid arthritis, 20 sera from patients with sepsis and 20 from patients with pneumonia. Figure 10 clearly depicts that none of these sera had circulating antibodies cross reacting against NapA.

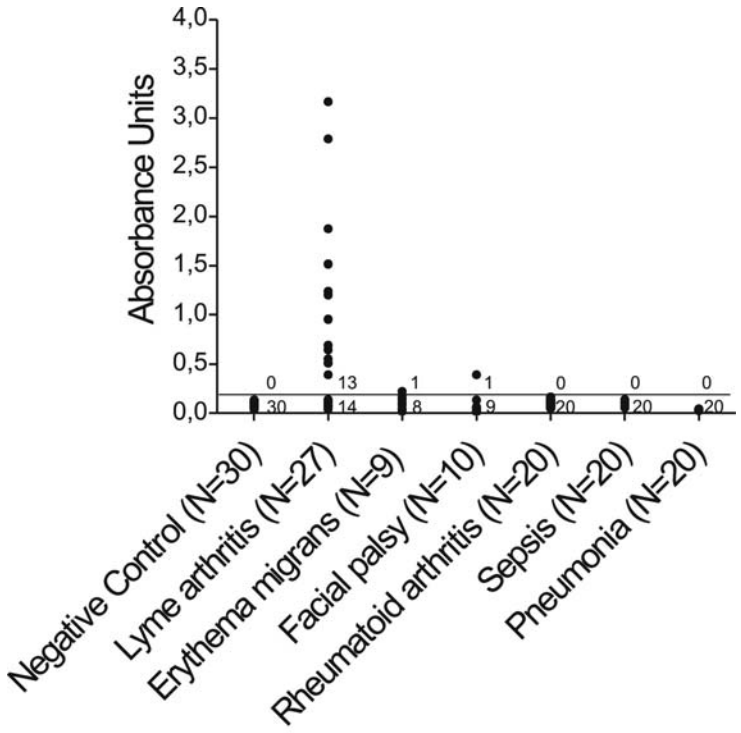

Figure 10: NapA immunogenicity in patients with Lyme arthritis. NapA-specific antibodies in 46 patients with Lyme disease, 20 patients with rheumatoid arthritis, 20 patients with sepsis, 20 patients with pneumonia, and 30 healthy controls were determined using a specific enzyme-linked immunosorbent assay (ELISA). NapA-specific antibodies were detected in serum samples from 48% of the Lyme disease patients with Lyme arthritis, whereas Lyme disease patients with erythema migrans or facial palsy had minimal or no serologic reactivity with NapA. The healthy control subjects and patients with rheumatoid arthritis, sepsis, and pneumonia were seronegative for NapA. ELISA results were considered positive at $\alpha 3$ SD above the mean value in normal control subjects, and 0.2 absorbance units (horizontal line) was considered the threshold.

It is important to note that anti-NapA antibody response was particularly relevant in patients with Lyme arthritis, a late manifestation of the illness, whereas they are rarely detected in *Borrelia*-infected patients with erythema migrans, or facial palsy, which are earlier manifestations of the disorder.

5.1.2 *NapA drives IL-17 secretion by synovial fluid Th cells from patients with Lyme arthritis*

The orchestration of T cells in the development of Lyme arthritis is well documented (Steere & Glickstein, 2004). Th1 cells, producing high levels of IFN-γ, are considered crucial for the pathogenesis of human and experimental Lyme arthritis (Yssel *et al.*, 1991, Gross *et al.*, 1998a), but recent data show that Lyme arthritis can occur and propagate even in IFN-γ-deficient mice (Brown & Reiner, 1999), (Christopherson et al., 2003)), suggesting that other Th cells and factors are involved in the genesis of arthritis.

On the basis of these observations we examined whether synovial T cells from patients with Lyme arthritis produced IL-17. In preliminary experiments, we used synovial fluid mononuclear cells (SFMC) to generate T-cell lines and analyzed their IL-17 and TNF-α production in culture supernatants. After 72 hours stimulation with immobilized anti-CD3 mAb, SFMCs produced high level of IL-17 and of TNF-α (Figure 11 A). Subsequently, synovial fluid-derived anti-CD3 mAb-induced T cell lines were expanded by addition of IL-2 every 3 days. At day 15, T cell blasts of each synovial fluid T-cell line were stimulated, in a concentration dependent curve, with NapA, Tetanus Toxoid (TT) or Purified Protein Derivative (PPD), in the presence of autologous Antigen Presenting Cells (APCs) for 48 hours in ELISPOT microplates coated with anti-IL-17 antibody. At the end of culture period the number of IL-17 spot-forming cells (SFCs) were counted (Figure 11 B).

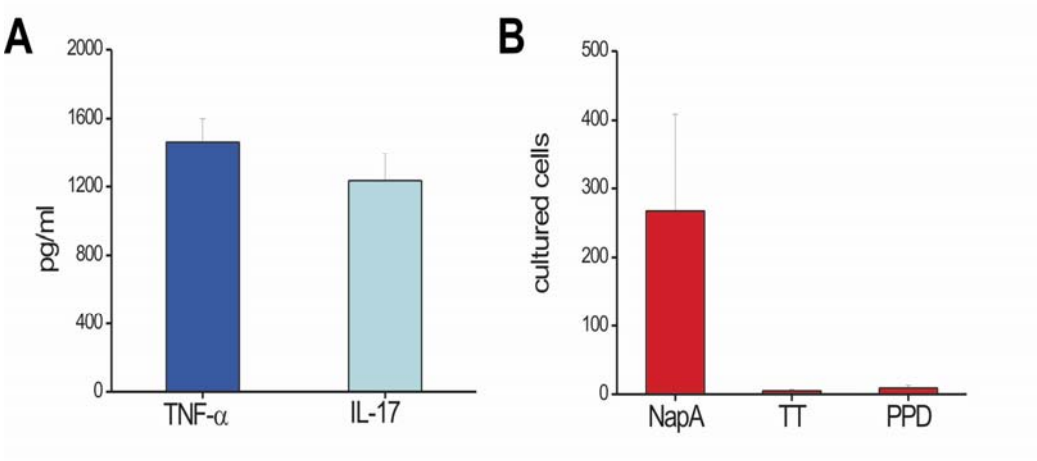

Figure 11: NapA drives IL-17 secretion by synovial fluid Th cells in patients with Lyme arthritis. (A) TNF-α and IL-17 release by fresh Synovial Fluid Mononuclear Cells (SFMCs) after 72 hours stimulation with immobilized anti-CD3 mAb. (B) SFMCs were stimulated with immobilized anti-CD3 mAb, and subsequently synovial fluid-derived T-cell lines were expanded with IL-2. At day 15, T-cell blasts of each line were stimulated with NapA, TT, or PPD, in the presence of irradiated autologous APCs for 48 hours in ELISPOT microplates coated with anti-IL17 antibody. At the end of the culture period, IL-17 SFCs were counted using an automated ELISPOT reader. After specific stimulation with NapA, a significant proportion of synovial fluid-derived Th cells produced IL-17. Results represent mean values ± SD of SFCs per 10^5 cultured cells over background levels.

A significant proportion of all synovial fluid-derived T-cell lines, under MHC restricted conditions, reacted specifically to NapA by producing IL-17 in a dose-dependent fashion (Figure 12), whilst TT and PPD were ineffective at any concentration used.

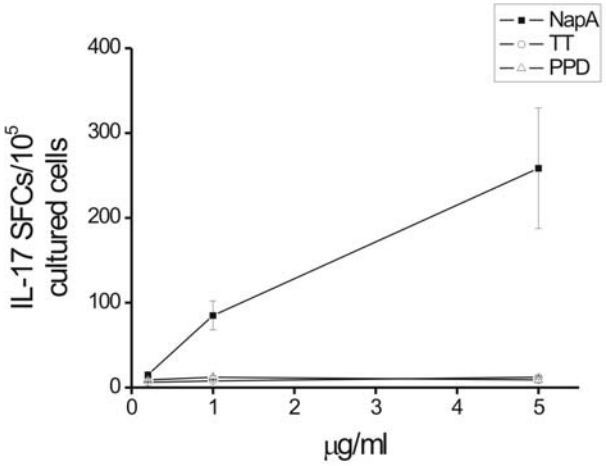

Figure 12: Dose-response effect of graded concentrations of antigens on the IL-17 SFCs counts. SFMCs were stimulated as described in figure 5. Results represent mean values + SD of SFCs per 105 cultured cells over background levels.

Collectively, these data demonstrate that not only in synovial fluid of Lyme arthritis-affected patients there are Th17 lymphocytes, but also that a significant percentage of these cells are NapA specific.

5.1.3 *NapA activates neutrophils and monocytes to release cytokines essential for the differentiation of Th17 cells*

IL-17 exerts many different activities on both immune and non-immune cells. IL-17 links synovial inflammation to bone destruction. It recruits and activates neutrophils and is considered a key cytokine driving autoimmune-like inflammation, mainly consisting of neutrophils and T cells (Iwakura & Ishigame, 2006). IL-17 is a potent stimulator of osteoclastogenesis and bone destruction and its activity is synergistically enhanced by TNF-α (Kotake et al., 1999, Schwandner *et al.*, 2000). Th17 cells were shown to play a key role in the induction of experimental autoimmune arthritis and in rheumatoid arthritis (Kotake et al., 1999, Nakae *et al.*, 2003, Shen *et al.*, 2005). Mice treated with an IL-17 neutralizing antibody were resistant to the induction of experimental arthritis, and did not show joint inflammation and erosion (Koenders *et al.*, 2005).

On the basis of these observations, we asked whether NapA is able to drive the differentiation of T lymphocytes towards a Th17 phenotype, through the induction of a pro-Th17 cytokine *milieu*.

In order to address this point, we treated freshly monocytes isolated from healthy donors with NapA or Outer surface protein A (OspA), a major antigen produced by *Borrelia burgdorferi* which has been shown to trigger IL-1β, IL-6 and TNF-α release by monocytes (Haupl *et al.*, 1997), and we have analyzed their ability in inducing synthesis and release of cytokines known to be involved in Th17 differentiation, such as IL-6, TGF-β and IL-1β.

As shown in figure 13, IL-6 mRNA was found to increase up to 90 times after 6 hours of NapA treatment, and, accordingly, a significant accumulation of the mature protein was observed in the culture supernatants of the cells harvested for the mRNA quantification.

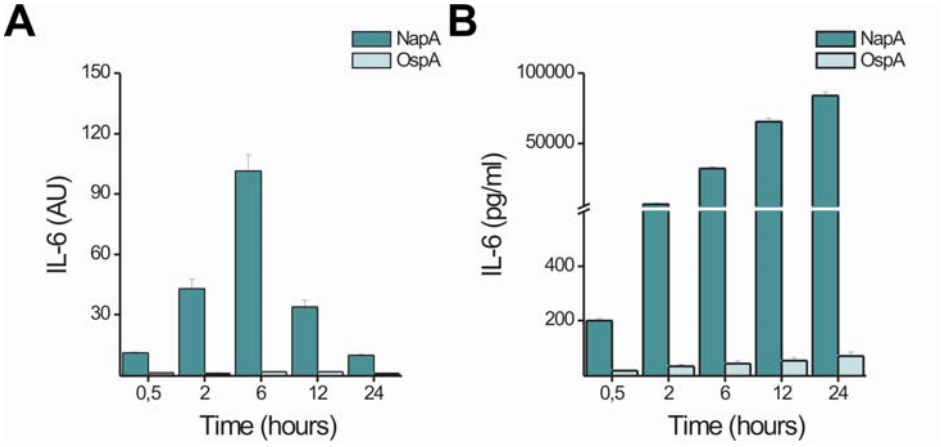

Figure 13: Kinetics of IL-6 synthesis and production in monocytes stimulated with NapA or OspA. IL-6 mRNAs were determined by quantitative Real time PCR at the indicated time points after NapA administration (A). IL-16 protein levels were measured in the culture supernatants of the same monocytes (B) harvested for messengers evaluation. Data represent the average of four independent experiments with triplicate samples. (AU, arbitrary units).

NapA also induced a high expression of TGF-β m-RNA which started to increase after 2 hours and peaked after 12 hours from NapA addition, leading to accumulation of TGF-β protein in the supernatant (Figure 14 A and B).

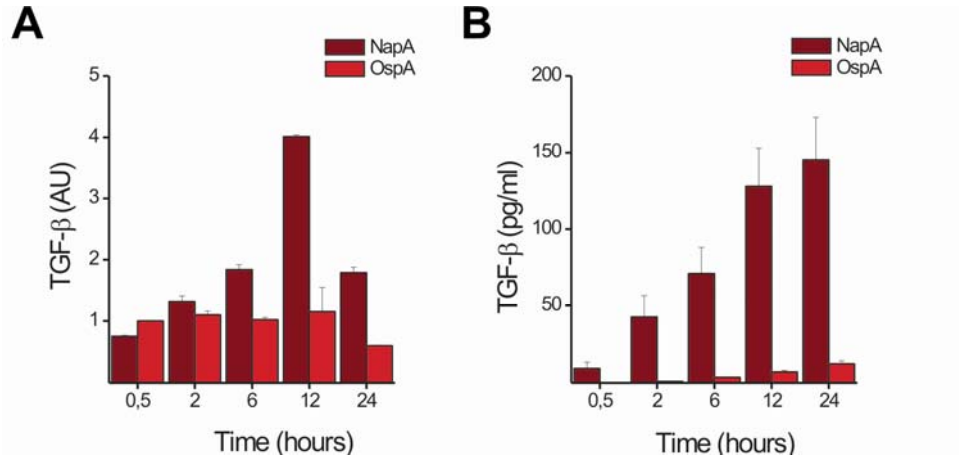

Figure 14: Kinetics of TGF-β synthesis and production in monocytes stimulated with NapA or OspA. TGF-β mRNAs were determined by quantitative Real time PCR at the indicated time points after NapA administration (A). TGF-β protein levels were measured in the culture supernatants of the same monocytes (B) harvested for messengers evaluation. Data represent the average of four independent experiments with triplicate samples. (AU, arbitrary units).

Finally, IL-1β was found to significantly increase following NapA stimulation (Figure 15 A and B).

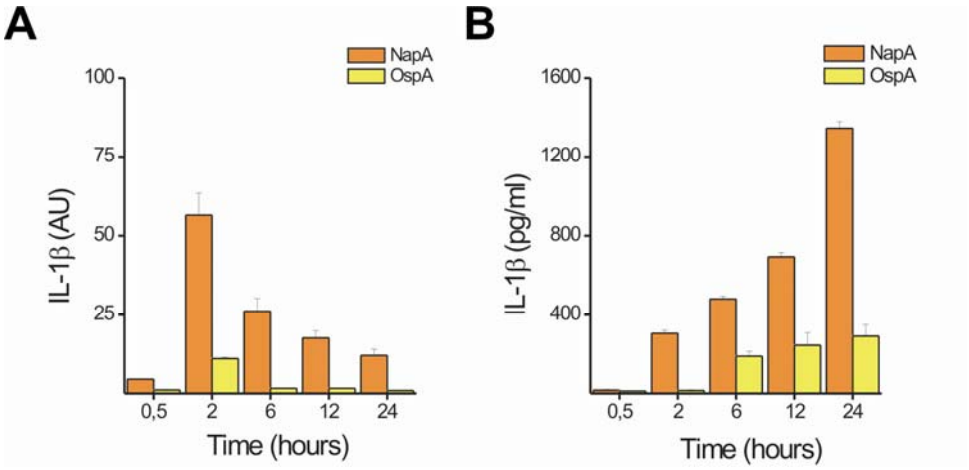

Figure 15: Kinetics of IL-1β synthesis and production in monocytes stimulated with NapA or OspA. IL-1β mRNAs were determined by quantitative Real time PCR at the indicated time points after NapA administration (A). IL-1β protein levels were measured in the culture supernatants of the same monocytes (B) harvested for messengers evaluation. Data represent the average of four independent experiments with triplicate samples. (AU, arbitrary units).

Interestingly, OspA was significantly less efficient than NapA in inducing TGF-β release by monocytes; it was able to trigger the release of IL-1β and IL-6, however also the latter were induced to a lesser extent.

Another important cytokine involved in the proliferation of differentiated Th17 cells is IL-23. IL-23 consists of a heterodimeric protein, formed by a p19 and a p40 subunit; the p40 subunit is shared with IL-12 (Oppmann *et al.*, 2000).

IL-23 is an essential survival factor for Th17 cells, since these cells are not present in mice lacking IL-23 (Stockinger & Veldhoen, 2007). Accordingly, IL-23 blocking monoclonal antibodies completely abrogated the ability of *B. burgdorferi*-stimulated dendritic cells to induce the release of IL-17 by T cells (Knauer *et al.*, 2007).

We have observed that NapA was able to induce neutrophils isolated from healthy donors to express both IL-12p40 and IL-23p19 (Figure 16 A and B). The kinetics of expression of the two subunits were consistent with those of protein accumulation in culture supernatants (Figure 16 C). Interestingly, no expression and release of IL-23 was observed when neutrophils were exposed to OspA.

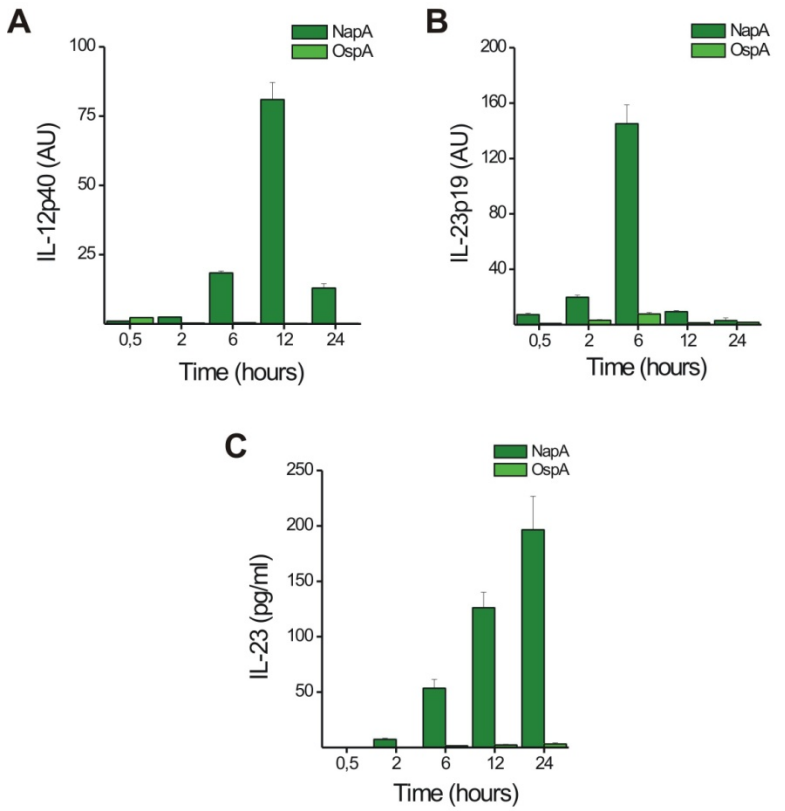

Figure 16: Kinetics of IL-23 synthesis and production in neutrophils stimulated with NapA or OspA. IL12-p40 and IL-23p19 cytokine mRNAs (A and B) were determined by quantitative Real time PCR. The experiments shown are representative out of seven experiments conducted with different cell preparations. (AU, arbitrary units). IL-23 protein levels (C) were measured, by ELISA, in the culture supernatants of the same cells harvested for mRNA evaluation. The kinetics of production were comparable among different experiments whereas the amounts varied among different donors. Data represent the average of four independent experiments with triplicate samples.

Similarly, NapA induces monocytes to express both IL-12p40 and IL-23p19 (Figure 174 A and B) and the release of the mature protein IL-23 in culture supernatants (Figure 17 C).

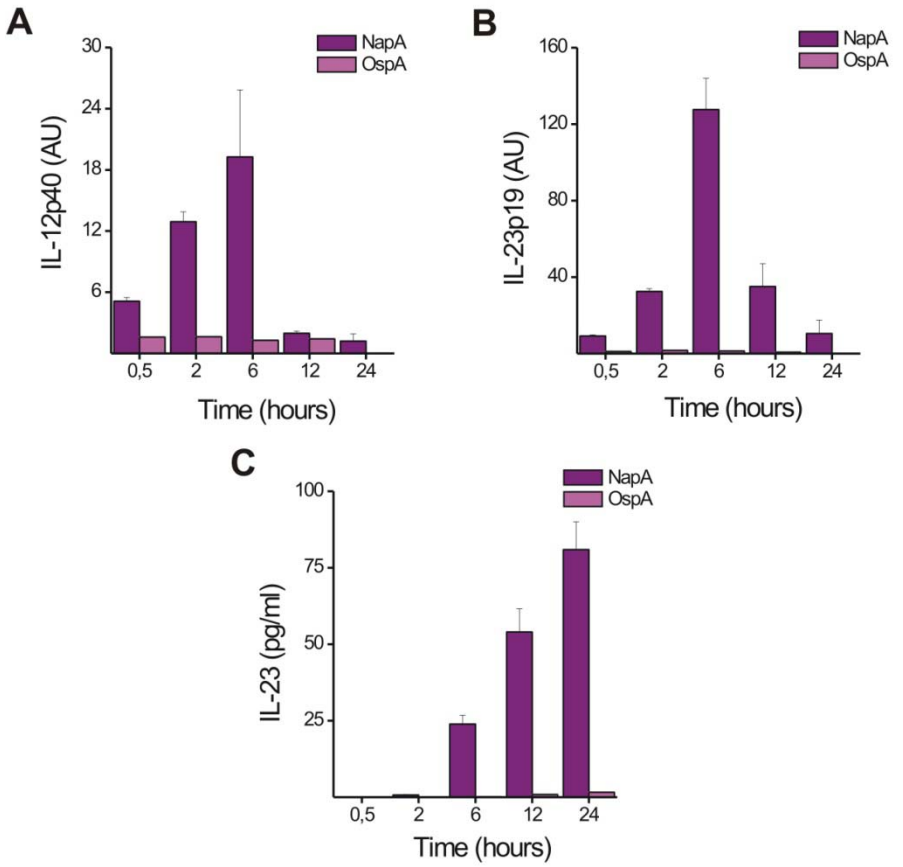

Figure 17: Kinetics of IL-23 synthesis and production in monocytes stimulated with NapA or OspA. IL12-p40 and IL-23p19 cytokine mRNAs (A and B) were determined by quantitative Real time PCR. The experiments shown are representative out of seven experiments conducted with different cell preparations. (AU, arbitrary units). IL-23 protein levels (C) were measured, by ELISA, in the culture supernatants of the same cells harvested for mRNA evaluation. The kinetics of production were comparable among different experiments whereas the amounts varied among different donors. Data represent the average of four independent experiments with triplicate samples.

Alltogether, these findings indicate that NapA, acting on monocytes and neutrophils, contributes to create a cytokine *milieu* enriched in IL-6, TGF-β, IL-1β and IL-23 with a strong potential of driving the differentiation of T cells towards the Th17 subset.

The present results suggest that NapA of *B. burgdorferi* plays a major role in promoting a Th17 inflammation of the joints of patients with Lyme arthritis that

may lead, if not arrested, to bone erosion and remodelling (Lawson & Steere, 1985). *B. burgdorferi* was reported to induce IL-23 (Knauer et al., 2007), but the nature of the bacterial molecule(s) able to stimulate innate immune cells was not known. Here, we have documented that NapA activates neutrophils and monocytes to increase mRNA expression and secretion of IL-23, IL-6, IL-1β and TGF-β proteins, which are crucial for the induction of Th17 responses (Stockinger & Veldhoen, 2007). These findings do not exclude the possibility that additional bacterial factors contribute to the generation of the Th17 response found in the synovial fluids of Lyme arthritis patients.

5.1.4 *IL-23 secretion follows NapA binding to TLR2*

TLRs are host cell receptors which recognize conserved molecular patterns on microbial components such as lipoproteins, lipopolysaccharide, proteoglycans, flagellin, and nucleic acids. Binding of these components to TLRs initiates a signalling cascade that culminates in the NF-κB-mediated expression of various proinflammatory cytokines and chemokines. Most TLR signaling is directed through the adapter molecule myeloid differentiation factor 88 (MyD88). Recent studies have shedded light upon the role of TLR signaling in the control of borrelial infection and the development of arthritis (Nardelli *et al*., 2008).

To investigate the mechanism by which NapA activates monocytes, we evaluated the possibility that NapA engages a TLR. Considering that bacterial proteins preferentially activate monocytes and dendritic cells via TLR2 or TLR4, we used human embryonic kidney (HEK) 293 cells transfected with plasmids encoding for either of them. HEK293 cell lines lack expression of endogenous TLRs, although their TLR signalling machinery is fully functional. The common pathway leading to NF-κB activation requires phosphorylation and degradation of the cytosolic inhibitor of NF-κB, IκB-α. The engagement of a specific TLR, expressed on HEK293, was monitored by evaluating the phosphorylation of IκB-α after exposure to NapA. As shown in Figure 18, phosphorylation of IκB-α was observed in cells expressing TLR2, but not in those expressing TLR4. The phosphorylation of IκB-α was evident already after 10 min of NapA treatment

and the time-course of the band intensity is compatible with the degradation of the NF-κB inhibitor by proteasome.

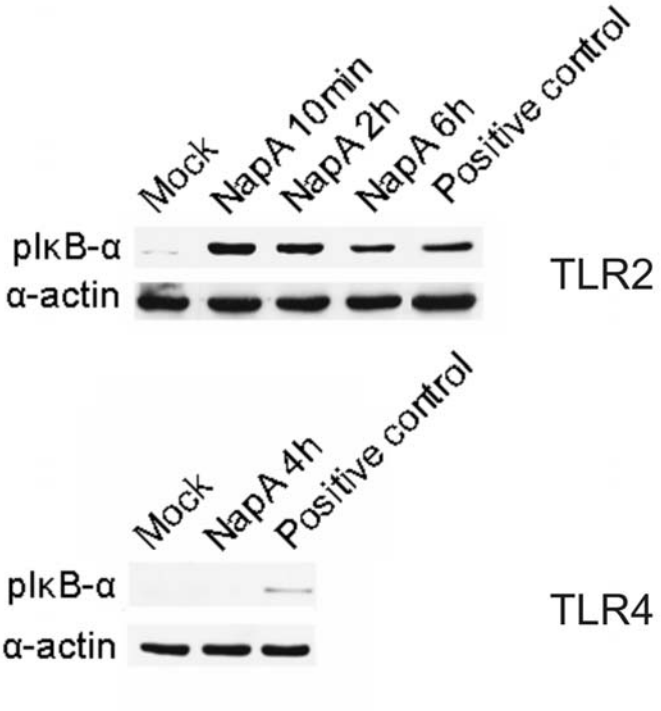

Figure 18: Engagement of TLR2 by NapA. HEK 293 cellsexpressing either TLR2 or TLR4 were incubated with NapA; HP-NAP for TLR2-expressing cells and LPS for TLR4-expressing cells, were used as positive controls respectively. Mock cells represented the negative control. After different times, cell lysates were subjected to SDSPAGE/ immunoblotting with anti-phospho IκB-α. Blot with total α-actin antibody was used as a control for equal loading.

In order to link the NapA ability to create a cytokine environment promoting Th17 differentiation with the TLR2 engagement, monocytes were stimulated with NapA in the presence of a blocking anti-TLR2 antibody.

As demonstrated in Figure 19, in monocytes pretreated with anti-TLR2 blocking antibody and then stimulated with NapA, both the syntesis and secretion of IL-23 were abrogated.

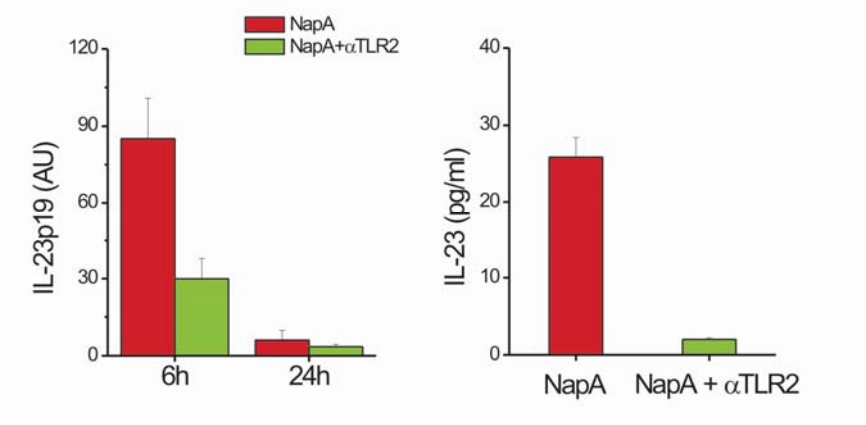

Figure 19: TLR2 involvement in IL-23 production. Monocytes were pre-incubated or not for 2 h with 20 µg/ml of an anti TLR2 blocking antibody before being exposed to NapA. After 6 and 24 h cells were harvested and mRNA levels of IL-23p19 quantified by Real time PCR. The 24 h-culture supernatants of the same cells were collected and used for the ELISA evaluation of the secreted IL-23 cytokine. Data represent the average of four independent experiments with triplicate samples. (AU, arbitrary units).

Taken together, the results we obtained demonstrate that the *B. burgdorferi* NapA protein elicits Th17 inflammation which may be crucial for the induction and maintenance of Lyme arthritis. We would like to suggest that NapA and the Th17 pathway may represent novel therapeutic targets for the prevention and treatment of the disease.

These data have been published in (Codolo *et al.*, 2008).

5.2 Structure and immunological properties of NapA of *Borrelia burgdorferi*

NapA belongs to the family of Dps proteins (DNA binding proteins from starved cells); these are proteins which form dodecameric complexes capable of sequestring iron, and together the property of bind DNA, they are able to protect DNA.

NapA shares with Dps proteins the dodecameric structure but not the ability to bind and protect DNA (our unpublished observations). Moreover, NapA possesses other peculiarities that might be implicated in its specific immunological activity: it is longer at both the N-terminus and C-terminus. On the basis of these differences, we were concerned to investigate whether in these two portions of the molecule could reside the peculiar Th17-orienting property of NapA. Moreover, the fact that synovial fluid-derived NapA-specific T cells of patients with Lyme arthritis produced IL-17, further supported the hypothesis that NapA contains Th17 motifs in its non-conserved parts (Codolo *et al*., 2008).

5.2.1 *Structure of NapA monomer and dodecamer*

The NapA monomer (Figure 20 A) was found to adopt the typical folding of the Dps-like family members (Grant et al., 1998, Papinutto et al., 2002, Zanotti et al., 2002, Ceci et al., 2003) characterized by a four-helix bundle.

Both the N- and C-terminus extensions cannot be seen in the electron density map, indicating that they are flexible and/or disordered. The C-terminus is rich in cysteine residues (6 Cys in 20 residues), but the electron density map for our model stops at residue 161, and only Cys 160 is visible. A possible reason is that the protein, during purification and crystallization, was permanently kept in the presence of 10 mM DTT: in fact, since these Cys residues are exposed on the protein surface, they can be oxidized with the formation of intermolecular disulfide bridges, giving rise to aggregates. The presence of covalent dimers was, in fact, observed during the purification process of the protein. Moreover, the presence of reducing conditions could have hindered a correct folding of this part of the molecule.

The quaternary organization of NapA is that of a dodecamer with 23 symmetry. The macromolecular complex has a nearly spherical shape that tends to become a tetrahedron (Figure 20 B). The NapA crystal structure was solved by the group of professor Zanotti (unpublished data).

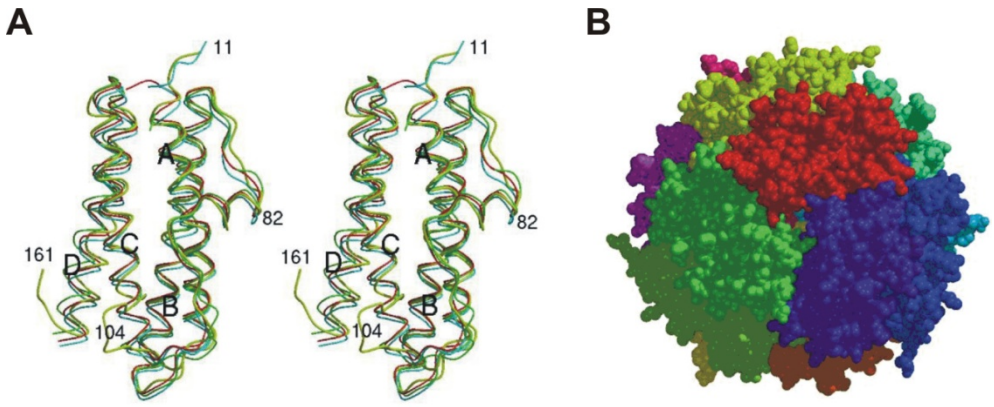

Figure 20: Three-dimensional model of the NapA monomer and oligomer. (A) Superposition of Cα chain trace of monomer of NapA (yellow) superimposed to that of other proteins of the family: Dps from *Bacillus brevis* (blue, PDB code 1N1Q) (Ren et al., 2003), HP-NAP from *Helicobacter pylori* (green, PDB code 1JI4) (Zanotti et al., 2002), Dlp-2 from *Bacillus anthracis* (red, PDB code 1JIG) (Papinutto et al., 2002). Residues are numbered according to (Li et al., 2007). Helices A (residues from 15 to 40) and B (from 46 to 53) are connected through a long strand to helices C (residues from 113 to 127) and D (residues from 133 to 153). (B) van der Waals representation of the NapA dodecamer.

5.2.2 *N- and C-terminal tails of NapA are not crucial for its pro-Th17 activity*

To elucidate whether the immuno-modulating activity of NapA resides either in its N-terminal or in its C-terminal tail, we produced two NapA mutants lacking 13 residues at the N-terminus and 20 at the C-terminus (as shown in Figure 21), respectively, and assayed them for their Th17-orienting property.

```
NapA       MYYNNYIKEIVMEKYLSYIKKDDLDAIQLKLQELLASLHIFYSNLRGIHW 50
HP-NAP     ----------------------MKTFEILKHLQADAIVLFMKVHNFHW 26
NapA       NIKDTNFFVIHKKTQKLYEYIEKIIDIVAERSRMLGYDSEFRYSEFMKKS 100
HP-NAP     NVKGTDFFNVHKATEEIYEGFADMFDDLAERIVQLGHHPLVTLSEALKLT 76
NapA       FIKELDIESTSNFLPSMESIVCSLTEILKNIFGMRKLIDTAGDYGTANIM 150
HP-NAP     RVKEETKTS-FHSKDIFKEILEDYKHLEKEFKELSNTAEKEGDKVTVTYA 125
NapA       DDIMSDLEKHLWMHKALLENCDCFCHDENESKCCECDAK 189
HP-NAP     DDQLAKLQKSIWMLQAHLA------------------- 144
```

Figure 21: NapA and HP-NAP alignment. In yellow are reported the N- and C-terminal longer tails; in red are reported the amino acids substituted with a methionine and with a stop codon, respectively.

To this aim, monocytes isolated from healthy donors were incubated with NapA, either wt or deleted, and at different time points mRNA was extracted, retro-transcribed and amplified by Real Time PCR in the presence of primers specific for the cytokines crucial for the differentiation and expansion of Th17 cells, such as IL-23, IL-1β, IL-6 and TGF-β

As expected, the amount of mRNA for both IL-12p40 and for IL-23p19 was increased in monocytes exposed to wt NapA; surprisingly, the exposure to both the deleted forms of NapA resulted in an even more pronounced induction of mRNA for both IL-12p40 and IL-23p19 (Figure 22).

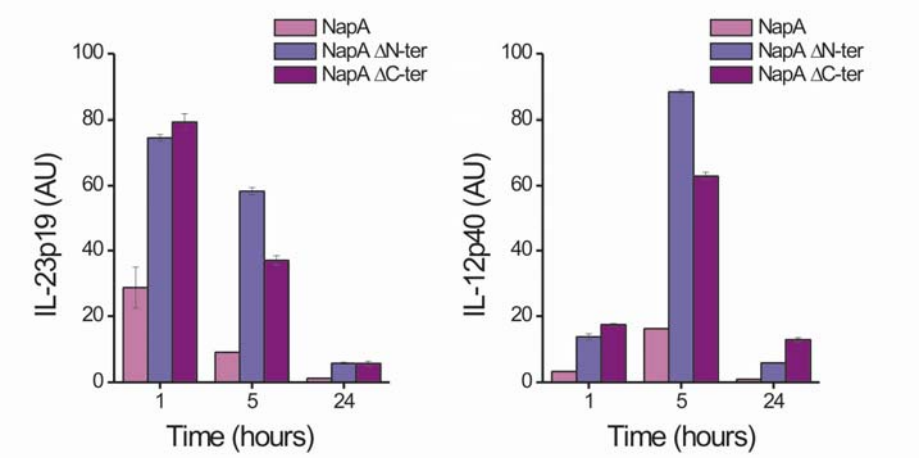

Figure 22: Kinetics of IL-23 synthesis in monocytes stimulated with NapA wt and NapA deletion mutants. IL-23p19 and IL-12p40 cytokine mRNAs were quantified by Real time PCR. The experiment shown is representative out of 4 experiments conducted with different cell preparations. (AU, arbitrary units).

These results, suggesting that neither the N-terminus, nor the C-terminus of NapA were crucial for its immuno-modulatory activity, were further confirmed by the evaluation of the IL-6, TGF-β and IL-1β mRNA (Figure 23). Indeed, also in this case the deletion of the two NapA domains did not abrogate the ability of the protein to activate monocytes.

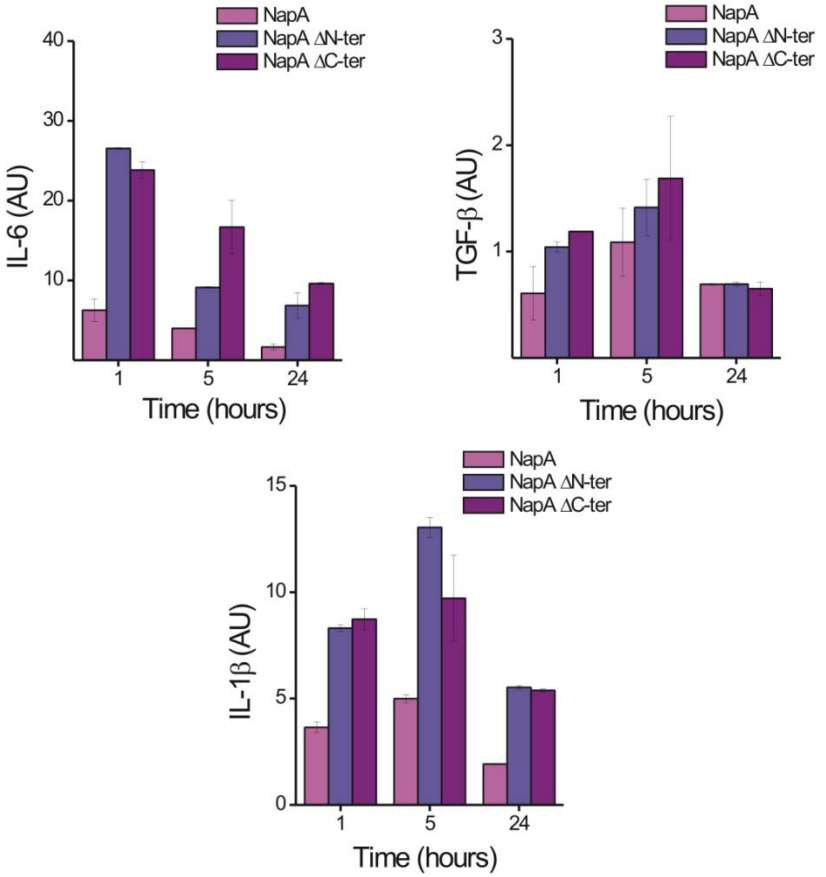

Figure 23: Kinetics of IL-6, TGF-β and IL-1β synthesis in monocytes stimulated with NapA wt and NapA deletion mutants. Cytokines' mRNAs were quantified by Real time PCR. The experiment shown is representative out of 4 experiments conducted with different cell preparations. (AU, arbitrary units).

5.2.3 *NapA mutants retain the ability to engage TLR2*

Finally, to further investigate whether NapA mutants retain the ability to engage the TLR2, which is crucial for the immuno-modulatory properties of the wild type protein (Codolo et al., 2008), we evaluated the induction of IL-23 in the presence of a blocking anti-TLR2 antibody. As clearly depicted in Figure 24 the presence of the antibody significantly reduced the ability of NapA, both wt and mutant, to promote the synthesis and release of IL-23, suggesting that the portion of the protein responsible for the binding to the receptor does not localize within the two N- and C-terminal tails.

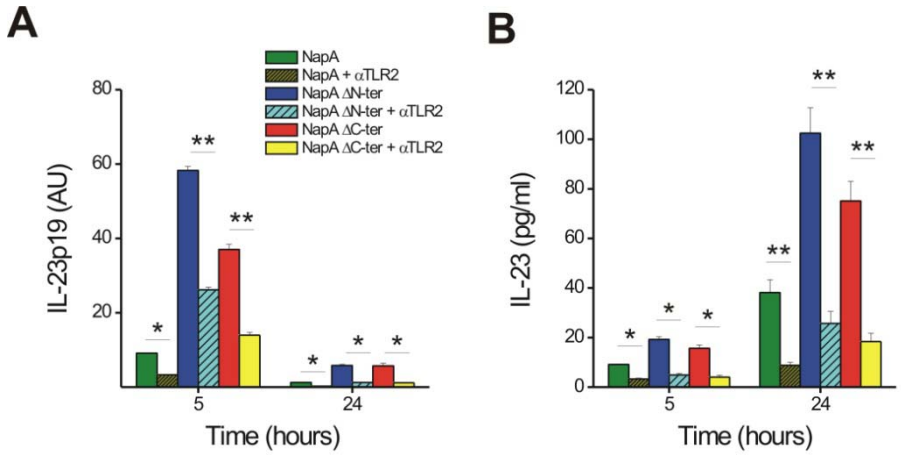

Figure 24: The immuno modulatory properties of NapA deletion mutants occur through the engagement of TLR2. Monocytes were pre-incubated or not for 2 hours with 20 µg/ml of an anti TLR2 blocking antibody before being exposed to NapA wt or to NapA mutants. After 5 and 24 hours cells were harvested and mRNA levels of IL-23p19 quantified by Real time PCR (A). The experiment shown is representative of three experiments conducted with different cell preparations. (AU, arbitrary units). The culture supernatants of the same cells were collected and used for the ELISA evaluation of the secreted IL-23 cytokine (B). Significance was determined by Student's t test; *, $p<0.05$ and **, $0.01< p<0.05$ versus anti TLR2 treated cells.

The surface of NapA we present in this study lacks 13 residues at the N-terminus and 20 at the C-terminus, but both these domains are not involved in the interaction, as demonstrated by the fact that the deletion mutants retain their immuno-modulatory properties as the wild-type protein or even more. Consequently, part of the external surface of our NapA model must contain the interaction site. The external surface of the extracellular region of TLR2 is quite hydrophilic, with a distribution of both positively and negatively charged amino acid side chains spread around the all surface. Something similar is true for both NapA and HP-NAP, whose exposed surface presents a large number of negative and positive charges, the latter outnumbering the former (Zanotti et al., 2002). It is consequently possible to hypothesize that NapA and HP-NAP directly interacts with the extracellular region of TLR2 via polar and electrostatic interaction and, in doing so, they favor TLR2 dimerization in a way perhaps reminiscent to the dimerization of TLR4 mediated by *E. coli* MD-2 (Park *et al.*, 2009). However, the charge distributions on NapA and HP-NAP are quite different (Figure 25), making it hard to find a conserved area that could represent the surface of interaction with the receptor. This is in line with the evidence that the two

bacterial proteins induce different immunological responses and this could be the consequence of two different way of interaction with TLR2.

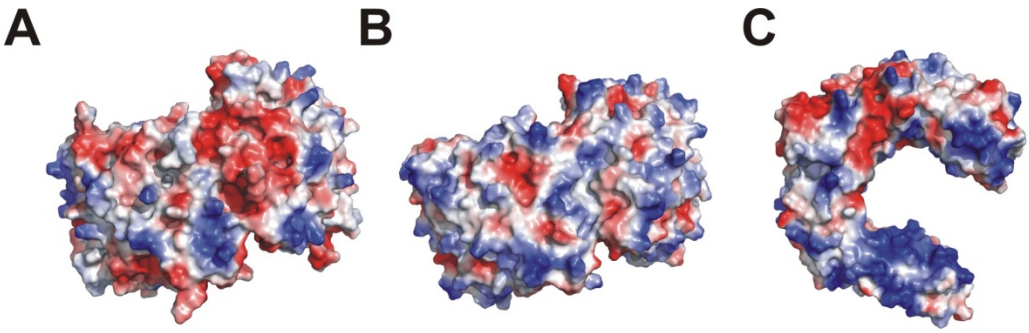

Figure 25: Molecular surface of NapA (A), HP-NAP (B) and TLR2 (C) colored according to the Coulomb electrostatic potential (red, negative; blue, positive; minimum and maximum between -88 and +88 for A and B, from -60 to +60 for C). Only the tetramers of NapA and HP-NAP, oriented in the same way, are shown.

5.3 Evaluation of the possible role for NapA in triggering inflammation in synovial cavity

Lyme arthritis is characterized by progressive joint damage that is mediated by several mechanisms (Figure 26). The inflammatory process is characterized by infiltration of inflammatory cells into the joints, leading to the proliferation of fibroblast-like synoviocytes (FLS) and the destruction of cartilage and bone, which is associated with the formation of a proliferating pannus. Pannus is a new synovial tissue that grows over the articular surface and erodes into the cartilage and underlying bone, contributing to joint destruction (Walsh, 1999). The interface between pannus and cartilage is occupied predominantly by activated macrophages and synovial fibroblasts that express matrix metalloproteinases and cathepsins.

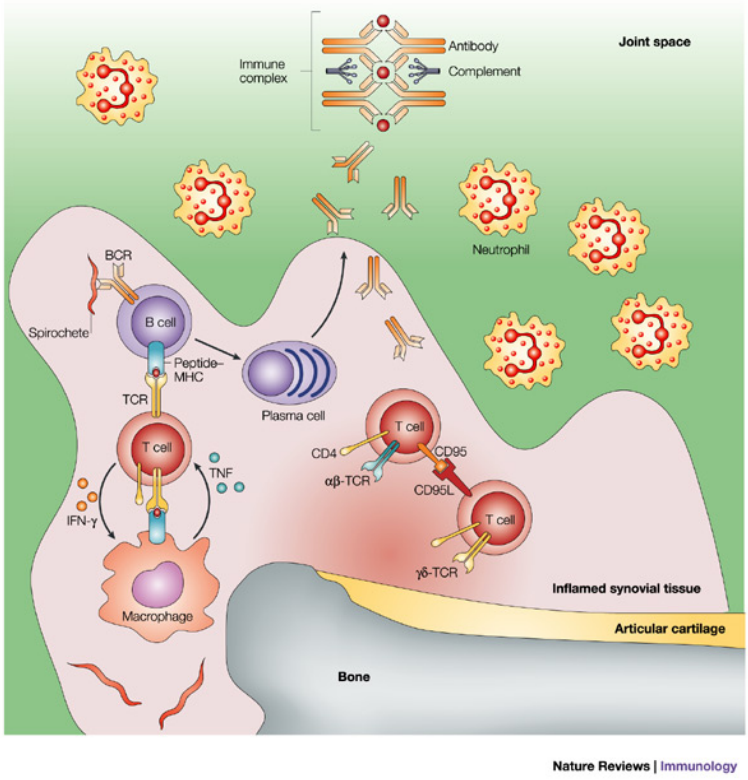

Figure 26: Immune responses to Borrelia burgdorferi in an inflamed joint. After dissemination of the spirochete from the skin to the joint, the synovial tissue is infiltrated by mononuclear cells, including macrophages, T cells, B cells and plasma cells. Joint fluid, contains large numbers of neutrophils, T lymphocytes and inflammatory cytokines (from(Steere & Glickstein, 2004).

The inflammatory infiltrate in the joints consists mainly of neutrophils and monocytes, as reported in mouse model studies (Barthold *et al.*, 1990), and the high levels of chemokines found in the joint tissue well correlate with the recruitment of these two cell types into the site of infection. As demonstrated by Brown C.R. and colleagues (Brown *et al.*, 2003), the recruitment of neutrophils into the infected joint appears to be a prerequisite for the development of Lyme arthritis and neutrophils play a nonphagocytic immunoregulatory role in the development of pathology (Figure 27).

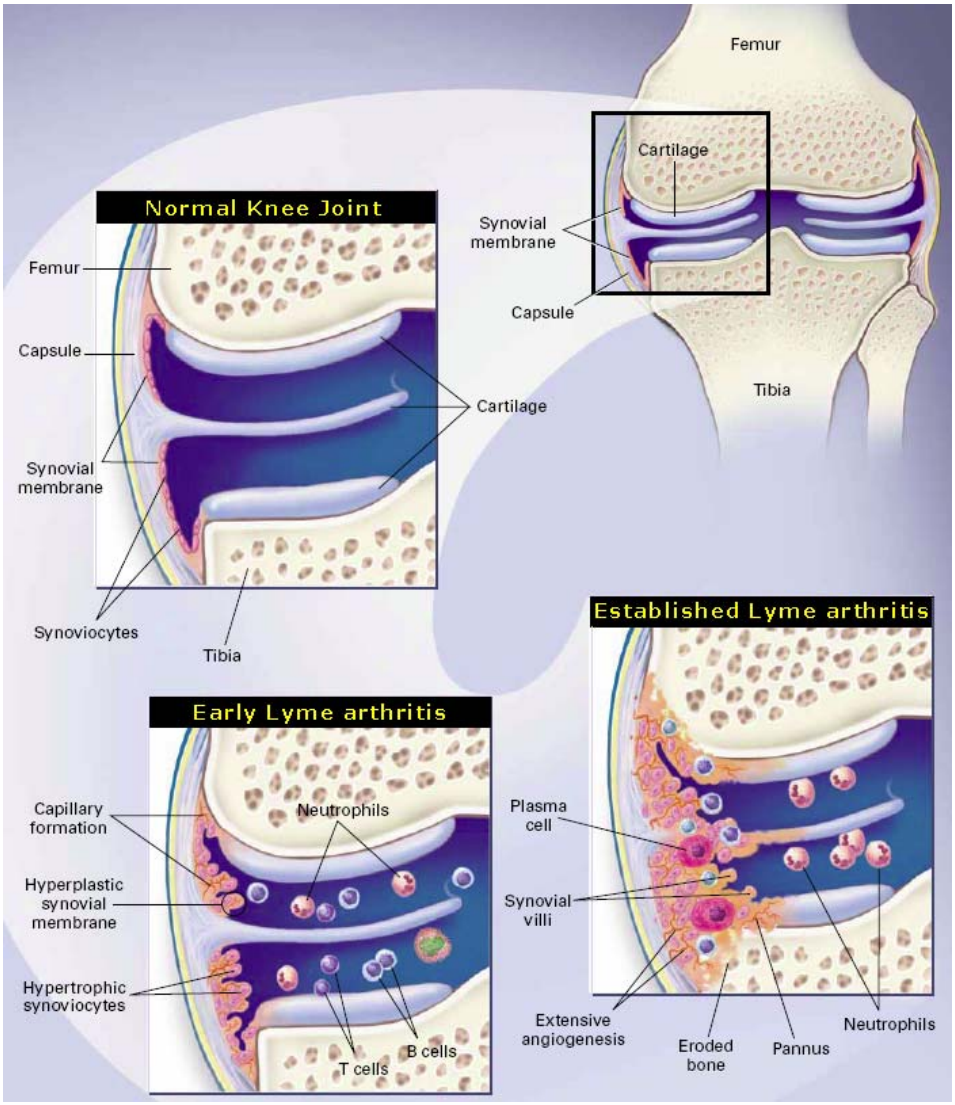

Figure 27: Pathogenesis of Lyme Arthritis. In the normal knee joint, the synovium consists of a synovial membrane (usually one or two cells thick) and underlying loose connective tissue. Synovial-lining cells are designated type A (macrophage-like synoviocytes) or type B (fibroblast-like synoviocytes). In early Lyme arthritis, the synovial membrane becomes thickened because of hyperplasia and hypertrophy of the synoviallining cells. An extensive network of new blood vessels is formed in the synovium. T cells (predominantly CD4+) and B cells (some of which become plasma cells) infiltrate the synovial membrane. These cells are also found in the synovial fluid, along with large numbers of neutrophils. In the early stages of Lyme arthritis, the synovial membrane begins to invade the cartilage. In established Lyme arthritis, the synovial membrane becomes transformed into inflammatory tissue, the pannus. This tissue invades and destroys adjacent cartilage and bone. The pannus consists of both type A and type B synoviocytes and plasma cells modified from (Choy & Panayi, 2001).

Migration of leukocytes into the synovium is a regulated multistep process involving interactions between leukocytes, endothelial cells and adhesion molecules express on cell surface, as well as between leukocytes and chemokines (Szekanecz *et al.*, 2006). Chemokines are small, chemoattractant cytokines that play a key role in the accumulation of inflammatory cells at the site of infection. Therefore, chemokines and chemokine receptors are considered to be therapeutic targets in several chronic inflammatory disorders. In humans there are more than 50 types of chemokines that were originally identified by their chemotactic activity on bone marrow-derived cells (Charo & Ransohoff, 2006). They are classified into four families according to the location of cysteine residues. The four chemokine groups are CC, C, CXC and CX3C, where C is a cysteine and X any aminoacid residue, and their receptors are consequently classified as CCR, CR, CXCR and CX3CR. The chemokine receptors are inserted in the cell membrane through seven transmembrane helical segments coupled to a G-protein that transduces the intracellular signal. The two major subclasses include the CC chemokines (where the cysteines are neighboring) and the CXC chemokines (where the cysteines are separated by one amino acid). The CXC chemokines mainly act on neutrophils and lymphocytes, whereas the CC chemokines mainly act on monocytes and lymphocytes without affecting neutrophils (Luster, 1998). One characteristic feature of chemokines is the redundancy of the system. Several chemokines bind to more than one receptor, and the majority of chemokine receptors have multiple ligands, leading to the generation of multiple pathways directing similar cellular responses.

Synovial tissue and synovial fluid from Lyme arthritis patients, as well as other chronic inflammatory arthritis, contain high concentrations of several chemokines. The chemokine system is considered to be implicated in LA definire pathogenesis *via* the recruitment and retention of neutrophils, monocytes and T lymphocytes into the joints (Brown et al., 2003).

It is known that dissemination of *Borrelia* through the blood stream, ultimates in the invasion of tissues, such as the joints and the heart, where it expressess several proteins: Osps, variable surface antigen, heat shock proteins

and also NapA (Akin *et al.*, 1999, Codolo et al., 2008, Shanafelt *et al.*, 1991, Guerau-de-Arellano & Huber, 2005).

On the basis of our previous observation that NapA is involved in the pathogenesis of Lyme arthritis through the induction of Th17 mediated immune response (Codolo et al., 2008), we focused our attention to understand the possible involvement of NapA in cells recruitment in synovia of Lyme arthritis patients, through the induction of chemokines production.

5.3.1 *NapA is able to recruit neutrophils and T lymphocytes in rat synovia*

In an effort to elucidate the involvement of NapA in the recruitment of inflammatory cells during Lyme arthritis manifestations we decided, in collaboration with the group of Prof. Tedesco in Trieste, to study NapA activity in a rat model of arthritis (Fischetti *et al.*, 2007).

Animals knees were intra-articularly treated with NapA or sterile saline as control and after 2 hours or 2 days of treatment the infiltrated cells in synovia were collecetd and analyzed. 2 hours after the administration of the protein, we observed a neutrophil accumulation in the joint cavity, whereas after 2 days of treatment lympho-monocytes were the most abundant population (Figure 28).

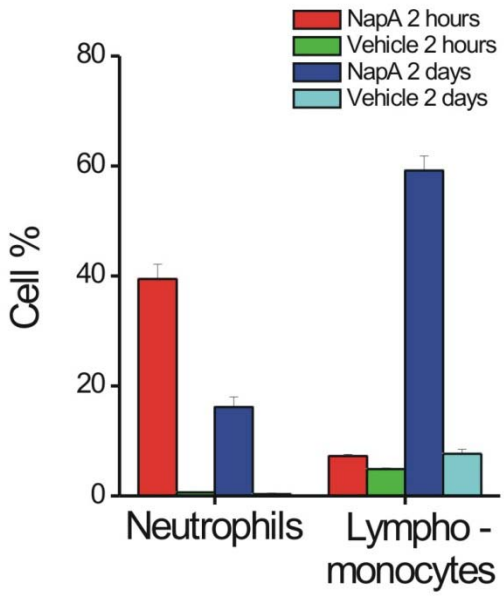

Figure 28: Cell recruitment in rat synovia. Arthritis was induced by intra-articular administration of NapA (10 μM/50μl of sterile saline) into the right knee; an equivalent volume of saline was injected into the left knee, as control. After 2 hours and 2 days two distinct groups of animals were killed and intra-articular cavity was washed with 2 ml of saline. Cells collected were stained: neutrophils were identified for their myeloperoxidase content, and lympho-monocytes were counted using May Grunwald- Giemsa staining.

In order to better define the phenotype of lympho-monocytes infiltrated in rat synovia after 2 days of treatment, synovial tissue was analyzed by immunohistochemistry using anti- CD3 and anti-IFN-γ antibodies; IFN-γ is the hallmark cytokine produced by $CD4^+$ Th1 cells, which dominates the late stage of Lyme disease (Keane-Myers & Nickell, 1995, Matyniak & Reiner, 1995).

Figure 29 shows that synovial tissue of rat treated with NapA is positive for CD3 (brown) and IFN-γ (blue) staining, whereas control samples are negative for both the staining.

Collectively, these data demonstrate that NapA is able to recruit neutrophils and lymphocytes in synovia, and a proportion of the latter belongs to the Th1 subset. These data correlate with previous observations reported by Gergel and Furie (Gergel & Furie, 2004) who demonstrated that populations of T lymphocytes that migrated across *B. burgdorferi*-activated endothelial cells were significantly enriched in IFN-γ secreting cells.

According to our data, demonstrating that NapA is able to drive Th17 immune response in the pathogenesis of LA, we should have verified by immunohistochemistry also the presence of Th17 lymphocytes in rat synovia, but at the present there are not commercial anti-IL-17 antibodies specific for the rat molecule.

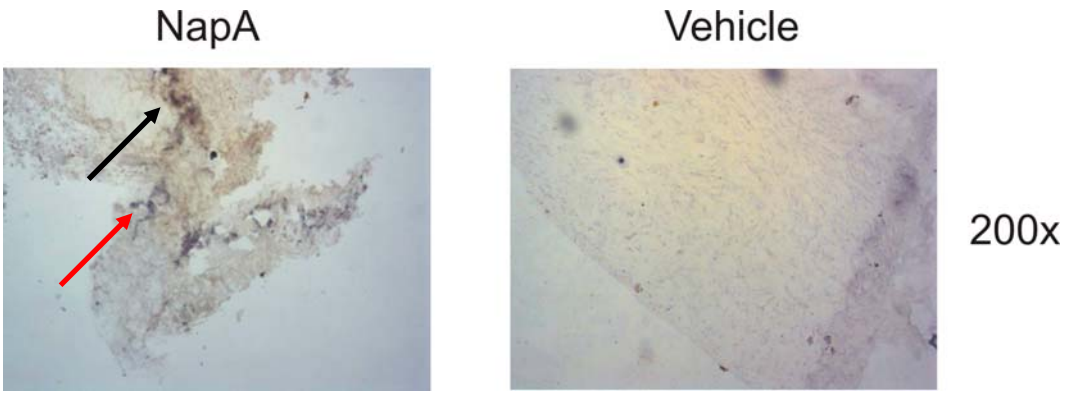

Figure 29: Immunohistochemical staining of CD3 and IFN-γ in synovial tissue. Synovia sections from rats, treated or not with NapA 10 μM for two days, were paraffin embedded and stained using anti-CD3 (brown), as indicated by black arrow, and anti-IFN-γ (blue) antibodies, as indicated by red arrow.

On the basis of these premises, we moved to investigate the mechanism by which NapA triggered the recruitment of immune and inflammatory cells. To this purpose we first characterized the chemokines accumulated in the same synovial fluids collected for the cell count.

Although probably many cytokines analyzed in samples derived from rats' articular lavage were under the threshold of detectability because of the strong sample dilution (rat intra-articular liquid and cells were collected by washing with 2 ml of saline), nevertheless, we observed CCL2 and CXCL1 accumulation after 2 hours of NapA administration (Figure 30); the level of both chemokines dropped down after 2 days of treatment.

CCL2 is a potent chemoattractant and activator of monocytes, while CXCL1 is a potent neutrophil chemoattractant (Rollins, 1997). Our observation is in agreement with that of Brown and colleagues who demonstrated that, in the joint tissue of mice infected with *B. burgdorferi*, high levels of CXCL1 and CCL2 correlate with the recruitment of neutrophils and monocytes into the site of infection. Moreover, they have demonstrated that CXCL1 and CCL2 are differentially expressed in joints of mice susceptible to develop Lyme arthritis, with respect to a resistant mouse strains, and that the accumulation of these specific chemokines correlated with the onset of arthritis (Brown *et al*., 2003).

We also observed, only in articular lavages from rats treated with NapA for 2 hours, a little accumulation of CCL20 (data not shown); this chemokine, produced by different cells, is chemotactic for lymphocytes and weakly attracts neutrophils.

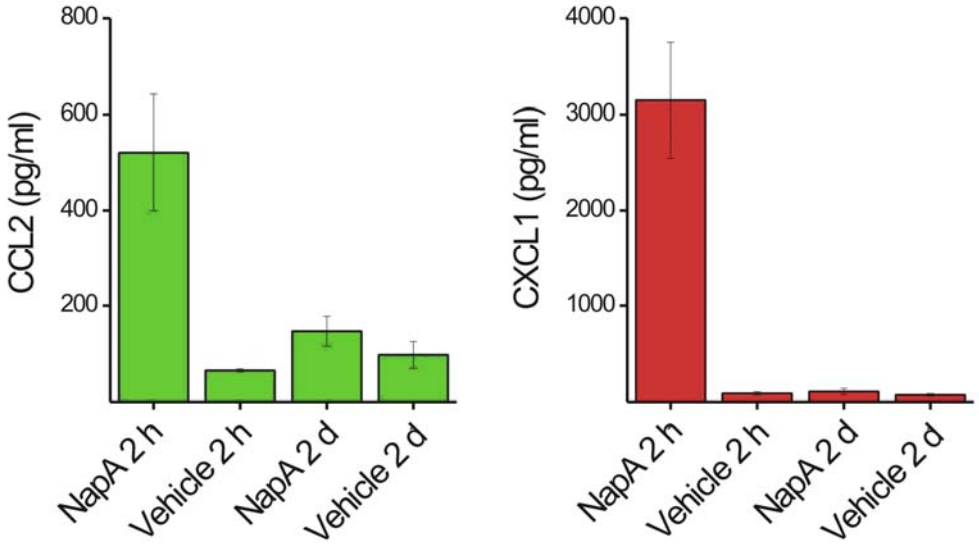

Figure 30: Chemokine accumulation in rat synovia. Samples of articular lavage fluid from NapA and vehicle treated rats were collected and the chemokine content was quantified in cell free articular lavage fluid by a Milliplex kit. Mean values (±SD) are reported.

On the basis of the results described above, we investigated the role of NapA in neutrophils and T lymphocytes recruitment. In a first set of experiments we observed that NapA, *per se*, is not able to recruit neutrophils and T lymphocytes. These observations suggest that, probably, NapA is not directly a chemotactict factor for immune cells. Therefore, it could be hypothesized that NapA acts indirectly inducing the production of factors able to attract neutrophils and T lymphocytes to the site of infection.

Particularly, on the basis of CCL2, CCL20 and CXCL1 accumulation in synovial fluids of rats treated with NapA, we wondered which cells could be the source of these mediators.

We performed preliminar experiments aimed to evaluate the production of chemokines in macrophages and neutrophils, after NapA stimulation. We focused our attention on these cells because macrophages are resident cells in synovia (Shahrara *et al.*, 2008), and for this reason, once activated, macrophages are able to produce pro-inflammatory molecules involved in the recruitment of other cells to the site of infection. Neutrophils, instead, are the first cells recruited in synovial cavity of Lyme arthritis-affected patients.

From our preliminary observations it seems that both cell types might be able to produce chemokines involved in neutrophils and T lymphocytes recruitment. This is an intresting observation that we will deeply investigate. These data could correlate with those reported by Pellettier and colleagues (Pelletier *et al.*) who have recently demonstrated that IFN-γ plus LPS-activated neutrophils release chemokines, such as CCL2 and CCL20 which, in turn, are responsible for the recruitment of Th17 cells. Moreover, they showed that activated neutrophils, *via* the release of CXCL10 and CCL2, may also trigger the chemotaxis of Th1 cells. Finally, they demonstrated that, upon activation, human Th17 cells can directly chemoattract neutrophils through the release of CXCL8.
Therefore, according to Pellettier *et al.*, neutrophils/Th cells interaction might create a pathogenic proinflammatory loop that ultimately amplifies the local accumulation of neutrophils and Th cells during inflammatory disease. We can speculate that such a loop could also occur during the inflammatory events in the pathogenesis of Lyme arthritis. In order to verify this hypothesis, we are currently performing experiments aimed to evaluate the phenotype of T lymphocytes recruited across a HUVEC-derived endothelium, under stimulation with supernatants harvested from either neutrophils or macrophages activated with NapA.

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