CEREBROSPINAL FLUID T-REGULATORY CELLS RECOGNIZE *BORRELIA BURGDORFERI* NAPA IN CHRONIC LYME BORRELIOSIS

A. AMEDEI[1], G. CODOLO[2,3], D. OZOLINS[4], C. BALLERINI[5], T. BIAGIOLI[5]
I. JAUNALKSNE[6], A. ZILEVICA[4], S. D'ELIOS[1], M. DE BERNARD[2,3] and M.M. D'ELIOS[1]

[1]Department of Experimental and Clinical Medicine, University of Firenze, Italy; [2]Department of Biology, University of Padova, Padova, Italy; [3]Venetian Institute of Molecular Medicine, University of Padova, Padova, Italy; [4]Microbiology, Faculty of Medicine, University of Latvia, Riga, Latvia [5]Department of Neurological Sciences, University of Firenze, Firenze, Italy; [6]Clinical Immunology Center, Riga, Latvia

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The first two authors contributed equally to this work

The NapA protein of *B. burgdorferi* is essential for the persistence of spirochetes in ticks. One of the most intriguing aspects of NapA is its potential to interfere with the host immune system. Here, we investigated the role of the acquired immune responses induced by NapA in the cerebrospinal fluids (CSF) of patients with chronic Lyme borreliosis. We evaluated the cytokine profile induced in microglia cells and CSF T cells following NapA stimulation. We report here that NapA induced a regulatory T (Treg) response in the CSF of patients with chronic Lyme borreliosis and it is able to expand this suppressive response by promoting the production of TGF-β and IL-10 by microglia cells. Collectively, these data strongly support a central role of NapA in promoting both Treg response and immune suppression in the CSF of patients with chronic Lyme borreliosis and suggest that NapA and the Treg pathway may represent novel therapeutic targets for the prevention and treatment of the disease.

Lyme borreliosis (LB) is the most frequent vector-borne infection in the United States and is endemic in Europe and parts of Asia (1). The infectious agent of LB, *Borrelia burgdorferi*, is transmitted to the host during the blood meal of an attached, infected Ixodes tick. The clinical outcomes of the infection differ between individuals and are related to both the type of host response and the bacterial subspecies (2, 3). After invasion into the skin, *Borrelia* can cause a local infection, resulting in an initial inflammatory response, called erythema migrans. During the second stage of Lyme disease, the bacterium can spread from the tick bite on the skin to various secondary organs throughout the body, including heart, joints, and peripheral and central nervous system (4). Major clinical findings of the neurological manifestation of acute Lyme neuroborreliosis include painful meningoradiculitis with inflammation of the nerve roots and lancinating, radicular pain (Bannwarth's syndrome), lymphocytic meningitis, and various forms of cranial or peripheral neuritis (5).

Although LB is treatable with antibiotics, there are patients who, despite completing treatment, have persistent symptoms, including musculosketal

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Mailing address: Prof. Marina de Bernard,
Venetian Institute of Molecular Medicine,
Via Orus 2,
35129 Padova, Italy
Tel.: +39 049 7923 223
Fax: +39 049 7923 250
e-mail: marina.debernard@unipd.it

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pain, paraesthesia, fatigue and subjective alteration of cognition; this constellation of symptoms has been referred to as "chronic LB" (6). Several mechanisms have been proposed to explain the persistence of *Borrelia* within the host (7), but how the bacterium manages to evade eradication and, in untreated/resistant individuals, trigger chronic infection, remains largely unknown. The mechanism behind the persisting symptoms is also unclear; however, it has been hypothesized that the chronic manifestations might be the result of injury caused by an immunopathological response (7).

A common strategy used by microorganisms to extend their survival involves the induction of strong regulatory responses that are usually physiologically associated with the fading of the host effector immune responses. Regulatory T cells (Tregs), characterized by the expression of CD4, $CD25^{high}$ and Foxp3, are hypothesized to play a significant role in the regulation of inflammatory responses via inhibition of T cells by both cell-cell interaction and by secretion of anti-inflammatory cytokines, such as IL-10 and transforming growth factor (TGF)-β (8, 9). Tregs require the transcription factor forkhead box 3 (Foxp3) for their development and function (10). Although it has been shown that *Borrelia* induces the secretion of IL-10 in mononuclear cells (11), and that the clearance of the bacterium in IL-10 deficient mice is ten times higher than in their wild-type littermates (12), the role of a T regulatory response in the persistence of *Borrelia* in the host, has never been addressed.

The chromosomal gene *bb0690* of *B. burgdorferi* encodes a protein termed Neutrophil Activating Protein A (NapA), that was shown to be essential for the persistence of spirochetae in ticks (13). The *B. burgdorferi* NapA protein is homologous to *Treponema pallidum* TpF1 and *Helicobacter pylori* HP-NAP that are endowed with several immunoregulatory and immunomodulatory properties (14-18). As *Treponema pallidum* and *Helicobacter pylori*, *Borrelia spp.* are bacteria able to cause chronic infections in humans. We have previously demonstrated that in *B. burgdorferi*-infected patients with Lyme arthritis there are NapA-specific T helper cells and that NapA is able to sustain, at least in part, experimental arthritis (19). Given that Treg-mediated suppression of cellular immunity has been observed in different chronic infections (18-19) we evaluated the *Borrelia*-induced Treg responses in patients with chronic LB. In this study, we report that NapA drives a cerebrospinal fluid (CSF) T regulatory response in patients with chronic LB. We show that CSF T cells from patients with chronic LB include a proportion of $CD4^+CD25^+Foxp3^+$ cells, which produce TGF-β and IL-10 in response to NapA. Furthermore, we report that NapA strongly stimulates microglia to release IL-10 and TGF-β, two key cytokines in driving Treg cell differentiation.

Taken together, these results suggest that NapA is a *Borrelia* virulence factor involved in the pathogenesis of chronic LB because it permits the bacterium to survive in the host by down-regulating the immune response.

MATERIALS AND METHODS

Patients

After approval of the local Ethics Committee and after informed consent, 5 patients (3 males and 2 females, mean age 57 years; range 43-62) with chronic LB and 4 with Multiple Sclerosis (MS) were enrolled in the study. LB was diagnosed according to the European clinical case definition (4), i.e. defined as an erythema migrans ≥ 5 cm or clinically relevant neurological symptoms, mononuclear pleocytosis in CSF ($\geq 5 \times 10^6$ cells/l) and *Borrelia*-specific antibodies in serum. The LB patients were treated with ceftriaxon and/or doxyciclin with no significant beneficial effect and displayed positive serological test for anti-Borrelia (Biorad). Patients diagnosed with chronic LB had had symptoms for longer than 6 months.

Reagents

Human recombinant IL-2 were provided by Chiron Corp. PHA (M form) was purchased from Gibco Laboratories, ionomycin and phorbol 12-myristate 13-acetate (PMA) from Sigma. Anti-CD3, anti-CD4, anti-CD8, and anti-CD25 were purchased from BD Biosciences. Foxp3 determination was carried out according to the manufacturer's instructions (eBiosciences). The repertoire of the TCR Vß chain of T cell clones was analyzed with a panel of 24 monocolonal antibodies specific to the following: Vß1, Vß2, Vß3, Vß4, Vß5.1,Vß5.2, Vß5.3, Vß7.1, Vß7.2, Vß8, Vß9, Vß11, Vß12, Vß13.1, Vß13.2, Vß13.6, Vß14, Vß16, Vß17, Vß18, Vß20, Vß21.3, Vß22 and Vß23 (TCR Vß Repertoire Kit, Beckman Coulter); isotype-matched non-specific Ig was used as negative control. NapA was cloned, expressed, and purified from *Bacillus subtilis*, as detailed elsewhere (19).

Production of DCs

Dendritic cells were generated by culturing purified $CD14^+$ cells, as previously described (17). Briefly, CD14+ cells were sorted from PBMCs and induced to differentiate into dendritic cells (DCs) by culturing in medium supplemented with 5% FCS (Hyclone), 50 ng/ml GM-CSF and 20 ng/ml IL-4. On day 7, all CD14+ cells lost CD14 and acquired the expression of the DC marker CD1a. Accordingly, in the same cells, CD80 and HLA-DR expression was increased, whereas CD86 expression was decreased.

Generation of T-cell clones from cerebrospinal fluid of LB patients

Upon approval of the local Ethics Committee, cerebrospinal fluid T cells were obtained, from all the 5 patients with chronic LB and from the 4 patients with MS after informed consent. Aspiration of cerebrospinal fluid was carried out by atraumatic lumbar puncture for diagnostic or therapeutic reasons.

Fresh cerebrospinal fluid-derived T cells $CD4^+$ T cells were purified by MACS using CD4 microbeads (Miltenyi Biotec.). The purity of the sorted population was 95-99%. $CD4^+$ T cells were then separated through the use of CD25 microbeads (Miltenyi Biotec.) in two different groups: $CD4^+CD25^-$ and $CD4^+CD25^+$ T cells.

$CD4^+CD25^-$ T cells were stimulated with autologus DCs (1:1 ratio) in presence of NapA (10 μg/ml). On day 6, reactive cells were expanded with recombinant IL-2 (20 U/ml), as previously described (19).

$CD4^+CD25^+$ T cells were stimulated with autologus DCs (1:1 ratio) loaded with NapA (10 μg/ml) in the presence of recombinant IL-2 (100 U/ml). T-cell blasts from NapA-specific T-cell lines obtained from $CD4^+CD25^-$ T cells and from NapA-specific $CD4^+CD25^+$ T cells of the same donor were cloned under limiting dilution (0.3 cell/well), as previously described (20). Briefly, single T-cell blasts were seeded in microwells (0.3 cells/well) in the presence of 2×10^5 irradiated (9000 rad) PBMC, PHA (0.5% V/V) and IL-2 (50 U/ml). At weekly intervals, 2×10^5 irradiated PBMCs and IL-2 were added to each microculture to maintain the expansion of growing clones. T-cell clones were screened for responsiveness to NapA by measuring [^{3}H]thymidine (Amersham Pharmacia Biotech) uptake after 60 h co-culture with irradiated autologous mononuclear cells in the presence of medium or NapA (10 μg/ml). The mitogenic index (MI) was calculated as the ratio between mean values of cpm (counts per minute) obtained in stimulated cultures and those obtained in the presence of medium alone. A MI ≥ 5 was considered as positive, as previously reported (17).

Cytofluorimetric analysis of T-cell markers and characterization of the cytokine profile of T-cell clones

Cell surface marker analysis of T cells was carried out by flow cytometry using fluorochrome-conjugated anti-CD3, anti-CD4, anti-CD25, anti-Foxp3. Cells were analyzed by a BD FACScan cytofluorimeter using Diva software (BD Biosciences). A total of 10^4 events for each sample were acquired.

To induce the cytokine production by NapA-specific T-cell clones, 10^6 T-cell blasts of each clone were co-cultured for 48 h in 1 ml medium with 5×10^5 irradiated autologous PBMCs as antigen presenting cells (APC) with or without NapA (10 μg/ml).

To elicit cytokine production by non-NapA-specific T-cell clones, T-cell blasts were resuspended at 10^6/ml medium and cultured for 36 h in the presence of PMA (10 ng/ml) and ionomycin (200 ng/ml). Cell-free supernatants were collected and assayed in duplicate for IFN-γ, IL-17, IL-10 and TGF-β by using commercial ELISA (BioSource International).

Foxp3 expression was tested in presumed clones first, and the cytokine and suppressive properties resulted from those lines that were Foxp3+.

Treg cell suppressive assays

The proliferation of NapA-specific Teff clones was evaluated with or without NapA and antigen presenting cells in co-culture with NapA-specific or non-NapA-specific Treg clone. 2×10^4 blasts of the of NapA-specific Teff clones were cultured with 4×10^3 irradiated (9000 rad) autologous antigen-loaded or antigen-unloaded DCs in the presence of 2×10^4 blasts of NapA-specific or non-NapA-specific Treg clones. On day 4, after 8 h of pulsing with 0.5 mCi 3H-thymidine/well, cultures were harvested and radionucleotide uptake measured.

Microglia cell culture and activation

Microglial cells from human source were purchased from Clonexpress Company and cultured according to the manufacturer's instructions. Microglial cells were cultured for 7 days and detached using trypsin (0.25%) and DNase (50 μg/ml) before being seeded at 5×105 cells/ml in their conditioned media. Cells were exposed to medium alone, NapA (10 μg/ml) or purified lipopolysaccharide (LPS) (1 μg/ml) (Sigma-Aldrich).

Cytokine detection in culture supernatants

Culture supernatants of microglia were collected at different time points, and the amount of IL-23, IL-6, IL-10, TGF-β and IL-1β was quantified by ELISA (BioSource International).

Statistical analysis

Data were expressed as mean values ± SD. Statistical significance was calculated by unpaired Student's *t*-test. A p-value≤ 0.05 was considered significant.

RESULTS

$CD4^+CD25^{+high}FoxP3^+$ T cells in the CSF of patients with chronic Lyme borreliosis

After isolation of CSF cells from patients, $CD4^+$ T cells were purified by MACS using CD4 microbeads, and $CD25^+$ or $CD25^-$ cells were selectively recovered. After separation, the purified fraction of $CD4^+CD25^+$ T cells expressed Foxp3, whereas $CD4^+CD25^-$ T cells were $Foxp3^-$ (Fig. 1). Both $CD4^+CD25^-$ and $CD4^+CD25^+$ T cell fractions were cloned by limiting dilution.

T cells were characterized for expression of CD4, CD25, Foxp3 and TCR Vβ chain by flow cytometry, for cytokine production, for suppressive activity and proliferation in response to NapA. $Foxp3^+$

Table I. *Antigen specificity of T-cell clones generated from CSF of patients with chronic Lyme borreliosis.*

	NapA-specificity of CSF-derived T-cell clones	
LB patients		
	N° of specific / Treg clones	N° of specific /Teff clones
1.	2/16	2/12
2.	1/11	3/15
3.	2/14	2/16
4.	2/18	2/13
5.	2/12	2/13
All LB patients	***9/71 (12%)***	***12/73 (16%)***
All MS patients	*0/12*	*0/4*

LB: chronic Lyme borreliosis; MS: multiple sclerosis

Table II. *Cytokine secretion by microglia cells stimulated with NapA or LPS.*

	stimulus for cytokine production		
Cytokine (pg/ml) production	Medium alone	NapA (10 μg/ml)	LPS (1 μg/ml)
TGF-β	179.4± 3.5	8970.3±236.4	540.2±25.6
IL-6	130.2± 1.3	260.6±17.3	3820.6±250.4
IL-23	17.6±4.3	145.4±15.1	155.6±16.5
IL-10	15.5±1.7	730.2±31.2	142.3±13.1
IL-1β	27.8±9.5	81.8±16.6	1060.3±105.4

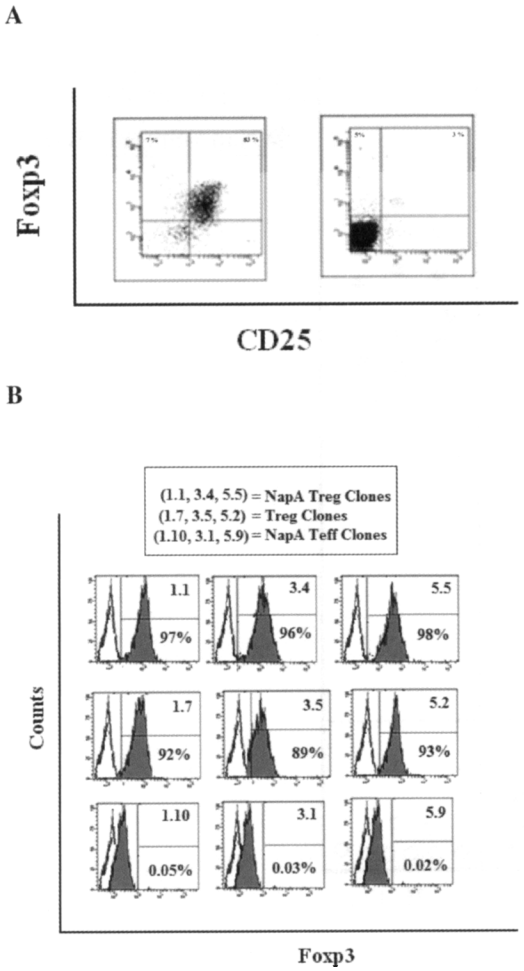

Fig. 1. *Flow-cytometric expression of CD25 and Foxp3 in $CD3^+CD4^+CD25^{+high}$ (Panel **A**, left) and $CD3^+CD4^+CD25^-$ (Panel **A**, right) purified cell fractions obtained from CSF infiltrating lymphocytes. Panel **B** shows the Foxp3 (grey) and isotype control (white) expression of Nap-A specific (NapA Treg Clones), or non specific (NapA Treg clones) or NapA-specific T effector clones (NapA Teff Clones). The figure shows one representative out of three experiments carried out in duplicate.*

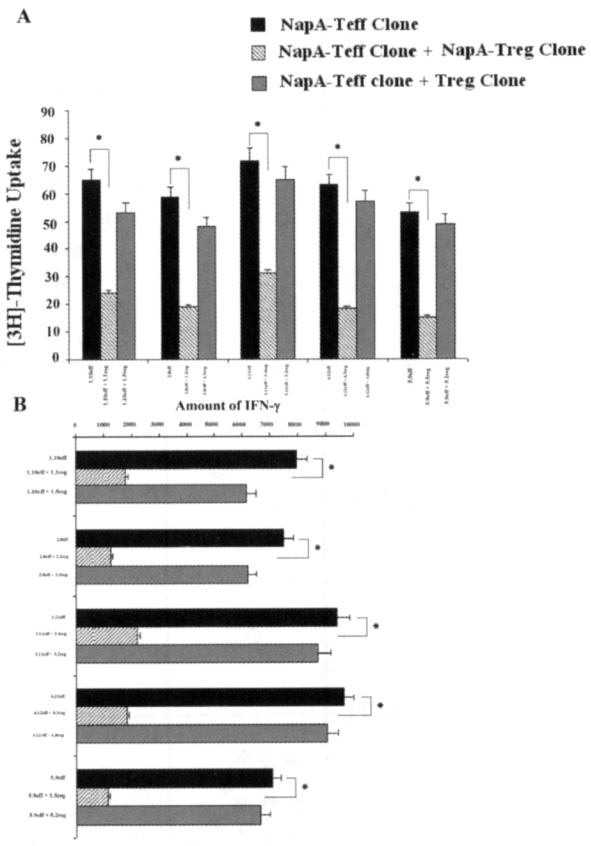

Fig. 2. *Inhibition of proliferation and cytokine production of NapA-specific Teff clones by NapA-specific Treg clones. **A)** Effect of NapA-specific- or NapA-non specific-Treg on the antigen-induced proliferation of NapA-specific Teff clones following expansion of antigen-specific $CD4^+CD25^+Foxp3^{+high}$ T lymphocytes by NapA-loaded autologous DCs. Proliferative data obtained (mean±SD) were subjected to statistical analysis. * $p < 0.01$. **B)** Inhibition of NapA-specific Teff clones antigen-induced IFN-g production by NapA-specific- or NapA-non specific-Treg clones. The figure shows the mean values±SD of three experiments carried out in duplicate. The number before the dot identifies the patient, while the number after the dot defines the clone.*

clones producing TGF-β and/or IL-10 were named as T regulatory (Treg) clones whereas $Foxp3^-$ clones producing IFN-γ and/or IL-17 were named as T effector (Teff) clones. Among the 71 CSF-derived T regulatory clones (Treg), none showed proliferation when cultured in medium alone whereas 9 (12%) proliferated in response to NapA and irradiated autologous DCs (Table I). On the other hand, from the CSF of the same patients we isolated 73 T-cell effector clones (Teff). Twelve of them (16%) proliferated to NapA protein. In the same experimental conditions, no $CD4^+$ T-cell clones, isolated from the CSF of the 4 MS patients, proliferated in response to NapA.

Treg clones exhibited suppressive phenotype

The suppressive phenotype of all Treg clones, isolated from CSF of LB patients, was assessed by evaluating: i) their ability to inhibit the proliferative response of autologous $CD4^+CD25^-$ to allogenic stimulation, ii) the expression of Foxp3, and iii) the

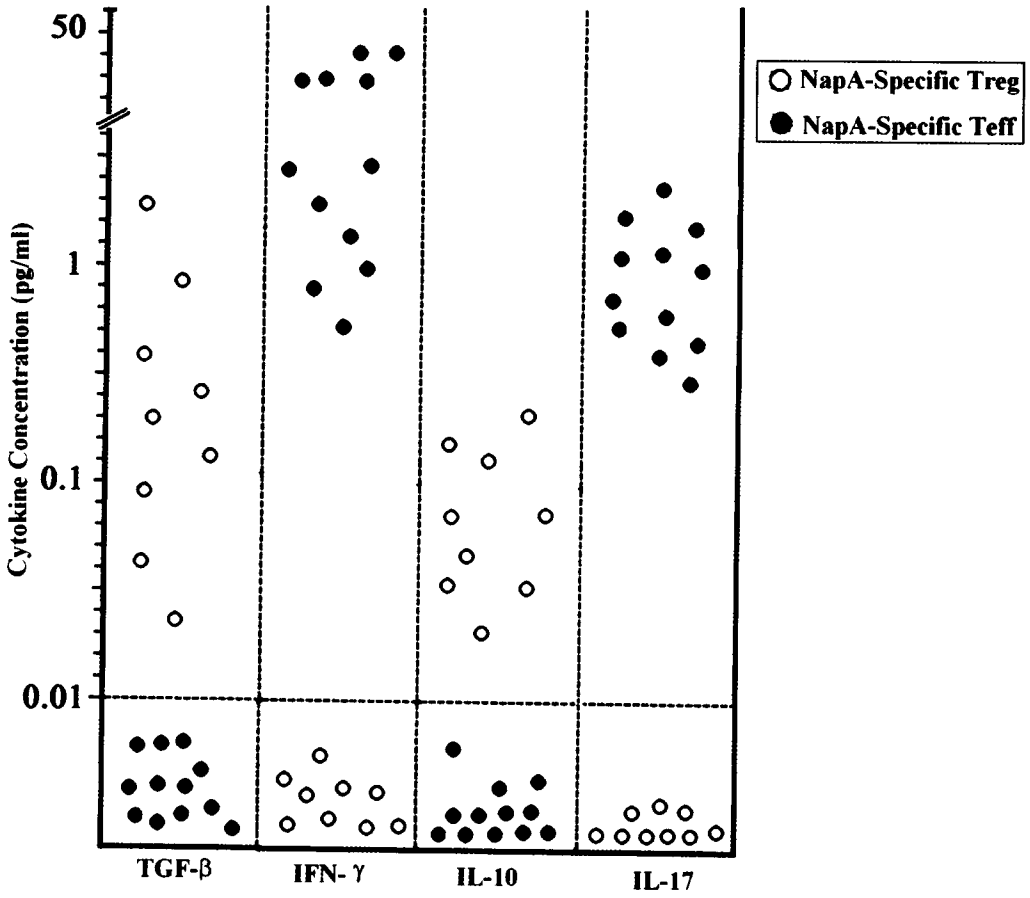

Fig. 3. *Ag-induced cytokine production by T-cell clones derived from CSF of patients with chronic Lyme borreliosis. Both Teff clones and Treg clones were stimulated with NapA (10 mg/ml); TGF-b, IFN-g, IL-10 and IL-17 production was measured in culture supernatants by specific ELISA kits. In supernatants of control cultures stimulated without antigen, with medium alone, levels of cytokines were <0.01 ng/ml. The figure shows the mean values±SD of three experiments carried out in duplicate. Dotted lines represent 3 SD over the mean levels of cytokines measured in control cultures.*

ability to produce cytokines after NapA stimulation. As shown in Fig. 2A, the NapA-specific Teff clones vigorously proliferated to NapA stimulation; notably, the addition of NapA-specific-Treg clones to NapA-specific-Teff clones significantly (in a ratio of 1:1) suppressed the proliferation of the Teff clones, whereas the addition of non-NapA-specific Treg did not (Fig. 2A). Thus, all nine Treg clones exhibited a detectable inhibitory activity (30-70%) on the proliferation of autologous NapA-specific T-cell effector clones.

Moreover, Treg and Teff clones were characterized for Foxp3 expression and cytokine production. Treg clones displayed both high Foxp3 expression (Fig. 2 B) and the ability to produce TGF-β, and IL-10 but not IL-17 nor IFN-α (Fig. 3). On the other hand, all T-cell effector clones were negative for Foxp3 and contained variable proportions of T-cell blasts able to produce IFN-γ and/or IL-17, when stimulated with NapA and autologous APCs (Fig. 3). It is of note that the production of IFN-γ and/or IL-17 was strongly suppressed by the presence of autologous

NapA specific Treg cells (Fig. 2). Using non-NapA-specific Treg no inhibition of cytokine production was present.

NapA promotes microglia TGF-β and IL-10 production

Constitutive synthesis of cytokines by quiescent microglia is relatively limited, but activation is accompanied by a significant increase of cytokine production (21). Considering the evidence that *Borrelia* induces the secretion of IL-10 in mononuclear cells (11) we examined the possible capacity of NapA in creating a cytokine milieu polarizing towards the T regulatory phenotype by acting on microglia isolated from healthy donors. Therefore, we assayed the secretion of TGF-β and IL-10 by microglia, following stimulation with NapA or LPS as control. The release of IL-6, IL-23 and IL-1β was also evaluated: indeed, the expression of the latter cytokines is expected to be down-modulated in case microglia was switched to a pro-Treg profile (21). As shown in Table II, in respect to non-stimulated cells, LPS-activated microglia exhibited substantial increase in the release of IL-1β and IL-6 (range 30–40-fold), and only a moderate increase in the secretion of IL-10 and IL-23 (range 10–20-fold). The levels of TGF-β were only minimally affected by LPS activation (<3 fold). On the other hand, the stimulation with NapA induced a strong increase of TGF-β and IL-10 secretion (range 40–50-fold) and only a limited increase of the other cytokines, such as IL-1β, IL-6, IL-23. These findings indicate that NapA, acting on microglia, contributes to create a cytokine *milieu*, enriched in TGF-β and IL-10, able to promote the development of Treg response (21).

DISCUSSION

Despite adequate antibiotic treatment, Lyme borreliosis (LB) can develop into a chronic condition with persisting symptoms such as musculoskeletal pain, subjective alteration of cognition and fatigue. The mechanism behind this is unclear, but it has been postulated that an aberrant immunological response might be the cause (1, 19). Accordingly, chronic LB patients displayed a lower number of *Borrelia*-specific IFN-γ-secreting cells compared to previously *Borrelia*-exposed asymptomatic individuals (22), thus supporting the notion that a strong Th1 immune response in the course of the disease is for the host the best strategy to face *Borrelia* infection (1, 19, 23-25). Interestingly, chronic LB patients had higher amounts of *Borrelia*-specific FoxP3 mRNA than healthy controls (22). Therefore, the possibility exists that regulatory T cells might also play a role, by immunosuppression, in the development of chronic LB. The activation of Treg function may provide a successful evasion strategy for *Borrelia* species. In diseases caused by different microorganisms such as parasites (26), bacteria (27-28) or viruses, Treg-mediated suppression of cellular immunity has been observed, especially in the course of chronic infections. For example in syphilis, the miniferritin TpF1 produced by *T. pallidum* displays a strong ability to promote the development of regulatory T cells (18).

In this study, we characterized the CSF $CD4^+$ T cell-response in chronic LB patients; we also evaluated the potential involvement of the antigen NapA, produced by *B. burgdorferi*, in driving a specific profile of the adaptive immune response in neuroborreliosis. The latter idea stemmed from our previous observations that *B. burgdorferi*-infected patients with Lyme arthritis were able to display both antibody- and synovial fluid T-cell specific for NapA (19).

For the first time, we were able to isolate $CD4^+CD25^{+high}FoxP3^+$ Treg cells from CSF of patients with chronic LB. Notably, among 71 CSF-derived T regulatory clones, 12% of them proliferated in response to NapA.

We derived nine NapA-specific Treg clones from patients with chronic LB. All these Treg, in dose-dependent fashion, exhibited inhibitory activity on the proliferation of autologous NapA-specific T effector cells clones. Moreover, Treg clones were found to be highly expressing Foxp3 and to produce IL-10 and TGF-β but not IL-17 and IFN-γ. On the contrary, Teff clones derived from a $CD4^+CD25^-$ T-cell fraction, were negative for Foxp3, but were able to produce, following NapA stimulation, a high amount of IFN-γ and/or IL-17. These two cytokines have been reported to accumulate also in synovial fluid of patients affected by Lyme arthritis (19, 29, 30), and this indicates that eliciting a Th1/Th17 profile within the effector adaptive immune response

is a peculiarity of *Borrelia*.

TGF-β together with IL-10 are potent cytokines able to activate Treg cell responses and it is plausible that the relative amount of the different cytokines (i.e. TGF-β, IL-10, IL-6, IL-1β and IL- 23) present in the local microenvironment might dictate the T cell profile and therefore the progression or the blockage of the infection.

Therefore, we evaluated the microglia production of TGF-β, IL-10, IL-6, IL-23 and IL-1β following activation with NapA. We have found that the stimulation with NapA induced a relevant secretion of TGF-β and IL-10 by microglia but a moderate release of other cytokines, such as IL-1β, IL-6 and IL-23. These findings indicate that NapA, acting on microglia, contributes to create a cytokine milieu enriched in TGF-β and IL-10 that may drive the differentiation of T cells towards the Treg phenotype.

Taken together, the present results demonstrate that in patients with chronic LB, *B. burgdorferi* NapA protein represents a possible mechanism able to generate specific T regulatory cells and also to make them stable by stimulating microglia to produce a strong amount of TGF-β and IL-10 cytokines.

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