Borrelia burgdorferi NapA–Driven Th17 Cell Inflammation in Lyme Arthritis

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Objective. Human Lyme arthritis caused by *Borrelia burgdorferi* is characterized by an inflammatory infiltrate that consists mainly of neutrophils and T cells. This study was undertaken to evaluate the role of the innate and acquired immune responses elicited by the neutrophil-activating protein A (NapA) of *B burgdorferi* in patients with Lyme arthritis.

Methods. Serum anti-NapA antibodies were measured in 27 patients with Lyme arthritis and 30 healthy control subjects. The cytokine profile of synovial fluid T cells specific for NapA was investigated in 5 patients with Lyme arthritis. The cytokine profile induced by NapA in neutrophils and monocytes was also investigated.

Results. Serum anti-NapA antibodies were found in 48% of the patients with Lyme arthritis but were undetectable in the healthy controls. T cells from the synovial fluid of patients with Lyme arthritis produced interleukin-17 (IL-17) in response to NapA. Moreover, NapA was able to induce the expression of IL-23 in neutrophils and monocytes, as well as the expression of IL-6, IL-1β, and transforming growth factor β (TGFβ) in monocytes, via Toll-like receptor 2.

Conclusion. These findings indicate that NapA of *B burgdorferi* is able to drive the expression of IL-6, IL-1β, IL-23, and TGFβ by cells of the innate immune system and to elicit a synovial fluid Th17 cell response that might play a crucial role in the pathogenesis of Lyme arthritis.

Three pathogenic *Borrelia* species are primarily responsible for Lyme disease, the most common tick-transmitted illness of the boreal hemisphere (1,2). In Europe, Lyme disease is caused by *Borrelia burgdorferi*, *Borrelia afzelii*, and *Borrelia garinii*, whereas in the US, *Borrelia burgdorferi* is the sole cause (3,4). Lyme disease usually begins with a characteristic expanding skin lesion, erythema migrans (5). After several days or weeks, the spirochete may spread to different sites, causing meningitis, cranial or peripheral neuritis, carditis, atrioventricular node block, or migratory musculoskeletal pain. Months later, Lyme arthritis may develop in untreated patients with *Borrelia* infection, with a rapid onset of marked swelling and pain of affected joints, most commonly the knee (6). In these patients, synovial fluid contains polymorphonuclear and mononuclear cells (7). In most patients, Lyme arthritis responds to appropriate oral or intravenous antibiotic therapy. However, in rare cases, proliferative synovitis persists after

>2 months of treatment with oral antibiotics or >1 month of treatment with intravenous antibiotics, or both. This condition is called antibiotic-refractory Lyme arthritis (3).

The host immune response to *B burgdorferi* influences the clinical outcome of the infection. T lymphocytes, particularly interferon-γ (IFNγ)–secreting Th1 cells, have been proposed to play a central role in Lyme arthritis (8–10). However, in mice, Th1 cells have been shown not to be essential in the induction of Lyme arthritis (11,12), suggesting the involvement of mediators different from IFNγ and interleukin-12 (IL-12) and of other T cell subsets in the pathogenesis of the disease. Th17 cells, a new subset of T helper cells, play a crucial role in the induction of autoimmune tissue injury via the release of IL-17 (13). IL-6, transforming growth factor β (TGFβ), IL-1β, and IL-23 are key cytokines in the differentiation and expansion of Th17 cells (14,15). In turn, IL-17 induces the release of several proinflammatory cytokines and chemokines by stromal cells, synoviocytes, chondrocytes, fibroblasts, and macrophages, and recruits and activates neutrophils (16). Intriguingly, IL-17 levels are elevated in synovial fluid from patients with rheumatoid arthritis (17), where it promotes the formation of osteoclasts (18), and several experimental observations suggest its involvement in the pathogenesis of murine Lyme arthritis (19,20).

A major unanswered question about the chemical nature of the bacterial factor(s) responsible for the induction of Th17 cells remains. Although a role of the outer membrane lipoproteins has been suggested (19), bacterial products are known to possess immunomodulatory properties and to drive different types of innate and adaptive immune responses. We previously characterized the neutrophil-activating protein of *Helicobacter pylori* (HP-NAP), a bacterium that causes chronic infections in humans, as does *B burgdorferi*. This protein is a strong immunogen and is endowed with immunomodulatory properties (21–25). The chromosomal gene *bb0690* of *B burgdorferi* encodes neutrophil-activating protein A (NapA), which is homologous to HP-NAP (22,26), and NapA was shown to be essential for the persistence of spirochetes within ticks (27).

In the present study, we found that in patients with Lyme arthritis, NapA induces a humoral response and drives Th17 cell inflammation. We show that T helper cells from the synovial fluid of patients with Lyme arthritis produce IL-17 in response to NapA. Furthermore, we report that NapA is a Toll-like receptor 2 (TLR-2) agonist that is able to stimulate monocyte expression of IL-23, IL-6, IL-1β, and TGFβ, key cytokines for Th17 cell differentiation. Together, these re-

sults suggest that NapA is a *B burgdorferi* virulence factor involved in the pathogenesis of Th17 cell inflammation in patients with Lyme arthritis.

PATIENTS AND METHODS

Reagents. Recombinant human IL-2, tetanus toxoid (TT), and purified protein derivative (PPD) were provided by Chiron (Emeryville, CA). The TLR2.1 blocking monoclonal antibody (mAb) against TLR-2 was obtained from eBioscience (San Diego, CA). Polyclonal anti-NapA antibody was raised in rabbits using the purified recombinant protein as antigen. Anti-CD3 IgG from OKT3 hybridoma supernatants were affinity purified on MabTrap (GE Healthcare, Piscataway, NJ).

Cloning and protein purification. NapA was cloned, expressed, and purified from *Bacillus subtilis*. The NapA gene was amplified by polymerase chain reaction (PCR) from *B burgdorferi* strain B31, using standard methods. The following primers were used: 5′-CCCGAGCTCATAAAGGAGATAG-TTATG-3′ and 5′-CCCAAGCTTCTATTTTGCATCACA-CTC-3′ (underlining indicates *Sac* I and *Hind* III restriction enzyme sites, respectively). The amplified fragment was digested and inserted into the expression vector pSM214G, resulting in plasmid pSM214G-NapA. *B subtilis* strain SMS 118 containing the plasmid pSM214G-NapA was grown for 15 hours in YT medium and 15 μg/ml of chloramphenicol. After centrifugation, cells were resuspended in 30 m*M* Tris HCl, pH 7.8, and lysed through 3 French press passages. Debris was removed by centrifugation, and ammonium sulfate 60% was added to the supernatant.

After centrifugation at 32,000g, the resulting supernatant was dialyzed and loaded onto a prepacked anion-exchange column (Mono Q fast-protein liquid chromatography column; GE Healthcare). Using a linear gradient from 0.1*M* to 0.4*M* NaCl in buffer A, NapA was eluted in a range of 0.41–0.51*M* NaCl. The fractions containing the protein were pooled, and dithiothreitol (DTT) was added to a final concentration of 10 m*M*. NapA was further purified by gel-filtration chromatography (Superdex 200 HR 10/30; GE Healthcare) with phosphate buffer, pH 7.8, and 10 m*M* DTT. Protein was concentrated using a Centricon ultrafiltration system (Millipore, Bedford, MA). The final product was checked for purity in a Coomassie brilliant blue–stained gel and analyzed by Western blotting with a specific polyclonal antibody.

A lipidated preparation of *B burgdorferi* sensu stricto (strain ATCC 35211; American Type Culture Collection, Manassas, VA) outer surface protein A (OspA), purified as previously reported (28), was tested for its ability to promote cytokine release by neutrophils and monocytes. In all of the experiments we performed, OspA lipoprotein was used at a concentration of 100 pg/ml. At this concentration, OspA was able to activate NF-κB, as evaluated with the use of an electrophoretic mobility shift assay kit specific for NF-κB (Panomics, Redwood City, CA).

Patient and control samples and NapA-specific antibody assay. Serum samples examined for the presence of NapA-specific antibodies were obtained from 46 patients with Lyme disease who were living in the northeastern US. Of these 46 patients, 27 had Lyme arthritis, 9 had erythema migrans, and 10 had facial palsy. All patients with Lyme disease met the Centers for Disease Control and Prevention (CDC) case

definition for the condition (29). For comparison, serum samples from 30 healthy control subjects, 20 patients with rheumatoid arthritis, 20 patients with sepsis, and 20 patients with pneumonia were also tested.

The 9 patients with erythema migrans had 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 (30). 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 2-test approach of enzyme-linked immunosorbent assay (ELISA) and Western blotting, interpreted according to the CDC criteria (31). The serum samples from patients with facial palsy or Lyme arthritis were selected randomly from patients seen during the 25-year period from 1977 through 2001. However, a high proportion of patients in the Lyme arthritis group had an antibiotic-refractory course; 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. These patients had participated in a study on immunity in Lyme arthritis (32). Clinical information about these patients was obtained by review of their medical records.

For the ELISA, serum samples were added to a 96-well plate that had been coated with purified recombinant NapA (1 μg/well). Horseradish peroxidase–conjugated anti-human IgG subclass antibody was added to each well, and color was developed with ABTS. Absorbance was read at 450 nm.

Synovial fluid T cells from Lyme arthritis patients. Upon approval of the local ethics committee and after patients gave their informed consent, synovial fluid T cells were obtained from 5 patients with Lyme arthritis (3 men and 2 women with a median age of 51 years [range 42–58 years]) who were seropositive for anti-NapA antibodies. Synovial fluid was aspirated at the time of knee joint biopsy performed for diagnostic or therapeutic reasons. Fresh synovial fluid–derived T cells (1×10^5) were stimulated with immobilized anti-CD3 (OKT3) mAb (5 μg/ml) in round-bottomed microwell plates (33). After 72 hours of stimulation, supernatants were collected and assayed for IL-17 and tumor necrosis factor α (TNFα) using ELISA kits from R&D Systems (Minneapolis, MN) and BioSource (Camarillo, CA), respectively.

After removal of the supernatant, IL-2 was added to the synovial fluid–derived anti-CD3–induced T cell line every 3 days. On day 15, T cell blasts from each synovial fluid T cell line and T cells derived from peripheral blood mononuclear cells were stimulated for 48 hours with NapA, TT, or PPD in the presence of autologous antigen-presenting cells (APCs) in enzyme-linked immunospot (ELISpot) microplates that had been coated with anti–IL-17 antibody (eBioscience). Cells stimulated with medium alone served as negative controls. At the end of the culture period, the number of IL-17 spot-forming cells was counted.

Preparation of neutrophils and monocytes. Neutrophils and monocytes from healthy donors were prepared as described previously (34). The neutrophils and monocytes were cultured in RPMI 1640 containing 10% fetal calf serum (FCS) in the presence of 1.7 μg/ml of NapA, 100 pg/ml of OspA, or phosphate buffered saline (PBS; control). When

required, cells were preincubated for 2 hours with 20 μg/ml of anti–TLR-2 blocking mAb before exposure to NapA.

Real-time PCR analysis. Total RNA was isolated from 2×10^6 monocytes using TRIzol solution (Invitrogen, San Diego, CA) according to the manufacturer's instructions. RNA was reverse-transcribed and amplified with the following primers: for GAPDH, 5'-AGCAACAGGGTGGTGGAC-3' and 5'-GTGTGGTGGGGGACTGAG-3'; for IL-23p19, 5'-TCC-ACCAGGGTCTGATTTTT-3' and 5'-TTGAAGCGGAGA-AGGAGACG-3'; for IL-12p40, 5'-ACAAAGGAGGCGAG-GTTCTAA-3' and 5'-CCCTTGGGGGGTCAGAAGAG-3'; for IL-6, 5'-AACCTGAACCTTCCAAAGATGG-3' and 5'-TCT-GGCTTGTTCCTCACTACT-3'; for TGFβ, 5'-AGTGGTTG-AGCCGTGGAG-3' and 5'-CCATGAGAAGCAGGAAAG-G-3'; and for IL-1β, 5'-CTGTCCTGCGTGTTGAAAGA-3' and 5'-TTGGGTAATTTTTGGGATCTACA-3'. After the amplification, data analysis was performed using the second derivative method algorithm. For each sample, the amount of messenger RNA (mRNA) of the cytokines (IL-23p19, IL-12p40, IL-6, TGFβ, and IL-1β) was expressed as the n-fold of the normalized amount of mRNA in untreated cells (1 arbitrary unit = cytokine mRNA concentration/GAPDH mRNA concentration [both in fmoles/μl]).

Detection of IL-23, IL-6, TGFβ, and IL-1β in culture supernatants. Culture supernatants from the neutrophils and monocytes that had been harvested for quantification of mRNA levels were collected at the same time points after NapA or OspA administration for quantification of cytokine protein levels. The amount of IL-23 in supernatants from both cell types and the amounts of IL-6, TGFβ, and IL-1β in supernatants from monocytes were quantified by ELISA, using kits obtained from BioSource.

TLR screening. Human embryonic kidney (HEK) 293 cells that constitutively express TLR-2/CD14 (HEK 293-TLR-2/CD14) or TLR-4/CD14/myeloid differentiation 2 (MD-2) (HEK 293-TLR-4/CD14/MD-2) (InvivoGen, San Diego, CA) were grown in low-glucose Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated FCS. Before the experiments, HEK 293 cells were plated in 24-well tissue culture plates at a density of 5×10^5 cells/ml. Adherent cells were collected at various time points after the addition of 100 ng/ml of lipopolysaccharide (as a positive control ligand of TLR-4), 1.3 μg/ml of HP-NAP (as a positive control ligand of TLR-2) (34), or 1.7 μg/ml of 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 orthovanadate, phenylmethylsulfonyl fluoride, leupeptin, pepstatin, and aprotinin) by incubation on ice for 5 minutes.

Lysates were cleared by centrifugation, and proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. After blocking with Tris buffered saline–Tween (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, Beverly, MA). After stripping, blots were reprobed with an anti–α-actin antibody (GE Healthcare), which was used as a control for equal loading.

Statistical analysis. Data are reported as the mean $\pm$ SD. Student's t-test was used for statistical analysis of the

differences between experimental groups. *P* values less than or equal to 0.05 were considered significant.

RESULTS

NapA antibody response in patients with Lyme arthritis. To evaluate the role of NapA in patients with *B burgdorferi*–associated pathologic conditions, we tested by ELISA whether serum samples from infected individuals with different clinical outcomes of Lyme disease contained specific antibodies to this protein. Of the 27 patients with Lyme arthritis, a late manifestation of the disorder, 13 (48%) had circulating antibodies specific for NapA (Figure 1). 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 subjects.
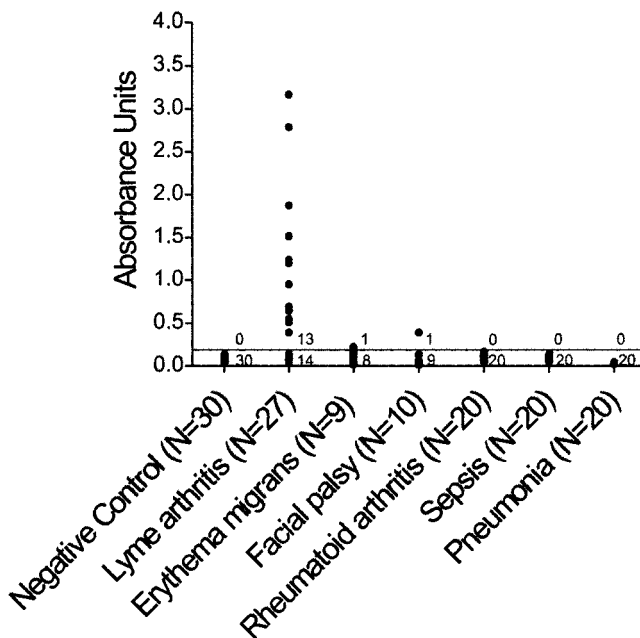

Figure 1. NapA immunogenicity in patients with Lyme arthritis. NapA-specific antibodies in 46 patients with Lyme disease, 20 patients with rheumatoid arthritis, 20 patients with sepsis, 20 patients with pneumonia, and 30 healthy controls were determined using a specific enzyme-linked immunosorbent assay (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 >3 SD above the mean value in normal control subjects, and 0.2 absorbance units (horizontal line) was considered the threshold.

Table 1. Comparison of clinical features in 27 patients with Lyme arthritis, according to the presence or absence of NapA antibody

	NapA positive (n = 13)	NapA negative (n = 14)
Age, median (range) years	18 (7–62)	46 (11–65)
Sex, no. male/female	10/3	10/4
Time from arthritis onset to start of treatment, median (range) months	4 (0.25–37)	9 (0.25–84)
Time from treatment to sampling date, median (range) months	3.5 (0.25–12)	0.5 (0–24)
Time from treatment to resolution of arthritis, median (range) months	7 (0.5–22)	3 (1–48)
Antibiotic response group, no. of patients*		
Antibiotic-refractory arthritis	9	6
Antibiotic-responsive arthritis	3	7

* One patient in each antibody status group did not receive antibiotic therapy.

Among the 27 patients with Lyme arthritis, those with positive responses to NapA antibody tended to be younger, to have a longer duration of arthritis after antibiotic treatment, and to have an antibiotic-refractory course compared with those with negative responses to NapA antibody (Table 1). However, these differences were not statistically significant.

In order to define the relevance of the NapA serologic findings in Lyme arthritis patients and to exclude potential cross-reactivity, we tested serum samples from 20 patients with rheumatoid arthritis, 20 patients with sepsis, and 20 patients with pneumonia for NapA reactivity. Figure 1 shows that none of these sera had circulating antibodies that cross-reacted with NapA.

NapA-driven IL-17 secretion by synovial fluid T helper cells from patients with Lyme arthritis. We next examined whether synovial fluid T cells from patients with Lyme arthritis produced IL-17. In preliminary experiments, we used synovial fluid mononuclear cells to generate T cell lines, and then we analyzed their release of IL-17 and TNFα in culture supernatants. After 72 hours of stimulation with immobilized anti-CD3 mAb, synovial fluid mononuclear cells produced high levels of IL-17 and TNFα (Figure 2A). Subsequently, synovial fluid–derived anti-CD3 mAb–induced T cell lines were expanded by the addition of IL-2 every 3 days. On day 15, T cell blasts from each synovial fluid T cell line were stimulated in a concentration-dependent curve with NapA, TT, or PPD in the presence of autologous APCs for 48 hours in ELISpot microplates that had been coated with anti–IL-17 antibody. At the end of the culture period, the number of IL-17 spot-forming cells was counted. A significant proportion of all synovial
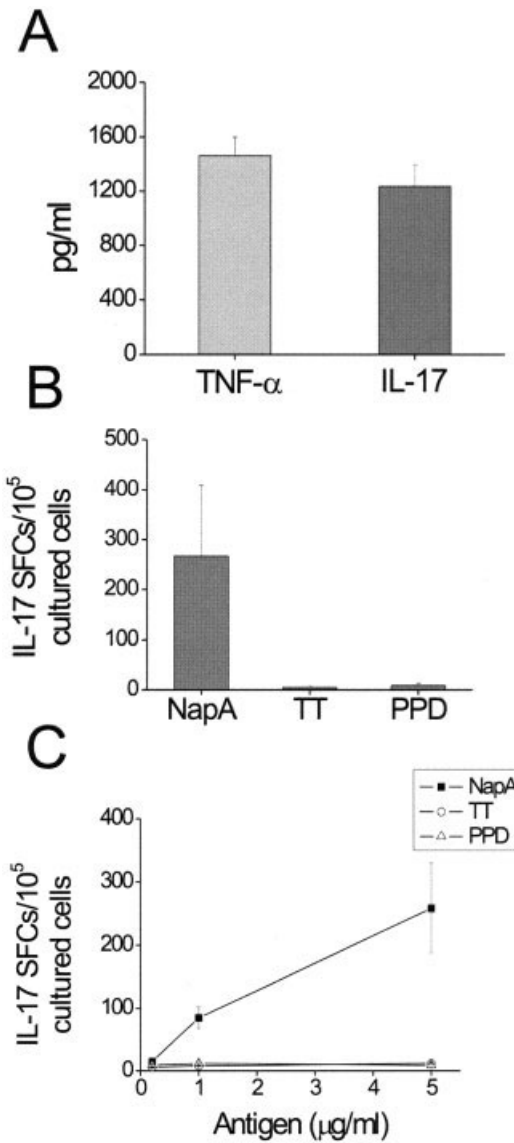
Figure 2. NapA-driven interleukin-17 (IL-17) secretion by synovial fluid T helper cells from patients with Lyme arthritis. **A,** Release of tumor necrosis factor α (TNFα) and IL-17 from fresh synovial fluid mononuclear cells after 72 hours of stimulation with immobilized anti-CD3 monoclonal antibody (mAb). **B,** Numbers of IL-17 spot-forming cells (SFCs) following stimulation of synovial fluid mononuclear cells with NapA, tetanus toxoid (TT), or purified protein derivative (PPD). Synovial fluid mononuclear cells were stimulated with immobilized anti-CD3 mAb, synovial fluid–derived T cell lines were expanded with IL-2, and on day 15, T cell blasts from each line were stimulated for 48 hours with NapA, TT, or PPD in the presence of irradiated autologous antigen-presenting cells in enzyme-linked immunospot microplates coated with anti–IL-17 antibody. IL-17 spot-forming cells were then counted using an automated reader. After specific stimulation with NapA, a significant proportion of synovial fluid–derived T helper cells produced IL-17. **C,** Dose-response effect of graded concentrations of antigens on the numbers of IL-17 spot-forming cells. Values are the mean ± SD number of spot-forming cells per 10^5 cultured cells over background levels. Values in **A** and **B** are the mean and SD.

fluid–derived T cell lines, under major histocompatibility complex–restricted conditions, reacted specifically to NapA by producing IL-17 in a dose-dependent manner (Figures 2B and C), while TT and PPD were ineffective at any of the concentrations we used.

NapA activation of neutrophils and monocytes to release cytokines essential for the differentiation of Th17 cells. *B burgdorferi* activates bone marrow–derived dendritic cells to release IL-23, which in turn promotes T cells to release IL-17 (35). IL-23 is a heterodimeric cytokine that shares the p40 subunit with IL-12 but has a distinct p19 subunit (36). Figure 3 shows that NapA was able to induce the expression of both IL-12p40 and IL-23p19 in neutrophils and monocytes isolated from
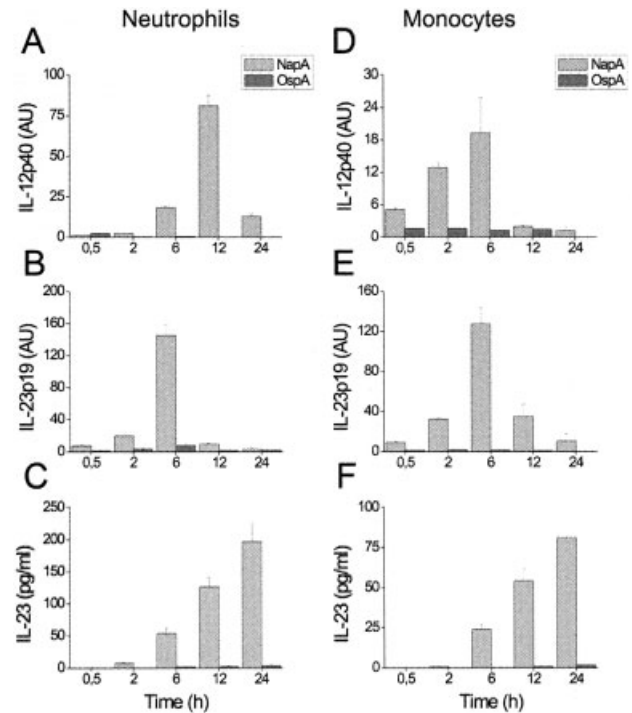

Figure 3. Kinetics of the synthesis and production of interleukin-23 (IL-23) by neutrophils and monocytes stimulated with NapA or OspA. Levels of IL-12p40 and IL-23p19 cytokine mRNA in neutrophils (**A** and **B**) and monocytes (**D** and **E**) from healthy donors were determined at the indicated time points after NapA or OspA stimulation, by quantitative real-time polymerase chain reaction analysis. Shown are representative results from 1 of 7 experiments conducted with different cell preparations. Values are the mean and SD arbitrary units (AU). Levels of IL-23 protein in neutrophils (**C**) and monocytes (**F**) from healthy donors were determined at the indicated time points after NapA or OspA stimulation, by enzyme-linked immunosorbent assay of culture supernatants from the same cells that had been harvested for mRNA evaluation. The kinetics of production were comparable among the different experiments, whereas the amounts produced varied among the different donors. Values are the mean and SD of 4 independent experiments using triplicate samples.
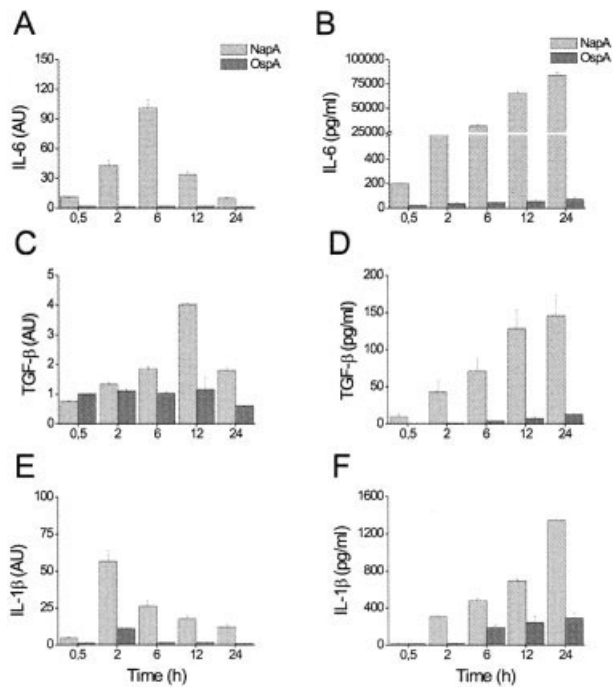
Figure 4. Kinetics of the synthesis and production of interleukin-6 (IL-6), transforming growth factor β (TGFβ), and IL-1β by normal monocytes stimulated with NapA or OspA. **A, C,** and **E,** Levels of IL-6, TGFβ, and IL-1β mRNA were determined by quantitative real-time polymerase chain reaction at the indicated time points after NapA or OspA stimulation. AU = arbitrary units. **B, D,** and **F,** Levels of IL-6, TGFβ, and IL-1β protein were determined by enzyme-linked immunosorbent assay at the indicated time points after NapA or OspA stimulation. Culture supernatants from the same monocytes that were harvested for mRNA evaluation were used for these analyses. Values are the mean and SD of 4 independent experiments using triplicate samples.
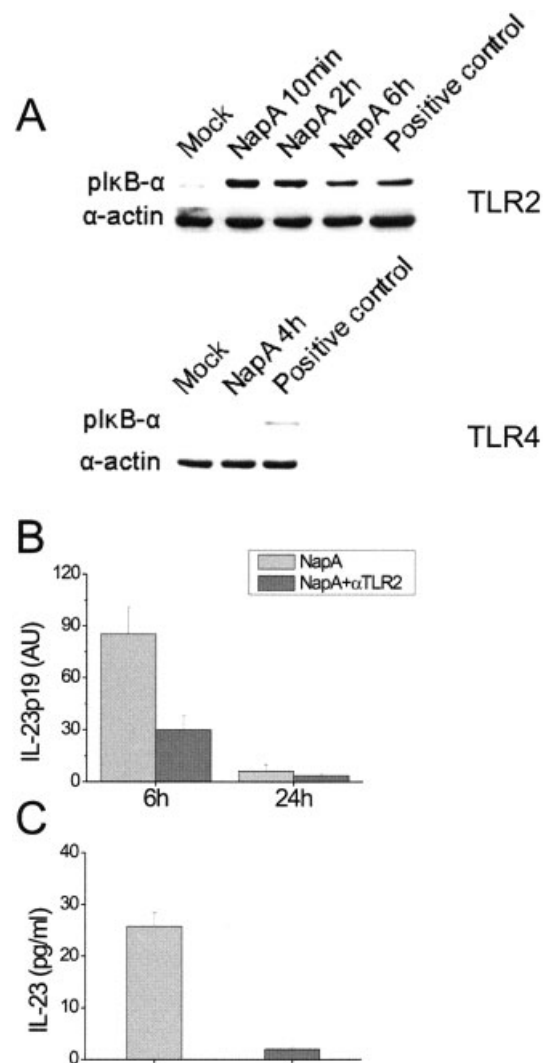

Figure 5. Engagement of Toll-like receptor 2 (TLR-2) by NapA and its involvement in the production of interleukin-23 (IL-23). **A,** HEK 293 cells expressing either TLR-2 or TLR-4 were incubated with NapA. As positive controls, neutrophil-activating protein of *Helicobacter pylori* (HP-NAP) was used for TLR-2–expressing cells, and lipopolysaccharide was used for TLR-4–expressing cells. Mock cells represent the negative control. At the indicated time points, cell lysates were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis/immunoblotting with anti–phospho-IκBα. Total α-actin antibody was used as a control for equal loading. **B,** Monocytes were either not preincubated or were preincubated for 2 hours with 20 μg/ml of an anti–TLR-2 blocking antibody and then exposed to NapA. After 6 hours or 24 hours, cells were harvested, and levels of mRNA for IL-23p19 were quantified by real-time polymerase chain reaction analysis. Results are expressed in arbitrary units (AU). **C,** The 24-hour culture supernatants from the same cells used for mRNA analysis were collected and analyzed by enzyme-linked immunosorbent assay for levels of the secreted IL-23 cytokine. The bar on the left shows IL-23 secretion after NapA stimulation, and the bar on the right shows IL-23 secretion after a 2-hour preincubation with 20 μg/ml of an anti–TLR-2 blocking antibody followed by stimulation with NapA. Values in **B** and **C** are the mean and SD of 4 independent experiments using triplicate samples.

healthy donors (Figures 3A, B, D, and E), although more so in neutrophils than in monocytes. The kinetics of expression of the 2 subunits were consistent with those of protein accumulation in culture supernatants (Figures 3C and F). Interestingly, no expression and release of IL-23 were observed when both cell types were exposed to OspA, another major antigen of *B burgdorferi*, which has been shown to trigger the release of IL-1β, IL-6, and TNFα from monocytes (37).

IL-23 is an essential survival factor for Th17 cells, since these cells are not present in mice that lack IL-23 (13). Accordingly, IL-23 blocking mAb completely abrogated the ability of *B burgdorferi*–stimulated dendritic cells to induce the release of IL-17 from T cells (35). However, more recent findings suggest that IL-23 is not required during the early differentiation of Th17 cells, whereas IL-6 is the crucial factor, together with TGFβ and IL-1β; IL-23 would serve to expand previously

differentiated Th17 cells (15). Thus, the mRNA and protein levels of these cytokines were determined after NapA stimulation of monocytes from normal subjects. Following stimulation, IL-6 mRNA expression was found to be up to 90 times higher, and high levels of IL-6 protein were present in culture supernatants (Figures 4A and B). NapA also induced high levels of TGFβ mRNA expression, which started to increase 2 hours after the addition of NapA and peaked after 12 hours, leading to the accumulation of TGFβ protein in the supernatant (Figures 4C and D). Furthermore, IL-1β was found to be significantly increased following stimulation with NapA (Figures 4E and F). OspA was significantly less efficient than NapA in inducing the release of TGFβ from monocytes, although OspA was able to trigger the release of IL-1β and IL-6 from monocytes.

These findings indicate that NapA, acting on monocytes and neutrophils, contributes to the creation of a cytokine milieu that is enriched in IL-6, TGFβ, IL-1β, and IL-23 and has a strong potential for driving the differentiation of T cells toward the Th17 subset.

IL-23 secretion following NapA binding to TLR-2. To investigate the mechanism of the NapA activation of monocytes, we evaluated the possibility that NapA activates TLR-mediated signaling. Considering that bacterial proteins preferentially activate monocytes and dendritic cells via TLR-2 or TLR-4, we used HEK 293 cells that had been transfected with plasmids that encode for either of these TLRs. HEK 293 cell lines lack expression of endogenous TLRs, although their TLR signaling machinery is fully functional. The common pathway that leads to NF-κB activation requires phosphorylation and degradation of IκBα, the cytosolic inhibitor of NF-κB. The engagement of a specific TLR expressed on HEK 293 cells was monitored by evaluating the phosphorylation of IκBα after exposure to NapA. As shown in Figure 5A, phosphorylation of IκBα was observed in cells that expressed TLR-2, but not those that expressed TLR-4. The phosphorylation of IκBα was already evident after 10 minutes of NapA treatment, and the time course of the band intensity was compatible with degradation of the NF-κB inhibitor by proteasome. In addition, treatment with an anti–TLR-2 blocking mAb abrogated the induction of IL-23 by NapA (Figures 5B and C).

DISCUSSION

The NapA protein of *B burgdorferi* is essential for the persistence of spirochetes within ticks, but it was not known to be expressed during the infection in mammals (26,27). Thus, it was surprising that reactivity with this spirochetal protein was observed in several patients with Lyme arthritis, particularly those with an antibiotic-refractory course. The present study revealed several major findings that are relevant to the pathogenesis of the disease associated with *B burgdorferi* infection. First, NapA drives synovial fluid Th17 cell responses in Lyme arthritis patients. Second, it induces IL-23 production in neutrophils and monocytes, as well as IL-6, IL-1β, and TGFβ production in monocytes. Third, it promotes IL-23 expression via activation of TLR-2.

The immune response in patients with Lyme disease expands gradually to a wide array of *B burgdorferi* proteins. Patients with Lyme arthritis, a late manifestation of the disorder, show reactivity with many spirochetal proteins, such as OSP, variable surface antigen, and heat-shock proteins (38–41). Here, we report that *B burgdorferi*–infected patients with Lyme arthritis produce antibodies and T helper cells that are specific for NapA. The role of the anti-NapA antibodies during the course of chronic infection with *B burgdorferi* is unknown. However, it is important to note that the anti-NapA antibody response was particularly relevant in patients with Lyme arthritis, whereas these antibodies are rarely detected in *Borrelia*-infected patients with erythema migrans or facial palsy, which are earlier manifestations of the disorder.

The orchestration of T cells in the development of Lyme arthritis is well documented (42). Th1 cells, which produce high levels of IFNγ, are considered crucial for the pathogenesis of human and experimental Lyme arthritis (8,43), but recent data show that Lyme arthritis can occur and propagate even in IFNγ-deficient mice (11,12), suggesting that other T helper cells and factors are involved in the genesis of arthritis. Here, we show that T helper cells isolated from patients with Lyme arthritis produced high levels of IL-17 and TNFα following T cell receptor stimulation with immobilized anti-CD3 mAb. IL-17 is a cytokine produced by Th17 cells, a novel subset of CD4+ T cells (13). *B burgdorferi* induces the production of IL-17 in T cells (19), and inhibition of IL-17 prevents the development of experimental arthritis in *B burgdorferi*–infected mice (20). However, the *Borrelia* factor(s) responsible for IL-17 production has remained unidentified.

In the present study, we demonstrated that NapA can play this role, at least in some patients, because it stimulates IL-17 production in synovial fluid–derived specific T cells, as demonstrated by ELISpot analysis of cells isolated from several patients with Lyme arthritis. It is noteworthy that NapA-specific T cells were localized in synovial fluid because IL-17 production could not be detected by ELISpot after stimulation of peripheral

blood T cells from the same patients with Lyme arthritis (data not shown).

IL-17 exerts many different activities on both immune and nonimmune cells. IL-17 links synovial inflammation to bone destruction. It recruits and activates neutrophils and is considered a key cytokine that drives autoimmune-like inflammation, mainly consisting of neutrophils and T cells (16). IL-17 is a potent stimulator of osteoclastogenesis and bone destruction, and its activity is synergistically enhanced by TNFα (18,44). Th17 cells have been shown to play a key role in the induction of experimental autoimmune arthritis as well as in patients with rheumatoid arthritis (18,45,46). Mice treated with an IL-17 neutralizing antibody were resistant to the induction of experimental arthritis and did not show joint inflammation and erosion (47). The findings of the present study suggest that NapA of *B burgdorferi* plays a major role in promoting a Th17 cell inflammation of the joints of patients with Lyme arthritis that, if not arrested, may lead to bone erosion and remodeling (48).

B burgdorferi is known to induce IL-23 (35), but the nature of the bacterial molecule(s) that is 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 TGFβ proteins, which are crucial for the induction of Th17 cell responses (13). These findings do not exclude the possibility that additional bacterial factors contribute to the generation of the Th17 cell response found in the synovial fluid of patients with Lyme arthritis.

NapA is a TLR-2 agonist that was able to activate NF-κB in TLR-2–transfected HEK 293 cells. Involvement of the TLR-2 receptor in the activation of monocytes by NapA was also supported by the abrogation of NapA-induced IL-23 expression by a specific anti–TLR-2 blocking antibody.

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

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AUTHOR CONTRIBUTIONS

Drs. D'Elios and de Bernard had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.
Study design. Montecucco, D'Elios, de Bernard.
Acquisition of data. Codolo, Amedei, Steere, Papinutto, Cappon, Polenghi, Benagiano, Paccani, Sambri.
Analysis and interpretation of data. Codolo, Amedei, Steere, Benagiano, Paccani, Sambri, Del Prete, Baldari, D'Elios, de Bernard.
Manuscript preparation. Steere, Del Prete, Baldari, Zanotti, D'Elios, de Bernard.
Statistical analysis. Codolo, Amedei.

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