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Orchestration of Inflammation and Adaptive Immunity in *Borrelia burgdorferi*–Induced Arthritis by Neutrophil-Activating Protein A

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Objective. **Lyme arthritis (LA) is characterized by infiltration of inflammatory cells, mainly neutrophils (polymorphonuclear cells [PMNs]) and T cells, into the joints. This study was undertaken to evaluate the role of the neutrophil-activating protein A (NapA) of *Borrelia burgdorferi* in eliciting inflammation and in driving the adaptive immune response.**

Methods. **Levels of NapA, interferon-γ (IFNγ), interleukin-17 (IL-17), and T cell–attracting chemokines were assessed by enzyme-linked immunosorbent assay in synovial fluid from patients with LA. The profile of T cells recruited into the synovia of patients with LA was defined by fluorescence-activated cell sorting analysis. NapA was intraarticularly injected into rat knees, and the cells recruited in synovia were characterized. The role of NapA in recruiting immune cells was confirmed by chemotaxis assays using a Transwell system.**

Results. **NapA, IFNγ, IL-17, CCL2, CCL20, and CXCL10 accumulated in synovial fluid from patients with LA. Accordingly, T cells obtained from these patients produced IFNγ or IL-17, but notably, some produced both cytokines. NapA promoted neutrophil and T lymphocyte recruitment both in vitro and in vivo. Interestingly, the infiltration of T cells not only resulted from the chemotactic activity of NapA but also relied on the chemokines produced by PMNs exposed to NapA.**

Conclusion. **We provide evidence that NapA functions as one of the main bacterial products involved in the pathogenesis of LA. Accordingly, we show that, at very early stages of LA, NapA accumulates and, in turn, orchestrates the recruitment of inflammatory cells into the joint cavity. Thereafter, with the contribution of recruited cells, NapA promotes the infiltration of T cells producing IL-17 and/or IFNγ.**

Borrelia burgdorferi is a spirochetal bacterium responsible for human Lyme disease, a multisystem illness transmitted by ticks (1). Lyme disease usually begins with the appearance of erythema migrans, a characteristic expanding skin lesion (2). Within days or weeks after the onset of local infection, bacteria may spread through the bloodstream to different sites, causing meningitis, cranial or peripheral neuritis, carditis, or musculoskeletal pain. After several months, severe and chronic symptoms that mainly affect joints may develop in untreated or inadequately treated patients (3); this condition, defined as Lyme arthritis (LA), is characterized by joint swelling, synovial hypertrophy, vascular proliferation, and infiltration of inflammatory cells (4). The presence of *B burgdorferi* in connective tissue of the joints of infected individuals likely plays an important role in establishing the course of LA (5).

The recruitment of neutrophils (polymorphonu-

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clear cells [PMNs]) to the site of infection is a critical first step in the immune response to pathogens. In experimental LA, but also in patients with LA, PMNs are the primary cell type present during the acute inflammatory phase, and they appear to be critical for development of the disease (6,7). Accordingly, not only are PMNs likely to be responsible for the tissue damage that may occur upon extracellular release of granule content but, more importantly, they may also play an active role in driving an immune response that, instead of being beneficial, contributes to the induction of LA (7). However, the mechanism by which PMNs contribute to LA has not yet been clarified.

The inflammation characterizing LA has traditionally been defined as a Th1 cell–mediated response; however, recent studies have shown that LA is induced by cytokines other than interferon-γ (IFNγ), because experimental LA can occur and propagate even in IFNγ-deficient mice (8,9). The disease paradigm based solely on a Th1 cell–mediated inflammatory response has been extended to include Th17 cells, a new subset of helper T cells (7); indeed, inhibition of interleukin-17 (IL-17) prevents the development of arthritis in vaccinated mice challenged with *B burgdorferi* (10). Furthermore, T cells from the synovial fluid of patients with LA produce IL-17 in response to neutrophil-activating protein A (NapA), a major antigen produced by *B burgdorferi* (11). Consistent with the fact that 2 major types of T cells are involved in the pathogenesis of LA, there is a predominance of some chemokines crucial for their recruitment into the synovial fluid of patients, such as CXCL10, which is specific for Th1 cells, and CCL2, which is specific for both Th1 and Th17 cells (12). Moreover, although it has not yet been evaluated in LA, CCL20-mediated recruitment of Th17 cells into the inflamed joints of patients with rheumatoid arthritis (RA) has also been described (13).

The contribution of macrophages to the creation of a milieu rich in chemoattractants for T cells in the joint fluid of patients with LA has been established (11,12,14). However, the possibility exists that PMNs are also involved; these versatile cells, either spontaneously or following appropriate stimulation, have been shown to express and/or produce numerous cytokines and chemokines (15).

We previously demonstrated that the immune modulatory properties of the antigen NapA are relevant for the differentiation of T cells toward the Th17 phenotype (11). However, we did not elucidate whether NapA accumulates in the joint fluid of patients with LA. Therefore, the potential participation of NapA in orchestrating the entire process of joint inflammation also remained unexplored.

In this study, we demonstrate that the protein NapA, which accumulates in the joint cavity of patients with LA, recruits PMNs in the early stage of disease and subsequently recruits T lymphocytes. We show that NapA recruits T cells via the contribution of chemokines released by PMNs. Recruited T cells include both Th1 and Th17 cells, as well as a subset producing both IFNγ and IL-17. Taken together, our results show that NapA is a major bacterial factor driving the generation of a proinflammatory T cell response responsible for clinical onset and histopathologic changes in LA.

PATIENTS AND METHODS

Patients. Synovial fluid was obtained from patients with LA, patients with RA, and healthy individuals, with informed consent and approval of the local ethics committee. Synovial fluid was aspirated at the time of knee joint biopsy that was performed for diagnostic or therapeutic reasons. Five anti-NapA–seropositive European patients with LA (3 men and 2 women with a median age of 51 years [±7 years]), defined according to the Centers for Disease Control and Prevention case definition for the condition (16,17), were included in the study. Three patients had an antibiotic-refractory disease course**,** defined as persistent joint swelling for ≥3 months after the start of at least a 4-week treatment course with intravenous antibiotics, at least an 8-week regimen of oral antibiotics, or both. The samples were obtained during the period of active arthritis. Patients with antibiotic-refractory arthritis had negative polymerase chain reaction (PCR) results for *B burgdorferi* DNA in joint fluid or synovial tissue (11).

After antibiotic treatment, patients with antibiotic-refractory arthritis were treated with nonsteroidal antiinflammatory drugs, intraarticular corticosteroids, or disease-modifying antirheumatic drugs. Five women with mild RA (mean ± SD Disease Activity Score in 28 joints [18] using the C-reactive protein level of 3.6 ± 1.0), diagnosed according to the American College of Rheumatology 1987 criteria (19), were included in the study. The mean ± SD age of the patients was 55.6 ± 14.3 years, disease duration was 132.4 ± 181.7 months, and all except 1 patient were positive for IgM rheumatoid factor and anti–cyclic citrullinated peptide. Five age-matched healthy individuals were included as negative controls.

Synovial fluid T cells from patients with LA. Synovial fluid–derived T cells were purified as previously reported (11) and stimulated with NapA in the presence of irradiated autologous antigen-presenting cells; synovial fluid–derived T cell lines were then expanded with IL-2. On day 15, T cell blasts from each line were stimulated with 25 ng/ml phorbol myristate acetate plus 1 μg/ml ionomycin in the presence of brefeldin A (1 μg/ml). Cells were stained for surface and intracellular markers, using the following monoclonal antibodies for flow cytometry: PerCP-conjugated anti-CD4, phycoerythrin-conjugated anti–IL-17, and fluorescein isothiocyanate (FITC)–conjugated anti-IFNγ (Becton Dickinson).

Animals. Wistar rats were obtained from a colony kept in the animal house at the University of Trieste, Italy. Male rats weighing 230–260 gm were used in this study. The in vivo experiments were performed in compliance with the guidelines of European (86/609/EEC) and Italian (D.L.116/92) laws and were approved by the Italian Ministry of University and Research and by the University Institutional Committee. All treatments were performed under total anesthesia induced with 25 mg/kg bromoethanol (Sigma).

Cloning and protein purification. The cloning of NapA, its expression, and its purification have been reported previously (11). Briefly, the NapA gene was cloned and expressed in *Bacillus subtilis*, and NapA was purified by performing anion-exchange chromatography (Mono Q fast-performance liquid chromatography; GE Healthcare), followed by gel-filtration chromatography (Superdex 200 HR 10/30; GE Healthcare). Protein was concentrated using a Vivaspin ultrafiltration system (Sartorius), and the final product was checked for purity in a Coomassie brilliant blue–stained gel and analyzed by Western blotting with a specific polyclonal antibody (20).

Enzyme-linked immunosorbent assay (ELISA) for NapA quantification in synovial fluid. A 96-well plate was coated overnight with 50 μl/well of synovial fluid from healthy donors, patients with LA, and patients with RA (diluted 1:4 in phosphate buffered saline [PBS]). Each well was incubated with a rabbit anti-NapA polyclonal antibody (20) followed by horseradish peroxidase (HRP)–conjugated anti-rabbit IgG. Plates were developed with tetramethylbenzidine (TMB) and read at 450 nm using a Packard Fusion microplate analyzer. The amount of NapA in each sample was determined by referring to a standard curve.

ELISA for revealing anti-NapA antibodies in synovial fluid. Synovial fluid samples from 2 patients with LA and 1 patient with RA were added to a 96-well plate previously coated with purified recombinant NapA (1 μg/well). HRP-conjugated anti-human IgG was added to each well before development with TMB. Absorbance was read at 450 nm using a Packard Fusion microplate analyzer. The acquired values were referred to a standard curve obtained using known concentrations of rabbit IgG anti-NapA.

In vivo NapA and outer surface protein A (OspA) administration. NapA (50 μl of sterile saline containing 6 μg or 20 μg of the protein) or OspA (50 μl of sterile saline containing 1 ng of the protein) was administered to rats by intraarticular injection into the right knee, while vehicle (saline only) was injected into the left knee. The concentrations of NapA- or OspA-containing solutions (420 μg/ml or 20 ng/ml, respectively) were 20-fold higher than those that exerted the maximum stimulation of human monocytes in vitro, in terms of cytokine production. In the case of NapA, a lower amount of protein was also tested. At different times postinjection (2 hours and 2 days), the rats were killed, and the periarticular tissue of the knee joints was gently removed to expose the intraarticular cavity. The intraarticular cavity was washed with 2 ml of saline, and the total cell content in the synovial lavage was determined with a Coulter Counter ZB1 (Beckman Coulter). The amount of PMNs was estimated by measuring the myeloperoxidase content (21). After cytocentrifugation, the percentage of lymphocytes/monocytes was determined by hematoxylin and eosin staining.

Immunohistochemical analysis. Rat knee joints were fixed for 6 days in 10% buffered formalin, decalcified for 5 days in Decalcifier I (Bio-Optica), and embedded in paraffin. Sections (6–8 μm) of paraffin-embedded specimens were used for immunohistochemical analysis.

IFNγ- and IL-17–positive cells present in the joint synovium were revealed on paraffin-embedded sections of the knee articulation by rabbit anti-rat IFNγ IgG (Hycult) or by rabbit anti-rat IL-17 IgG (Santa Cruz Biotechnology) for 60 minutes at room temperature. Sections were further exposed to anti-rabbit IgG, using a Vectastain ABC kit (Vector) according to the manufacturer's instructions.

Cell purification and culture. PMNs were enriched (>99.7% purity) from Ficoll-Paque–isolated granulocytes (obtained from the peripheral blood of healthy donors) by positively removing all contaminating cells using an EasySep Human Neutrophil Enrichment Kit (StemCell Technologies) (22). PMNs were suspended in RPMI 1640 medium supplemented with 10% low-endotoxin fetal bovine serum (FBS; EuroClone), and stimulated with 20 μg/ml NapA at various time points. Cells were harvested for quantification of chemokines in the culture supernatants.

For evaluation of CD4+ T cell chemotaxis, PMNs were cultured in RPMI 1640 with 10% FBS in the presence of 20 μg/ml NapA or saline plus 100 ng/ml IFNγ. After 24 hours of culture, supernatants were collected and kept at −80°C until used in chemotaxis assays.

Human CD4+ T lymphocytes from healthy donors were purified by negative selection using a RosetteSep Kit (StemCell Technologies) according to the manufacturer's instructions. Briefly, RosetteSep enrichment cocktail was added to buffy coat (50 μl/ml) and incubated for 20 minutes at room temperature. The sample was diluted with an equal volume of PBS supplemented with 2% fetal calf serum (FCS) and layered on Ficoll-Paque density-gradient medium (GE Healthcare). After centrifugation (1,200*g*) for 20 minutes at room temperature without brakes, T lymphocytes were collected and washed twice with PBS supplemented with 2% FCS. Cells were then resuspended in RPMI 1640 medium supplemented with 10% FCS.

Endothelial cell culture. Human umbilical vein endothelial cells (HUVECs) were isolated from 3–5 normal human umbilical cords by collagenase digestion and grown in tissue culture plates (Costar) coated with 2% endotoxin-free gelatin (23). HUVECs from the first passage were used. Cells were kept in culture in Medium 199 supplemented with 20% newborn calf serum (Invitrogen), 50 μg/ml heparin, 100 units/ml penicillin, 100 μg/ml streptomycin (Sigma), and 50 μg/ml endothelial growth supplement (Immunological Sciences) (24).

Chemotaxis assay. Polymorphonuclear cells (2×10^6/ml) were added to the upper chamber of a polycarbonate (PET) insert of a 24-well Transwell apparatus (Costar), while RPMI 1640 medium (with or without 20 μg/ml NapA or 100 n*M* fMLP) was added to the lower chamber. After 1 hour of incubation, PMNs that had migrated into the lower chamber were collected and counted using a CyQuant cell proliferation assay kit (Invitrogen). To evaluate the chemokinetic activity of NapA, the protein was added at the same concentration (20 μg/ml) in both the upper and lower chambers. After 1 hour of incubation, PMNs that had migrated into the lower chamber

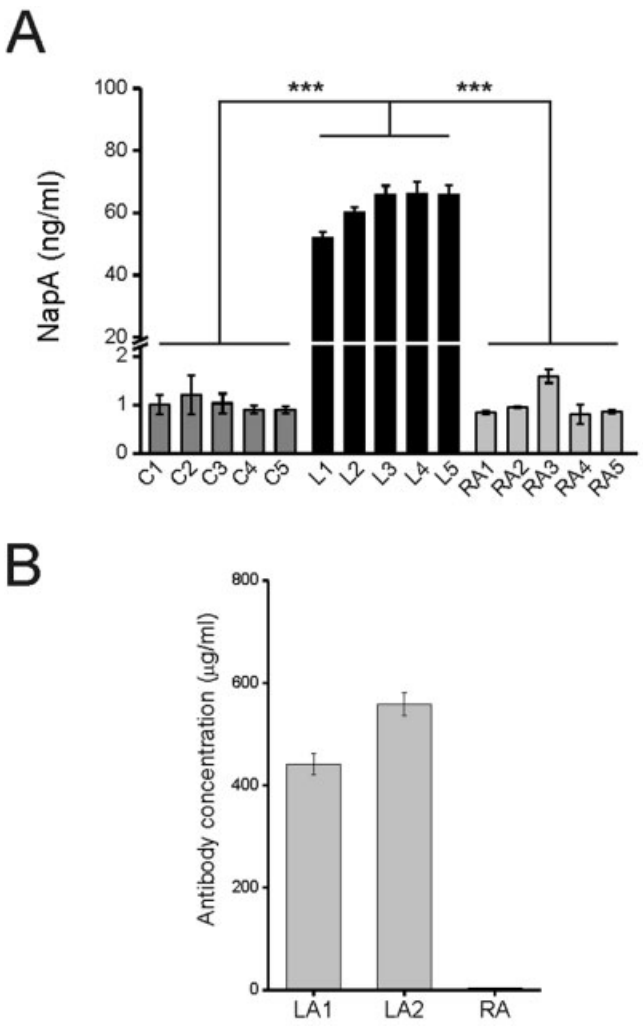

Figure 1. Quantification of neutrophil-activating protein A (NapA) and anti-NapA in the synovial fluid of patients with Lyme arthritis (LA). **A,** Presence of NapA in synovial fluid obtained from 5 patients with LA (L), 5 patients with rheumatoid arthritis (RA), and 5 healthy controls (C), as determined using a specific enzyme-linked immunosorbent assay (ELISA). The NapA content in each sample was determined by referring to a standard curve. *** = $P < 0.001$. **B,** Presence of anti-NapA antibodies as determined by ELISA in 2 patients with LA and 1 patient with RA. Bars show the mean ± SD.

were collected and counted. To verify the effect of anti-NapA antibodies on the chemotactic activity of NapA, we performed the chemotaxis assay by applying to the lower chamber 20 μg/ml NapA alone or 20 μg/ml NapA plus increasing concentrations of rabbit anti-NapA antibody (from 20 μg/ml to 500 μg/ml). For a positive control 100 n*M* fMLP was used. When synovial fluid was used as a source of anti-NapA antibodies, fluid samples diluted 1:4 in culture medium together with NapA were applied to the lower chamber. After 1 hour of incubation, PMNs that had migrated into the lower chamber were collected and counted.

CD4+ T lymphocytes (2×10^6/ml) were added to the upper chamber of the Transwell apparatus, while NapA or NapA-conditioned PMN culture medium was added to the lower chamber. The same experiment was performed in the presence of 1 μM cycloheximide (CHX). When required, NapA-conditioned PMN culture medium was preincubated for 30 minutes with the blocking antibodies anti-CCL2, anti-CXCL10 (5 μg/ml; PeproTech), or anti-CCL20 (5 μg/ml; R&D Systems) or with their isotype-related antibodies (5 μg/ml; BioLegend), and added to the bottom chamber. After a 2-hour incubation, migrated T lymphocytes were harvested from the lower chamber, and specific enzyme-linked immunospot (ELISpot) assay kits (eBioscience) were used to assess their ability to produce IFNγ, IL-17, or IL-4. When the chemotaxis assay was performed through an endothelial monolayer, HUVECs (2×10^5) were seeded onto 2% gelatin-coated polycarbonate (PET) inserts of a 24-well Transwell system and were used 5 days after plating. The formation of an intact monolayer on the insert was evaluated by adding FITC-labeled bovine serum albumin (BSA) (1 mg/ml) to the upper chamber, and after 5 minutes, the amount of labeled BSA passed into the lower chamber was measured using a FLUOstar microplate reader (SLT Labinstruments). Transwells were used only when the intensity of fluorescence in the lower chamber was negligible. The pore sizes of filters used for the chemotaxis assay were 3 μm for PMNs and 5 μm for T cells.

Chemokine and cytokine measurements. Chemokine content was quantified both in culture supernatants from the PMNs (10×10^6/ml) exposed to 20 μg/ml NapA (in the presence or absence of 100 ng/ml IFNγ) and in synovial fluid from patients with LA. Quantification of CCL2 and CXCL10 was performed using a specific multichemokine detection kit (Milliplex MAP; Millipore). The IFNγ and IL-17 content in synovial fluid from patients with LA and in synovial fluid from healthy subjects was measured using a specific multicytokine detection kit (Milliplex MAP). Data were analyzed using Bio-Plex Manager software (Bio-Rad). Quantification of CCL20 was performed using a specific ELISA (R&D Systems). For the multicytokine detection kit, the sensitivity threshold was 3 pg/ml, while it was 12 pg/ml for the CCL20 ELISA kit.

Statistical analysis. Data are reported as the mean ± 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

Accumulation of NapA within synovia of patients with LA. The presence of *B burgdorferi* in connective tissue of the joints is required for the development of arthritis (5). However, the chemical nature of the bacterial factor(s) responsible for the disease remains unknown. In order to verify whether NapA, a major antigen produced by *B burgdorferi*, might be involved in triggering and sustaining joint inflammation, we started by testing for the presence of NapA in the synovial fluid of patients with LA. Indeed, our recent finding that NapA-specific Th17 lymphocytes accumulate within the

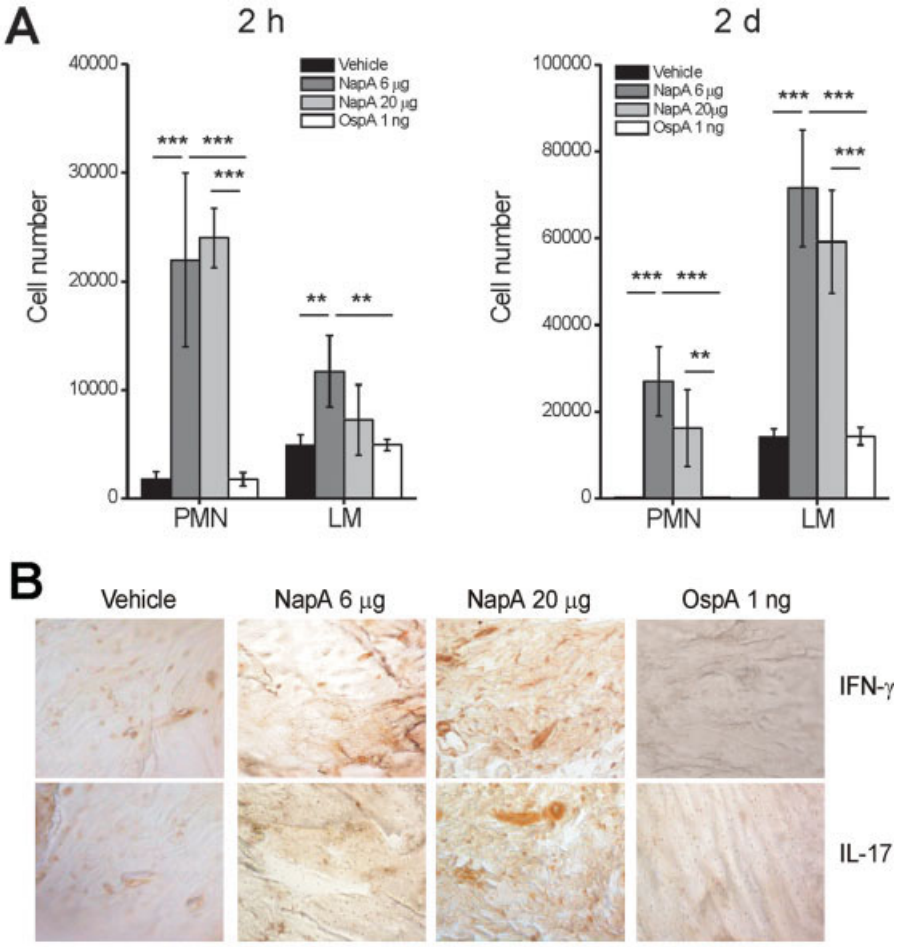

Figure 2. Leukocyte recruitment triggered by neutrophil-activating protein A (NapA) in rat synovia. **A,** Differential count of cells accumulated in the synovial cavity of rats treated with NapA, outer surface protein A (OspA), or saline (vehicle) for 2 hours (left) or 2 days (right). The numbers of polymorphonuclear cells (PMNs) were estimated by measuring the myeloperoxidase content, and lymphocytes/monocytes (LMs) were counted following hematoxylin and eosin staining. Bars show the mean ± SD. ** = $P < 0.01$; *** = $P < 0.001$. **B,** Immunohistochemical staining of rat synovial membranes for interferon-γ (IFNγ) and interleukin-17 (IL-17), 2 days postinjection. Original magnification × 200.

synovial cavity of patients with LA (11) suggested the possibility that the antigen was released by the bacterium in that niche.

An ELISA performed using synovial fluid isolated from 5 patients with LA revealed the presence of NapA at a concentration that was similar among the different samples (range 50–65 ng/ml). In contrast, NapA was not detectable in synovial fluid from either patients with RA or healthy control subjects (Figure 1A). The concentration of NapA detected in human synovia was probably underestimated: it reflected not only a certain degree of dilution, but most importantly, it was influenced by the presence of proteases. Indeed, a dominant feature of inflammatory tissue is the presence of proteolytic activity, due partly to the fibrinolytic system and partly to other proteases derived from plasma, leukocytes, or other types of cells in the area of inflammation (25). Notably, specific anti-NapA antibodies were observed in synovial fluid from patients with LA but were not detectable in synovial fluid from patients with RA (Figure 1B).

NapA-induced recruitment of PMNs and T lymphocytes in rat synovia. We next examined whether the administration of NapA into rat knees was able to provoke inflammation. For this purpose, the effect of intraarticular injection of 2 different amounts of NapA in rats was evaluated and compared with that induced by injection of OspA, another *Borrelia* immunodominant antigen (26), as well as with that induced by saline alone. Analysis of the cell content in lavage fluid from rat knees

injected with NapA revealed a distinct recruitment of PMNs 2 hours postinjection (Figure 2A), which is consistent with the fact that PMNs are the first cells recruited to the site of infection (27). Notably, 2 days after NapA administration, the majority of cells collected in lavage fluid were lymphocytes/monocytes (Figure 2A). No significant difference was appreciable between the 2 doses of NapA administered; interestingly, however, OspA was much less effective than NapA in recruiting cells into synovia.

Immunohistochemical analysis of rat synovial tissue revealed marked IFNγ and IL-17 staining in NapA-treated rats (the signal was slightly less intense in rats receiving the lower dose of NapA), while no staining was observed in tissue obtained from OspA-treated rats or saline-treated rats (Figure 2B). These observations are consistent not only with various studies indicating that IFNγ is a key modulator of arthritis development but also with more-recent evidence supporting IL-17 as a possible mediator of LA (11,12). These results demonstrate that once NapA is present in the joint cavity, it triggers the recruitment of both PMNs and lymphocytes and induces the production of IFNγ and IL-17.

Effect of PMNs recruited by NapA on migration of CD4+ T cells that produce IFNγ and IL-17. To evaluate whether NapA had chemotactic activity, we monitored the passage of PMNs and lymphocytes across a Transwell insert following application of NapA to the lower chamber. Counting the number of cells that migrated 1 hour after the application of the stimulus revealed that NapA promoted movement of PMNs toward the lower chamber, although at a lower level than that induced by fMLP, a classic PMN activator (28,29) (Figure 3A). Notably, no migration was appreciable when NapA was added to both the upper and lower chambers (data not shown), permitting the exclusion of random movement of PMNs due to an eventual chemokinetic effect of NapA.

Migration of PMNs promoted by NapA was not impaired by anti-NapA antibodies, neither those accumulated in the synovial cavity of patients with LA nor those raised in rabbits following immunization with recombinant NapA (data not shown). This observation suggests that the anti-NapA humoral immune response does not exert blocking action on the chemotactic activity of NapA.

The same experimental approach was used to verify whether NapA was also able to recruit T lymphocytes, as suggested by the results obtained with in vivo administration. We evaluated the chemotactic activity of NapA, but because T lymphocyte recruitment in vivo

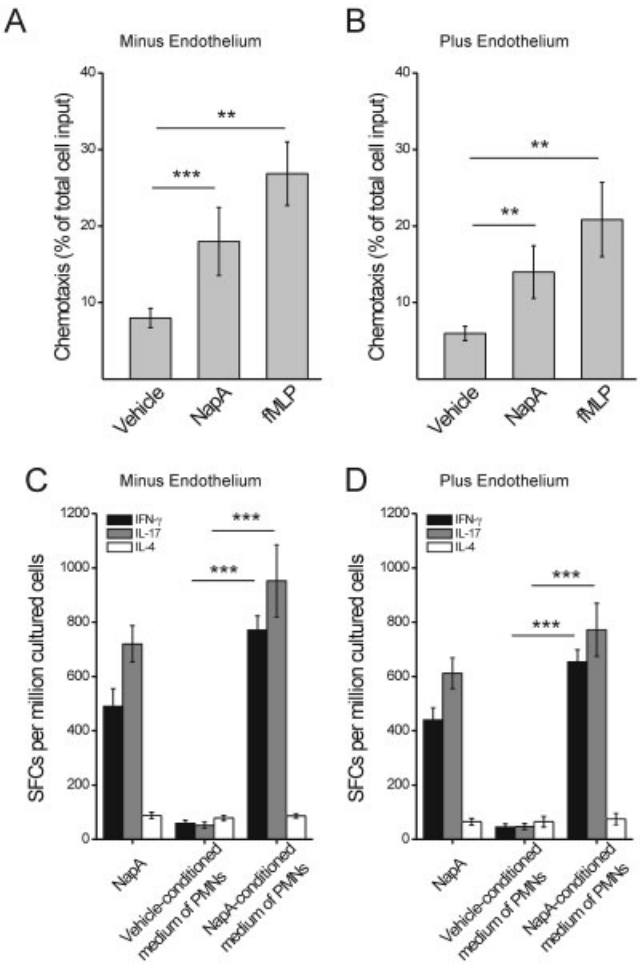

Figure 3. Chemotaxis of PMNs and CD4+ T lymphocytes induced by NapA. **A** and **B**, Quantification of the migration of PMNs and lymphocytes across a Transwell insert following application of NapA to the lower chamber, in the absence (**A**) or presence (**B**) of human umbilical vein endothelial cell–derived endothelium grown onto a Transwell filter. PMNs (2×10^6/ml) were added to the upper chamber of a Transwell system, and medium (with or without 20 μg/ml NapA or 100 n*M* fMLP) was added to the lower chamber. After 1 hour of incubation, the PMNs that had migrated into the lower chamber were collected and counted. **C** and **D**, Quantification of spot-forming cells (SFCs) in the absence (**C**) or presence (**D**) of endothelium. CD4+ T lymphocytes (2×10^6/ml) were added to the upper chamber of a Transwell system, and NapA, supernatants from NapA-stimulated PMNs, or supernatants from PMNs exposed to vehicle were added to the lower chamber. After 2 hours, CD4+ T lymphocytes were collected from the lower chamber, and their profiles were determined in enzyme-linked immunospot microplates coated with anti-IFNγ, anti–IL-17, or anti–IL-4 antibodies. Bars show the mean ± SD. ** = $P < 0.01$; *** = $P < 0.001$. See Figure 2 for other definitions.

was subsequent to that of PMNs, we also examined the effects of the supernatant collected from PMNs exposed to NapA for 24 hours (referred to as NapA-conditioned

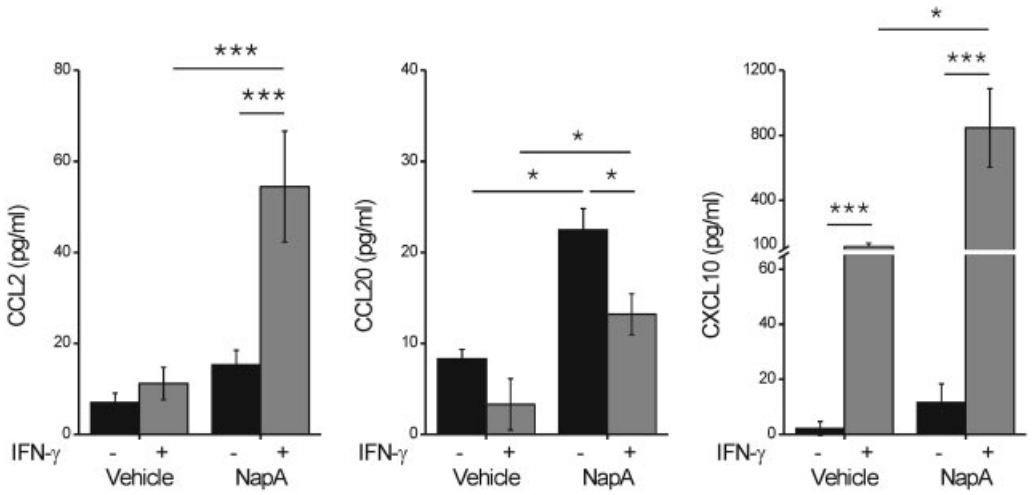

Figure 4. Chemokine production by polymorphonuclear cells (PMNs) stimulated with neutrophil-activating protein A (NapA). The production of CCL2, CCL20, and CXCL10 in the culture supernatant from NapA- or saline-treated PMNs (in the presence or absence of interferon-γ [IFNγ]) was determined by enzyme-linked immunosorbent assay after 20 hours of stimulation. Bars show the mean ± SD results of 3 experiments conducted with different cell preparations. * = $P < 0.05$; *** = $P < 0.001$.

medium of PMNs). The 24-hour stimulation of PMNs with NapA was carried out in the presence of IFNγ, to optimize the induction of chemokines for T cells (30–33). Notably, IFNγ accumulates in the synovial fluid of patients with LA (see below). Therefore, total CD4+ T cells were added to the upper chamber, and 2 hours after the administration of the stimuli, cells that had migrated were collected and analyzed for their profile. We observed that NapA was chemoattractant for T cells, directly and indirectly, by means of PMNs. Recruited T cells produced IFNγ or IL-17 but not IL-4 (Figure 3C); this observation suggested that both mechanisms triggered the recruitment of specific T cell subsets.

Notably, a similar migration of PMNs and lymphocytes also occurred in the presence of an endothelial monolayer grown onto the Transwell filter, suggesting that even if endothelium activation occurred in the presence of stimuli, it did not contribute to NapA-mediated cell recruitment (Figures 3B and D).

Finally, in order to exclude the possibility that the migration of CD4+ T lymphocytes was attributable to their endogenous production of chemoattractant factors, we evaluated T cell migration in the presence of CHX, to block protein synthesis in T cells. We did not observe any difference in T cell migration ability in response to NapA or NapA-conditioned supernatants from PMNs, regardless of the presence of CHX (data not shown).

Role of NapA in stimulating PMNs to release chemokines recruiting Th1 and Th17 cells. PMNs are known to produce biologically functional levels of CCL2, CCL20, and CXCL10 in response to lipopolysaccharide (LPS) plus IFNγ (30–32). It was previously demonstrated that PMN-derived CCL20 induces a migratory response of CCR6-expressing dendritic cells (30,33) and T lymphocytes (34), whereas PMN-derived CXCL10 induces migration of CXCR3-expressing lymphocytes (32). Importantly, CCR6 and CXCR3 receptors are typically expressed by Th17 and Th1 cells, respectively (14,35). Moreover, it was demonstrated that CCL2 cooperates with either CCL20 or CXCL10 in inducing Th17 or Th1 lymphocyte recruitment, supporting the notion that there is crosstalk between PMNs and T cells (34).

Therefore, based on the observation that NapA-conditioned supernatants from PMNs recruited CD4+ T cells producing IFNγ and/or IL-17 (Figure 3), we evaluated the possibility that NapA could activate PMNs (>99.7% purity) to produce CCL2, CCL20, and CXCL10. As shown in Figure 4, we observed that NapA promotes the release of all 3 chemokines to a comparable extent (range 15–25 pg/ml). When IFNγ was present, the NapA-induced release of CCL2 and CXCL10 was significantly enhanced, and the effect was synergistic (Figure 4). Interestingly, the addition of IFNγ reduced the secretion of CCL20, in contrast to what was observed with the other Toll-like receptor agonist, LPS (30). This unusual result, which suggests interplay between the signaling cascades triggered by NapA and IFNγ, deserves further investigation.

T cells recruited by NapA belong to the Th1, Th17, and Th1/Th17 cell subsets. In order to clarify whether CCL2, CCL20, and CXCL10 produced by PMNs treated with NapA plus IFNγ were involved in the recruitment of IFNγ- and IL-17–producing T cells, we preincubated NapA-conditioned supernatants of PMNs with specific blocking (or isotype-related) antibodies. These supernatants were then applied to the lower chambers of the Transwell system after total CD4+ T lymphocytes were added to the upper chambers. Migrated cells were analyzed for the production of IFNγ and IL-17 by ELISpot assay, as described in Patients and Methods. Neutralization of CCL2, CCL20, or CXCL10 reduced, at variable but significant levels, the recruitment of both IFNγ- and IL-17–producing CD4+ T cells. Notably, simultaneous blockade of all 3 chemokines resulted in the most extensive reduction in T cell chemotaxis (Figure 5). Isotype control antibodies were ineffective.

Collectively, these results provide support for a cooperative action by PMN-derived CCL2, CCL20, and CXCL10 in the recruitment of IFNγ- and IL-17–producing CD4+ T lymphocytes. Moreover, they suggest that T lymphocytes that are able to produce both IFNγ and IL-17 are involved in a NapA-mediated immune response related to the pathogenesis of LA. To verify such an hypothesis, T cell lines specific for NapA, obtained from mononuclear cells isolated from the synovial cavity of 5 patients with LA (11), were assayed for their IFNγ and IL-17 content by cytofluorometry (Figure 6A). Our analysis revealed that the major proportion of T cells were IL-17 producers (9% ± 2.3%), although IFNγ producers were also retrieved (6.4% ± 1.7%). Interestingly, however, a relevant proportion of T cells produced both cytokines (7.08% ± 1.38%). Consistently, we were able to quantify the documented accumulation of IFNγ in synovial fluid from patients with LA (12), but notably, we also observed the accumulation of IL-17 (Figure 6B).

Finally, in order to corroborate the in vivo data, we analyzed the chemokine content of synovial fluid obtained from the patients with LA. In accordance with a previous report (12), we detected CCL2 and CXCL10. Additionally, we demonstrated for the first time that CCL20 also accumulates in the synovial cavity of patients with LA (Figure 6C).

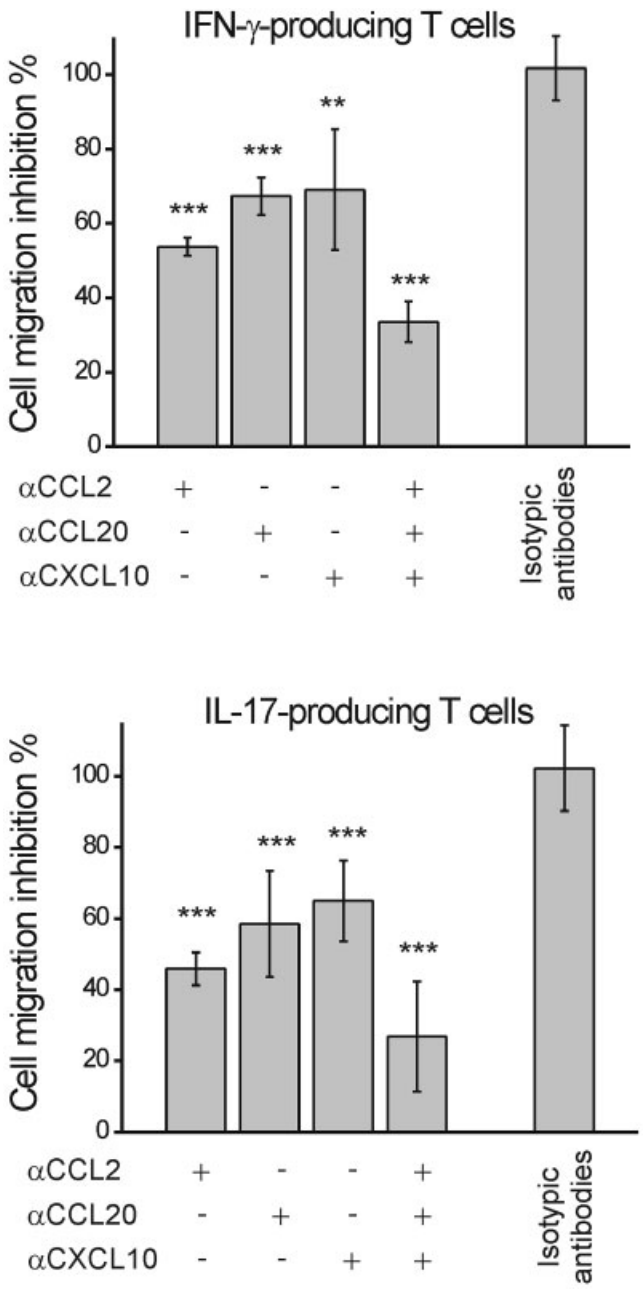

Figure 5. Recruitment of IFNγ- and interleukin-17 (IL-17)–positive CD4+ T lymphocytes by PMNs stimulated with NapA together with IFNγ or IL-17. CD4+ T lymphocytes (2×10^6/ml) were added to the upper chamber of a Transwell system, and supernatants from NapA/IFNγ-stimulated PMNs preincubated with chemokine-blocking antibodies (or with isotype-related antibodies) were added to the lower chamber. After 2 hours, CD4+ T lymphocytes were collected from the lower chamber, and their profiles were determined using enzyme-linked immunospot microplates coated with anti-IFNγ or anti–IL-17 antibodies. Bars show the mean ± SD results of 4 experiments conducted with different cell preparations. ** = $P < 0.01$; *** = $P < 0.001$ versus isotype control antibodies, by Student's *t*-test. See Figure 4 for other definitions.

DISCUSSION

Arthritis is a well-documented complication that follows infection with the tick-borne spirochete *B burg-*

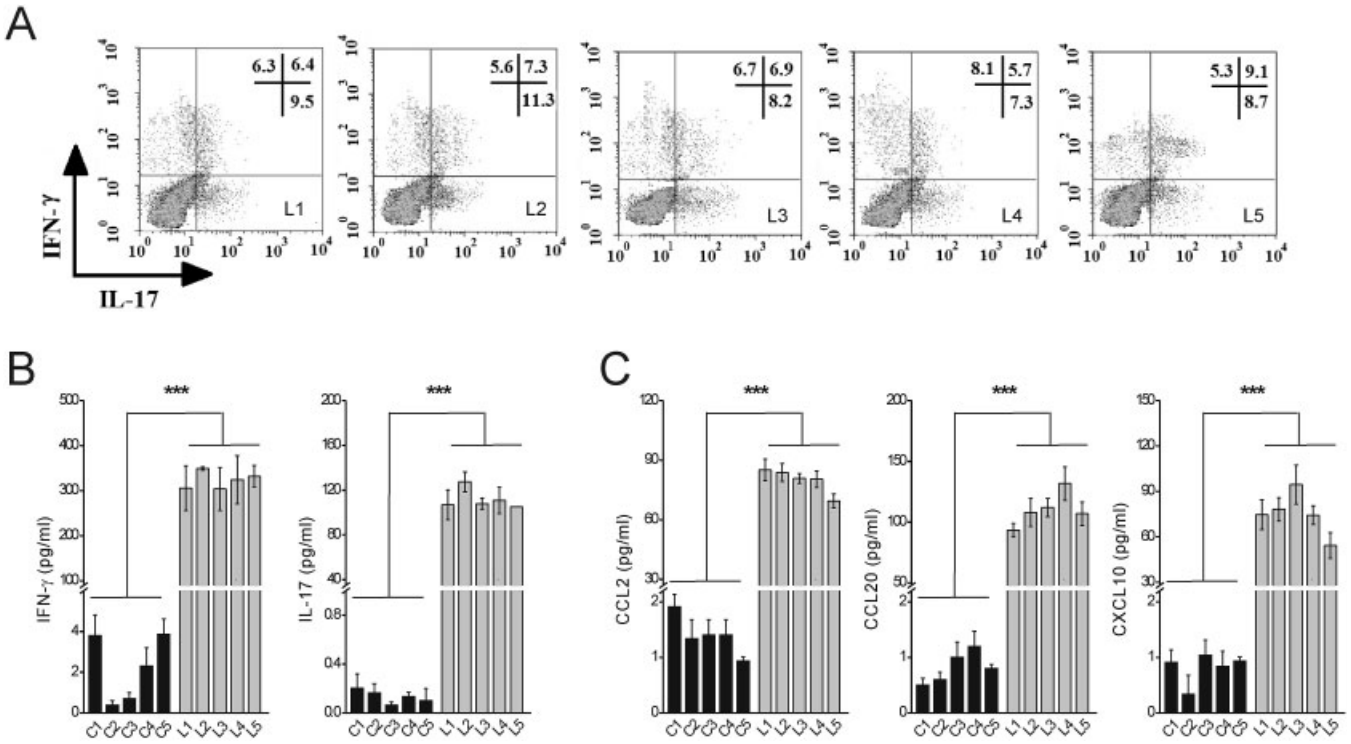

Figure 6. Involvement of T lymphocytes that produce both IFNγ and interleukin-17 (IL-17) in a NapA-mediated immune response related to the pathogenesis of Lyme arthritis (LA) **A,** NapA-driven IFNγ and IL-17 production by synovial fluid CD4+ T cells from patients with LA. Synovial fluid T cells from 5 patients with LA (L1–L5) were stimulated with NapA in the presence of irradiated autologous antigen-presenting cells. **B** and **C**, Accumulation of IFNγ and IL-17 (**B**) and chemokines involved in recruiting Th1 and Th17 cells (**C**) in the knee joint cavity of patients with LA. Total amounts of IFNγ, IL-17, CCL2, CCL20, and CXCL10 were measured in synovial fluid from the same 5 patients with LA as in **A** and from 5 healthy control subjects (C1–C5). Bars show the mean ± SD. *** = $P < 0.001$. See Figure 4 for other definitions.

dorferi. The severity of arthritis can range from mild to moderate inflammation of the joints that develops months after infection, to a chronic, debilitating osteoarthropathy with destruction of cartilage and erosion of bone that develops in a subset of patients within a few years. After dissemination of the spirochete from the skin to the joint, macrophages, T cells, B cells, and plasma cells infiltrate the synovial tissue, while joint fluid contains large numbers of PMNs.

The recruitment of PMNs into the infected joint appears to be a prerequisite for the development of LA, and these cells have been proposed to play a nonphagocytic immunoregulatory role (7,36). However, this issue remains quite controversial, because some studies showed that arthritis develops independently of the presence of PMNs (36,37). Another missing piece of the puzzle concerns the bacterial factor(s) that are responsible for the recruitment of PMNs.

Once *B burgdorferi* is disseminated from the site of inoculation, it establishes residence in the joint tissue; however, whether the bacterium survives for a long time in the synovial fluid is a matter of debate. Indeed, although almost all patients with LA have positive PCR results for detection of *B burgdorferi* DNA in synovial fluid (5,38), only 2 cases have been reported in which the bacterium itself was recovered from this site, and in neither case was it possible to subculture the isolate (39,40). Therefore, the idea that emerges is that in the synovial fluid of patients with LA, these bacteria are either moribund or dead (41), and the release of bacterial antigens in this context is absolutely expected. Interestingly, it has been recently proposed that retained spirochetal antigens might perpetuate synovial inflammation even after eradication of the spirochete from the joint (through antibiotic therapy), giving rise to chronic synovitis (6).

In a previous study, we demonstrated that patients with LA had a humoral immune response to the neutrophil-activating protein NapA, while patients with earlier manifestations of the disorder (erythema migrans

and facial palsy) had minimal or no reactivity with this antigen (11). Moreover, we showed that NapA is endowed with immune modulatory properties that reflect the activation of PMNs and monocytes, which are induced to release cytokines that are crucial for the induction of Th17 cell responses. It is noteworthy that NapA-specific T cells were mainly confined to the synovial fluid, because IL-17 production could not be detected after stimulation of peripheral blood T cells from the same patients with LA. These latter data, suggesting that NapA could be one of the spirochetal antigens that accumulate in the joint, have been confirmed in the present study. Indeed, we demonstrated that NapA is retained in the synovial fluid of patients with LA.

In addition, using a rat model of arthritis, we observed that intraarticular injection of NapA triggered the accumulation of inflammatory and immune cells: PMNs accumulated as early as 2 hours after injection, and lymphocytes/monocytes accumulated 2 days after injection. In addition to monocyte/macrophages, the latter population included IFNγ- and IL-17–producing cells, as revealed by relevant staining of the 2 cytokines on the synovial membrane of NapA-injected rats.

Notably, we also observed that NapA per se exerted chemotactic activity for PMNs and T lymphocytes without involving chemokines of endothelial origin. Moreover, despite the high concentration of anti-NapA antibodies in synovial fluid from patients with LA, these antibodies did not impair the NapA-induced recruitment of PMNs (data not shown).

The role of chemokines released by *Borrelia*-stimulated macrophages in the homing of T cells to infected joints is well established (14). We therefore investigated whether NapA could stimulate recruited PMNs to release chemoattractants that would participate in guiding the adaptive immune response in patients with LA. We observed that NapA-stimulated PMNs released CCL2, CCL20, and CXCL10. Together with CCL2, CCL20 is involved in the recruitment of Th17 lymphocytes, while CXCL10, together with CCL2, induces migration of CXCR3-expressing Th1 effector cells (34). Therefore, it is expected that NapA, via the involvement of PMNs, recruits both Th1 and Th17 cells; this is consistent with the synovial staining we observed in NapA-injected joints. Alternatively, the fact that these chemokines were crucial in recruiting IFNγ- and IL-17–producing cells was confirmed by evidence showing that the application of anti-CCL2, anti-CCL20, and anti-CXCL10 blocking antibodies significantly reduced T cell recruitment promoted by NapA-conditioned supernatants of PMNs. In accordance with these data, we observed the accumulation of not only IFNγ and IL-17 in synovial fluid of patients with LA, but also the accumulation of the 3 chemokines that we demonstrated to be produced by PMNs exposed to NapA. Finally, we report that some T cells recruited by NapA-stimulated PMNs belong to the subset that produces both IFNγ and IL-17, and notably, such a subset was detectable in patients with LA (42–44).

Collectively, our data highlight the role of the NapA antigen in orchestrating the innate and adaptive immune response during the development of LA. Moreover, our data support the notion that other than monocyte/macrophages (14), PMNs (whose role in LA pathogenesis had remained unclear until now) display the potential to guide the adaptive immune response during arthritis onset.

AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. de Bernard had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Codolo, Cassatella, M. D'Elios, de Bernard.

Acquisition of data. Codolo, Bossi, Durigutto, Della Bella, Fischetti, Amedei, Tedesco, S. D'Elios, Cimmino, Micheletti.

Analysis and interpretation of data. Codolo, Bossi, Durigutto, Della Bella, Fischetti, Amedei, Tedesco, S. D'Elios, Cimmino, Micheletti, Cassatella, M. D'Elios, de Bernard.

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