Borrelia burgdorferi Stimulates Macrophages to Secrete Higher Levels of Cytokines and Chemokines than Borrelia afzelii or Borrelia garinii

Klemen Strle,1 Elise E. Drouin,1 Shiqian Shen,1 Joseph El Khoury,1,2 Gail McHugh,1 Eva Ruzic-Sabljic,3 Franc Strle,4 and Allen C. Steere1

1Center for Immunology and Inflammatory Diseases, Division of Rheumatology, Allergy and Immunology, Infectious Diseases Division, Massachusetts General Hospital, Harvard Medical School, Boston, Massachusetts; and 3Institute of Microbiology and Immunology, University of Ljubljana, 4Department of Infectious Diseases, University Medical Center Ljubljana, Ljubljana, Slovenia

To delineate the inflammatory potential of the 3 pathogenic species of Borrelia burgdorferi sensu lato, we stimulated monocyte-derived macrophages from healthy human donors with 10 isolates each of B. burgdorferi, Borrelia afzelii, or Borrelia garinii recovered from erythema migrans skin lesions of patients with Lyme borreliosis from the United States or Slovenia. B. burgdorferi isolates from the United States induced macrophages to secrete significantly higher levels of interleukin (IL)-8, CCL3, CCL4, IL-6, IL-10, and tumor necrosis factor than B. garinii or B. afzelii isolates. Consistent with this response in cultured macrophages, chemokine and cytokine levels in serum samples of patients from whom the isolates were obtained were significantly greater in B. burgdorferi–infected patients than in B. afzelii– or B. garinii–infected patients. These results demonstrate in vitro and in vivo that B. burgdorferi has greater inflammatory potential than B. afzelii and B. garinii, which may account in part for variations in the clinical manifestations of Lyme borreliosis.

Lyme borreliosis, the most common vector-borne disease in North America, Europe, and Asia [1], is caused primarily by 3 pathogenic species of Borrelia burgdorferi sensu lato [2]. In the United States (US), B. burgdorferi is the sole agent of the disease, whereas Borrelia afzelii and Borrelia garinii are more prevalent in Europe. Variations in the clinical manifestations of the disease have been noted with each species [1]. For example, in the US, untreated B. burgdorferi infection often leads to arthritis, whereas in Europe, B. afzelii infection usually remains localized to the skin and B. garinii is associated with a range of neurologic complications.

With all 3 species, the first sign of infection is often an expanding skin lesion, called erythema migrans (EM), sometimes accompanied by non-specific symptoms, such as headache, fatigue, malaise, arthralgias, or myalgias [1, 3–6]. However, B. burgdorferi infection in the US is associated with faster lesion expansion, more disease-associated symptoms, and more frequent hematogenous dissemination, compared with B. afzelii infection in Europe [3, 7, 8]. In Europe, B. garinii causes faster expansion and larger size of EM lesions than B. afzelii [9, 10], but both species are thought to expand more slowly than B. burgdorferi in the US [7, 10]. Both microbial and host factors may play a role in these differences in disease expression.

In an effort to understand host responses to Borrelia species early in the infection, several previous studies examined cytokine or chemokine messenger (mRNA) profiles in the EM skin lesions of US and Austrian patients with Lyme borreliosis [3, 11–13]. Predominant
cytokines in EM lesions were the pro-inflammatory cytokine interferon (IFN)-γ and the anti-inflammatory cytokine interleukin (IL)-10 [11, 13]. However, in many cases, a number of additional proinflammatory cytokines were present, including IL-6, tumor necrosis factor (TNF), and IL-1β, which are associated with the activation of macrophages and other innate immune cells [11, 13]. Similarly, inflammatory chemokine levels were significantly greater in lesional skin than in normal skin, including the neutrophil chemoattractant CXCL1; the macrophage chemoattractants, CCL2, CCL3, and CCL4; and the T cell chemoattractants, CXCL9 and CXCL10 [12]. In a recent study, the EM skin lesions of B. burgdorferi–infected US patients had higher mRNA levels of cytokines and chemokines associated with macrophage activation than the lesions of B. afzelii–infected Austrian patients [3]. However, multiple resident and immune cells are involved in an effective immune response to Borrelia infection, and the inflammatory potential of the 3 pathogenic Borrelia species has not been studied directly in macrophages.

In this study, we stimulated macrophages from healthy human donors with isolates of B. burgdorferi, B. afzelii, or B. garinii recovered from EM skin lesions of patients with Lyme borreliosis from the US or Slovenia. B. burgdorferi induced normal macrophages to secrete higher levels of chemokines and cytokines than B. afzelii or B. garinii. This shows clearly that B. burgdorferi induces a greater inflammatory response in macrophages than B. afzelii or B. garinii.

MATERIALS AND METHODS

Patients. From 1998 through 2001, 93 isolates of B. burgdorferi were recovered from biopsy samples of EM skin lesions in a study of US patients with Lyme borreliosis from Rhode Island or Connecticut [14]. The Human Investigation Committee at Tufts Medical Center (1998–2001) and Massachusetts General Hospital (2002–2008) approved the study. After obtaining informed consent, the study physicians (Dr Nitin Damle [Rhode Island] and Dr Vijay Sikand [Connecticut]) performed a clinical assessment of signs and symptoms. In addition to skin biopsy samples for culturing, blood samples were obtained for polymerase chain reaction (PCR) and serologic testing, and serum samples were frozen for subsequent determinations.

In Slovenia, EM skin biopsies from patients with Lyme borreliosis were obtained from 1999 through 2006. Borrelia species were identified as described elsewhere [7]. Nearly 80% of these isolates were B. afzelii and ~15% were B. garinii [15]. The study physician made a clinical assessment of signs and symptoms. In addition to skin biopsy samples, serum samples were obtained for serologic testing and subsequent determinations. The Medical Ethics Committee of Slovenia approved the study.

For this study, in which normal macrophages were stimulated with patients’ isolates, 10 isolates of B. burgdorferi from EM skin lesions of US patients and 10 isolates each of B. afzelii or B. garinii from EM lesions of Slovenian patients were selected for testing. In an effort to assess isolates with possible differences in virulence, B. burgdorferi isolates from the US were chosen randomly from 5 patients who had hematogenous dissemination, on the basis of a positive PCR test for the RecA gene segment in blood, and from 5 patients who lacked evidence of disseminated disease [14]. In Slovenia, where B. afzelii and B. garinii less often cause hematogenous dissemination [16, 17], 5 isolates each of B. afzelii or B. garinii were selected randomly from patients who had ≥2 symptoms, and 5 each were selected from patients who lacked associated symptoms. The clinical findings regarding EM expansion and the number of associated symptoms in patients from whom the isolates were obtained were consistent with those in larger patient series [3–7].

Preparation of spirochetal isolates. Because it is difficult to count motile spirochetes reliably and because all 30 cultures needed to be ready at the same time, the numbers of organisms in each culture were determined by optical density (OD) and the concentration of organisms was adjusted based on a carefully constructed standard curve. To construct the curve, the numbers of organisms in a control culture (N40) were first counted 4 times and averaged using a Petroff-Hauser chamber with dark field microscopy. The number of organisms was validated by quantitative PCR targeting the RecA gene, as described elsewhere [18]. Serial dilutions of the control culture were then used to generate a standard curve of OD values, as determined by spectrophotometer (Beckman DU 520 spectrophotometer; Beckman Coulter) at 600 nm.

In preparation for experiments with macrophages, each of the 30 low-passage (≤5 passages) isolates were grown to late-log phase in complete BSK medium (Sigma Aldrich) containing 6% rabbit serum [19]. After washing twice, the OD value of each isolate was determined, and the standard curve was used to adjust the concentration of each isolate to 1.25 × 10^8 spirochetes/mL. As a final confirmation, the total protein concentration (D_600 Protein Assay; BioRad) of each isolate was shown to be similar.

Macrophage cell culture. Human macrophages were differentiated from peripheral blood mononuclear cells (PBMCs) obtained from 9 healthy donors by the Massachusetts General Hospital Blood-Component laboratory. To assure that the donor was healthy, he or she was required to answer a questionnaire, provide a vaccination report, and undergo a physical examination. In addition, the blood samples were tested for markers of infectious diseases, including syphilis, hepatitis B virus, hepatitis C virus, human immunodeficiency virus types 1 and 2, human T-lymphotropic virus types I and II, and West Nile Virus. If any of the test results were positive, the blood sample was not used.
Macrophages were derived as described elsewhere [20–22]. Briefly, PBMCs were resuspended in RPMI-1640 (Invitrogen) containing 10% human serum (Mediatech) and seeded in flasks for 1 h to allow attachment of monocytes. Detached cells were removed by washing, and adherent cells were allowed to differentiate into macrophages by 6-day culture in RPMI-1640 supplemented with 25% human serum, 2 mmol/L L-glutamine, 100 μg/mL streptomycin, and 100 U/mL of penicillin at 37°C and 5% CO₂. Before stimulation with borrelial isolates, adherent macrophages were washed 3 times, were transferred to 96-well culture plates at 1 × 10⁵ cells/well, and were deprived of serum and antibiotics for 12 h to remove growth factors. Because the inflammatory response in macrophages from frozen PBMC stocks was lower than in freshly derived macrophages, only fresh macrophages were used in subsequent experiments.

**Macrophage markers.** Pilot experiments using *B. burgdorferi* strain N40 demonstrated that maximum expression of the macrophage-derived cytokines and chemokines occurred within 48 h at a multiplicity of infection of 25 organisms per cell (data not shown). These conditions were used in all subsequent experiments. Monocyte-derived macrophages were >95% pure as evidenced by their expression of cell-surface markers HLA-DR (BioLegend), CD11b, and CD206 (BD Biosciences) following incubation in medium alone or lipopolysaccharide (50 ng/mL) for 36 h (FACSCalibur; BD Biosciences).

**Biologic activity of macrophages.** To assess whether live *Borrelia* were cytotoxic to macrophages in culture, metabolic activity, proliferation, and cell death were measured after 48 h culture with each of the 30 borrelial isolates. The global metabolic activity of macrophages was assessed using the CellTiter Aqueous Cell Proliferation Assay (Promega), according to manufacturer’s protocol. Proliferation was determined by culturing macrophages with each of the borrelial isolates for 30 h, prior to addition of 0.5 μCi of [³H]-thymidine (Perkin Elmer) for 18 h and measurement of [³H]-thymidine incorporation. Macrophage cell death was assessed via colorimetric CytoTox-96 Cytotoxicity Assay (Promega) following manufacturer’s Total-Cell-Number protocol. The percent cell death was a ratio of the absorbance values of lactate dehydrogenase released into culture medium, a sign of cell death, divided by the absorbance value of total lactate dehydrogenase released by homogenizing the remaining cells in the well.

**Detection of chemokines and cytokines from normal macrophages stimulated with patients’ *Borrelia* isolates.** Macrophages were cultured with each of the 10 isolates of *B. burgdorferi*, *B. afzelii*, or *B. garinii* (multiplicity of infection of 25) for 48 h in medium devoid of serum and antibiotics. All 30 isolates were tested with macrophages from each of the 9 healthy donors in 9 independent experiments. The expression of the macrophage-derived chemokines IL-8, CCL2, CCL3, and CCL4 and the cytokines TNF, IL-1β, IL-6, and IL-10 was assessed in culture supernatants (1:25) with use of 3 separate bead-based Multiplex assays from Millipore coupled with the Luminex-200 System (Luminex), following the manufacturer’s overnight protocol. Mean fluorescence intensity was converted to pg/mL with the Upstate Beadview software (Millipore). The results for each chemokine and cytokine from all 9 experiments were averaged for analysis.

**Detection of chemokines and cytokines in patient and control serum samples.** Serum samples, obtained at the time of EM skin biopsy, were available from 22 of the 30 patients from whom *Borrelia* isolates were recovered. Ten were from *B. burg-
**RESULTS**

**Characteristics and biologic activity of macrophages from healthy human donors.** Following differentiation, virtually all (>95%) monocyte-derived cells expressed high levels of HLA-DR, CD11b, and CD206 cell-surface markers, confirming their macrophage lineage (Figure 1A). Stimulation with lipopolysaccharide did not significantly alter the expression of these surface markers (Figure 1B). Thus, culturing of blood-derived monocytes in serum-enriched medium was sufficient to generate macrophages in absence of other stimuli [20–22].

As expected for mature, differentiated cells, no significant differences were observed in the total metabolic activity (Figure 2A), proliferation (Figure 2B), or viability (Figure 2C) of macrophages during the 48-h culture period with isolates from different species. Because *Borrelia* did not adversely affect the proliferation and viability of macrophages, we next assessed chemokine and cytokine secretion from these cells after stimulation with each of the 3 *Borrelia* species.

**Chemokine and cytokine secretion by normal human macrophages stimulated with *Borrelia* isolates.** All 30 borrelioid isolates induced the secretion of all chemokines and cytokines tested, but US *B. burgdorferi* isolates stimulated macrophages to secrete significantly higher levels of IL-8, CCL3, CCL4, IL-6, TNF, and IL-10 than European *B. afzelii* or *B. garinii* isolates (Figure 3). Among these inflammatory mediators, *B. burgdorferi* induced the greatest secretion of IL-8 (median level, 30,088 pg/mL; range, 26,248–42,700 pg/mL), CCL3 (median level, 12,270 pg/mL; range, 7,923–21,441 pg/mL), and IL-6 (median level, 12,540 pg/mL; range, 10,273–15,620 pg/mL). In contrast, the other inflammatory mediators did not differ in induction of CCL2 or IL-1β (Figure 3). Furthermore, no statistically significant differences in cytokine and chemokine secretion were observed between macrophages stimulated with *B. afzelii* or *B. garinii*. Among the US *B. burgdorferi* isolates, the levels of chemokines and cytokines did not correlate with dissemination, and among the *B. afzelii* or *B. garinii* isolates, the levels of these inflammatory mediators did not correlate with whether the patient had associated symptoms (data not shown). In comparison with *Borrelia*-activated macrophages, unstimulated cells secreted only low levels of IL-8 (median level, 173 pg/mL), CCL2 (405 pg/mL), and CCL3 (11 pg/mL) and undetectable levels of the other inflammatory mediators measured here (data not shown). We concluded that US *B. burgdorferi* isolates induced macrophages to secrete higher levels of inflammatory chemokines and cytokines than Slovenian *B. afzelii* or *B. garinii* isolates.

**Chemokine and cytokine levels in serum samples from patients from whom the *Borrelia* isolates were obtained.** Frozen serum samples, but not EM skin biopsy samples, were available from 22 of the 30 patients, including all 10 patients with *B. burgdorferi* infection, 8 with *B. afzelii* infection, and 4 with *B. afzelii*-infected US patients; 8 were from *B. afzelii*-infected and 4 were from *B. garinii*-infected Slovenian patients. In these samples, and in those from 9 healthy control subjects, the levels of chemokines (IL-8, CCL2, CCL3, CCL4, and CXCL-10) and cytokines (IL-6, IL-10, TNF, IL-1β, and IFN-γ) were assessed at the same time with use of the bead-based Multiplex assays (Millipore). Because of limited amounts of serum sample from patients, cytokine and chemokine levels in the samples were only determined once.

**Statistical analysis.** Differences between groups of borrelioid isolates were assessed using the Mann-Whitney rank-sum test. Statistical analyses were conducted using Sigma Stat, version 3.0.1 (SPSS).
Figure 3. Comparison of United States Borrelia burgdorferi isolates and Slovenian Borrelia afzelii and Borrelia garinii isolates. Normal human macrophages were stimulated for 48 h with 10 B. burgdorferi isolates from erythema migrans lesions of United States patients or 10 B. afzelii isolates or 10 B. garinii isolates from erythema migrans lesions of Slovenian patients. Chemokine (A) and cytokine (B) levels were determined in culture supernatants with use of bead-based multiplex assays. All 30 isolates were tested with macrophages from each of the 9 healthy donors. The values from the 9 experiments were then averaged. The box in the graph represent 25th and 75th percentiles, the line in the box is the median value, and the lines outside the box represent 5th and 95th percentiles. Except for CCL2 and IL-1β, B. burgdorferi isolates induced significantly higher levels of chemokines and cytokines than B. afzelii and B. garinii isolates. IFN, interferon; IL, interleukin; TNF, tumor necrosis factor. *; **; ***.

Consistent with the response of macrophages to Borrelia stimulation in culture, the levels of CCL3, CCL4, IL-6, IL-10, and TNF were significantly higher in serum of B. burgdorferi–infected US patients than in serum of B. afzelii– or B. garinii–infected Slovenian patients or normal control subjects. A similar trend was observed for CCL2 and IL-1β (Figure 4). In addition, serum levels of the T cell–derived cytokine IFN-γ and the IFN-γ–inducible chemokines CCL2 and CXCL10 were high, particularly in B. burgdorferi–infected patients. Among the American patients, there was not a significant correlation between the presence of B. burgdorferi DNA in blood and the levels of chemokines and cytokines (data not shown). Chemokine and cytokine levels in the serum samples of Slovenian patients were similar to those in normal control subjects. Thus, the presence of chemokines and cytokines in serum is a phenomenon associated with B. burgdorferi infection in the US.

garinii infection. Because the results were similar in Slovenian patients infected with B. afzelii or B. garinii and because of small numbers, particularly in the B. garinii group, the results in these patients were combined for presentation (Figure 4).
**DISCUSSION**

Previous studies have suggested that *B. burgdorferi* may be more virulent than *B. afzelii* or *B. garinii* [3, 7]. Compared with the 2 European *Borrelia* species, *B. burgdorferi* in the US causes faster expansion of EM skin lesions, is associated with more symptoms, and more often disseminates hematogenously [7, 8, 16, 17]. In addition, in our previous study, the EM lesions of *B. burgdorferi*-infected US patients had higher mRNA expression of inflammatory chemokines and cytokines, including those associated with macrophage activation, than the lesions of *B. afzelii*-infected patients [3]. In both human infection and the murine models, macrophages are critical in the early innate immune response [23–27]. However, the possibility that the 3 pathogenic *Borrelia* species differ in their ability to activate macrophages had not been assessed. We tested this hypothesis by stimulating macrophages from normal human donors with isolates of the 3 *Borrelia* species and measured chemokine and cytokine responses in culture supernatants.

A number of steps were taken to standardize and validate the study methods. Because individual isolates of each *Borrelia* species may vary in virulence, we tested 10 isolates of each of the 3 pathogenic *Borrelia* species obtained from patients' EM skin lesions. In preliminary experiments, we confirmed that monocytes differentiated into macrophages and showed that the global metabolic activity, proliferation, and viability of these cells were not adversely affected by culture with *Borrelia* species.
In an effort to hold host factors constant, we tested fresh macrophages from each of the 9 healthy donors with all 30 isolates. We timed the experiments so that isolates reached late-log phase at the same time that fresh macrophages differentiated to maturity. The numbers of organisms in each culture were determined by OD, and the concentration of organisms was adjusted based on a carefully constructed standard curve so that macrophages were stimulated with similar numbers of spirochetes. Because spirochetes begin to die within hours after removal from serum-enriched BSK medium [28], we do not think that the numbers of spirochetes increased in the macrophage cell cultures. Finally, we utilized bead-based multiplex assays to simultaneously measure the protein expression of multiple chemokines and cytokines from 1 complete experiment. This optimized culture system clearly showed that US B. burgdorferi isolates stimulate macrophages to secrete higher levels of IL-8, CCL3, CCL4, IL-6, TNF, and IL-10 than European B. afzelii or B. garinii isolates.

Consistent with the response of macrophages to stimulation with Borrelia species, the levels of these chemokines and cytokines in the serum samples of patients from whom the isolates were obtained were greater in B. burgdorferi–infected patients than in B. afzelii– or B. garinii–infected patients. This shows that the higher inflammatory potential of US B. burgdorferi isolates is not just an in vitro phenomenon observed with macrophages; it also occurs during the course of the natural infection. However, in contrast with cell culture, serum reflects global innate and adaptive immune responses of multiple cell types. In addition to the macrophage-derived chemokines and cytokines, serum samples from patients with B. burgdorferi infection contained significantly higher levels of the T cell-derived cytokine IFN-γ and the IFN-γ–inducible chemokines CCL2 and CXCL10 than serum samples from B. afzelii– or B. garinii–infected patients. We do not yet know whether B. burgdorferi activates other immune cells directly and to a greater degree than B. garinii or B. afzelii or whether macrophages mediate the greater global immune responses associated with B. burgdorferi infection. Regardless, the analysis of serum samples, which is consistent with the results from EM skin lesions [3, 12], shows that US B. burgdorferi isolates activate directly or indirectly both innate and adaptive immune responses to a greater degree than European B. afzelii or B. garinii isolates.

Why do US B. burgdorferi isolates have greater inflammatory potential than European B. garinii or B. afzelii isolates? Because B. burgdorferi induces greater secretion of the same cytokines and chemokines as B. afzelii or B. garinii, it may simply express higher levels of one or more of the same lipoproteins. Alternatively, B. burgdorferi may express one or more different lipoproteins, which could trigger additional intracellular signaling cascades, leading to higher cytokine and chemokine production. Several studies demonstrate the heterogeneity of lipoprotein expression among and within Borrelia species [29, 30]. We believe that our macrophage cell-culture system provides an important model to characterize the effects of these differences on immune cells.

The greater inflammatory potential of US B. burgdorferi isolates probably accounts for certain differences in the clinical manifestations of Lyme borreliosis in the US and Europe. For example, the greater inflammatory responses to B. burgdorferi in American patients may well explain the larger number of symptoms associated with EM skin lesions [3, 7] and the greater swelling of joints [1], compared with B. garinii or B. afzelii infection in Europe. However, it is not yet clear how these differences in host immune responses influence hematogenous dissemination, tropism to particular tissues, or persistence of the infection. B. burgdorferi in the US is more commonly associated with hematogenous dissemination than B. afzelii or B. garinii in Europe [3, 4, 7, 16, 17]. Surprisingly, in the 10 American patients in our study, the presence of B. burgdorferi DNA in blood did not correlate with the levels of chemokines and cytokines. However, not all patients with hematogenous dissemination have a positive PCR result in blood [8, 14], and the possible presence of patients with dissemination in the PCR-negative group may blur distinctions between groups. What is clear is that patients infected with US B. burgdorferi isolates, whether they had PCR evidence of dissemination or not, had significantly higher levels of chemokines and cytokines in blood than those infected with European Borrelia isolates.

In summary, our results show that B. burgdorferi directly stimulates macrophages to secrete higher levels of chemokines and cytokines than B. garinii and B. afzelii. In addition, the analysis of serum samples shows that B. burgdorferi activates both innate and adaptive immune responses to a greater degree than the 2 European Borrelia species. These results show unequivocally that B. burgdorferi has greater inflammatory potential than B. afzelii and B. garinii, which is likely to account in part for variations in the clinical manifestations of Lyme borreliosis.

Acknowledgments

We thank Dr Nitin Damle and Dr Vijay Sikand for obtaining the skin biopsy and serum samples from patients with EM, Dr Xin Li for help with culture of borrelial isolates, Dr Lisa Glickstein for review of the manuscript, and Colleen Squires for help with preparation of the manuscript.

References