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

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## Abstract

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*, *B. afzelii*, or *B. garinii* recovered from erythema migrans (EM) skin lesions of Lyme borreliosis patients from the United States or Slovenia. U.S. *B. burgdorferi* isolates induced macrophages to secrete significantly higher levels of IL-8, CCL3, CCL4, IL-6, IL-10 and TNF than *B. garinii* or *B. afzelii* isolates. Consistent with this response in cultured macrophages, the cytokine levels in sera 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.

### Keywords

Lyme disease; Borrelia; inflammation; macrophages; cytokines; chemokines

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 (U.S.), *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 U.S., untreated *B. burgdorferi* infection often leads to arthritis, whereas in Europe, *B. afzelii* infection usually

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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, U.S. *B. burgdorferi* infection is associated with faster lesion expansion, more disease-associated symptoms, and more frequent hematogenous dissemination compared with European *B. afzelii* infection [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 U.S. [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 mRNA profiles in the EM skin lesions of U.S. and Austrian patients with Lyme borreliosis [3,11-13]. Predominant cytokines in EM lesions were the pro-inflammatory cytokine IFN-γ and the anti-inflammatory cytokine IL-10 [11,13]. However, in many cases, a number of additional pro-inflammatory cytokines were present, including IL-6, TNF, and IL-1β, which are associated with the activation of macrophages and other innate immune cells [11,13]. Similarly, inflammatory chemokines 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 U.S. 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 U.S. 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

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

In Slovenia, EM skin biopsies from Lyme borreliosis patients were obtained from 1999 through 2006. *Borrelia* species were identified as previously described [7]. Nearly 80% of these isolates were *B. afzelii* and approximately 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 U.S. 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 U.S. were chosen randomly from 5 patients who had hematogenous dissemination, based on a positive PCR test for *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 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 darkfield microscopy. The number of oganisms was validated by QPCR targeting the *RecA* gene, as previously described [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 600nm.

In preparation for experiments with macrophages, each of the 30 low passage (≤5) 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 $1.25 \times 10^8$ spirochetes/ml. As a final confirmation, the total protein concentration ($D_C$ Protein Assay, BioRad) of each isolate was shown to be similar.

## Macrophage cell culture

Human macrophages were differentiated from peripheral blood mononuclear cells (PBMC) obtained from 9 healthy donors by the MGH 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, HBV, HCV, HIV 1 / 2, HTLV I/II and West Nile Virus. If any of the tests were positive, the blood sample was not used.

Macrophages were derived as previously described [20-22]. Briefly, PBMC were re-suspended 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, 2mM L-glutamine, 100μg/ml streptomycin and 100μ/ml of penicillin at 37°C and 5% $CO_2$. Before stimulation with borrelial isolates, adherent macrophages were washed 3 times, transferred to 96-well culture plates at $1\times10^5$ cells/well and 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 (MOI) 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 LPS (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 0.5μCi of $^{3}$H-thymidine (Perkin Elmer) for 18 h and measurement of $^{3}$H-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 LDH released into culture medium, a sign of cell death, divided by the absorbance value of total LDH 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* (MOI 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 macrophage-derived chemokines, IL-8, CCL2, CCL3 and CCL4, and cytokines, TNF, IL-1β, IL-6, and IL-10, was assessed in culture supernatants (1:25) using 3 separate bead-based Multiplex assays from Millipore coupled with the Luminex-200 System (Luminex), following manufacturer's overnight protocol. Mean fluorescence intensity was converted to pg/ml by 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; 10 were from *B. burgdorferi*—infected U.S. patients, 8 from *B. afzelii*-infected and 4 from *B. garinii*-infected Slovenian patients. In these samples, and in those from 9 healthy controls, the levels of chemokines (IL-8, CCL2, CCL3, CCL4 and CXCL-10) and cytokines (IL-6, IL-10, TNF, IL-1β and IFNγ) were assessed at the same time using the bead-based Multiplex assays (Millipore). Due to limited amounts of serum from patients, cytokine and chemokine levels in the samples were only determined once.

### Statistical Analysis

Differences between groups of borrelial isolates were assessed using the Mann-Whitney rank-sum test. Statistical analyses were conducted using the Sigma Stat version 3.0.1 software from SPSS.

# 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 (Fig. 1A). Stimulation with LPS did not significantly alter the expression of these surface markers (Fig. 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 (Fig. 2A), proliferation (Fig. 2B) or viability (Fig. 2C) of macrophages during the 48-hour culture period with isolates from different species. Since *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 borrelial isolates induced the secretion of all chemokines and cytokines tested, but U.S. *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 (Fig. 3). Among these inflammatory mediators, *B. burgdorferi* induced the highest secretion of IL-8 (median value, 30,088 pg/ml; range, 26,248-42,700), CCL3 (12,270 pg/ml; 7923-21441) and IL-6 (12,540 pg/ml; 10,273-15,620). In contrast, the 3 *Borrelia* species did not differ in induction of CCL2 or IL-1β (Fig. 3). Furthermore, no statistically significant differences in cytokine and chemokine secretion were observed between macrophages stimulated with *B. afzelii* or *B. garinii*. Among the U.S. *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 or not the patient had associated symptoms (data not shown). In comparison with *Borrelia*-activated macrophages, unstimulated cells secreted only low levels of IL-8 (median value, 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 U.S. *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. 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 (Fig. 4).

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 U.S. 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 (Fig. 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 sera 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.

## 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 U.S. 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 U.S. 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 the human infection and in 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. Since individual isolates of each *Borrelia* species may vary in virulence, we tested 10 isolates 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 all 30 isolates with fresh macrophages from each of the 9 healthy donors. 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 optical density (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 when removed 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 U.S. *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 U.S. *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, sera 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 sera 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 sera, which is consistent with the results from EM skin lesions [3,12], shows that U.S. *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 U.S. *B. burgdorferi* isolates have greater inflammatory potential than European *B. garinii* or *B. afzelii* isolates? Since *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. Alternately, *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 U.S. *B. burgdorferi* isolates probably accounts for certain differences in the clinical manifestations of Lyme borreliosis in the United States 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 U.S. 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 U.S. *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 sera 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.

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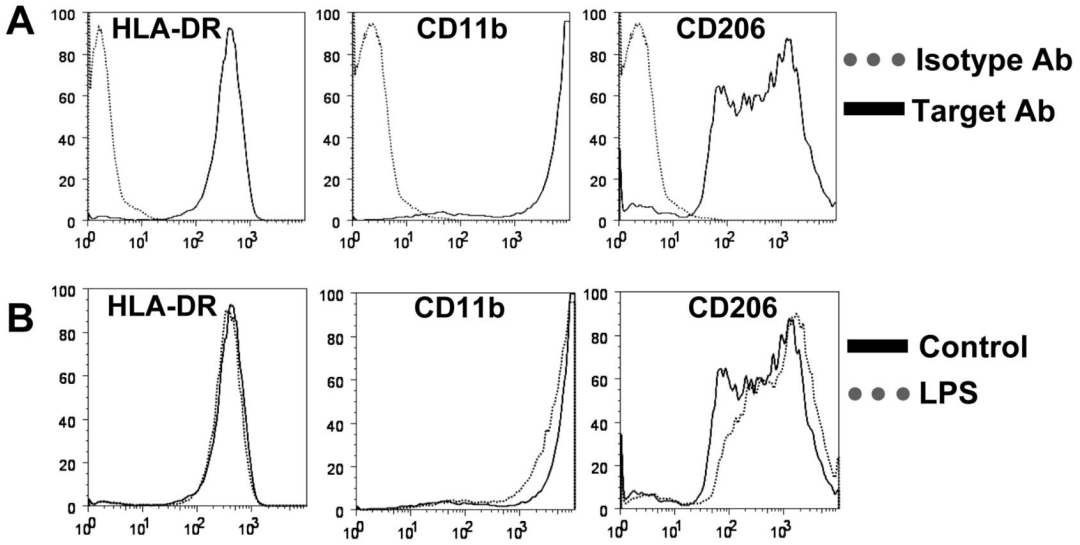


**Figure 1. Characterization of macrophage cell surface markers**

*A)* Monocyte-derived macrophages from normal human donors were cultured in medium alone for 12 h and then incubated with antibodies specific for cell surface markers HLA-DR, CD11b and CD206 (solid line), or with isotype control antibodies (dotted line). *B)* Macrophages from the same human donors were stimulated for 36 h with LPS (50ng/ml; dotted line), or left untreated (solid line) before incubation with antibodies specific for HLA-DR, CD11b and CD206. Approximately 95% of monocyte-derived macrophages from normal human donors expressed high levels of HLA-DR, CD11b and CD206, which is indicative of their macrophage lineage. The data shown are representative of 2 independent experiments.

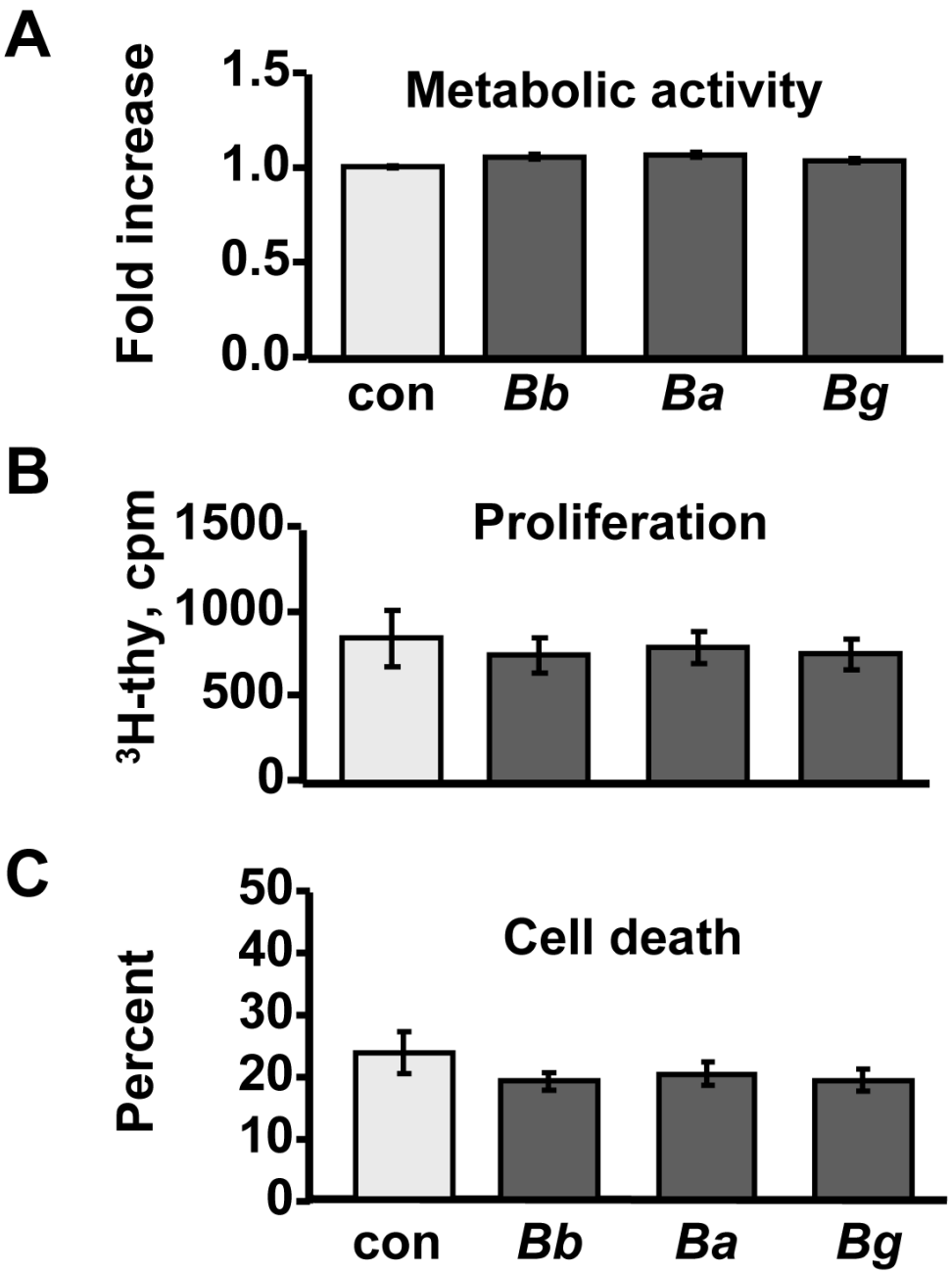


**Figure 2. Biological activity of macrophages**

Macrophages from normal human donors were stimulated for 48 h with 10 *B. burgdorferi* isolates from EM lesions of U.S. patients, or 10 isolates each of *B. afzelii* or *B.garinii* from EM lesions of Slovenian patients. Unstimulated cells served as a control (con). *A)* Metabolic activity was expressed as fold increase above unstimulated control cells. *B)* Proliferation was assessed by the amount (cpm) of $^{3}$H-thymidine incorporated into DNA. *C)* Percent cell death was determined as a ratio of LDH released into culture medium divided by the total LDH released after homogenizing all cells. The data shown are representative of 2 independent experiments.

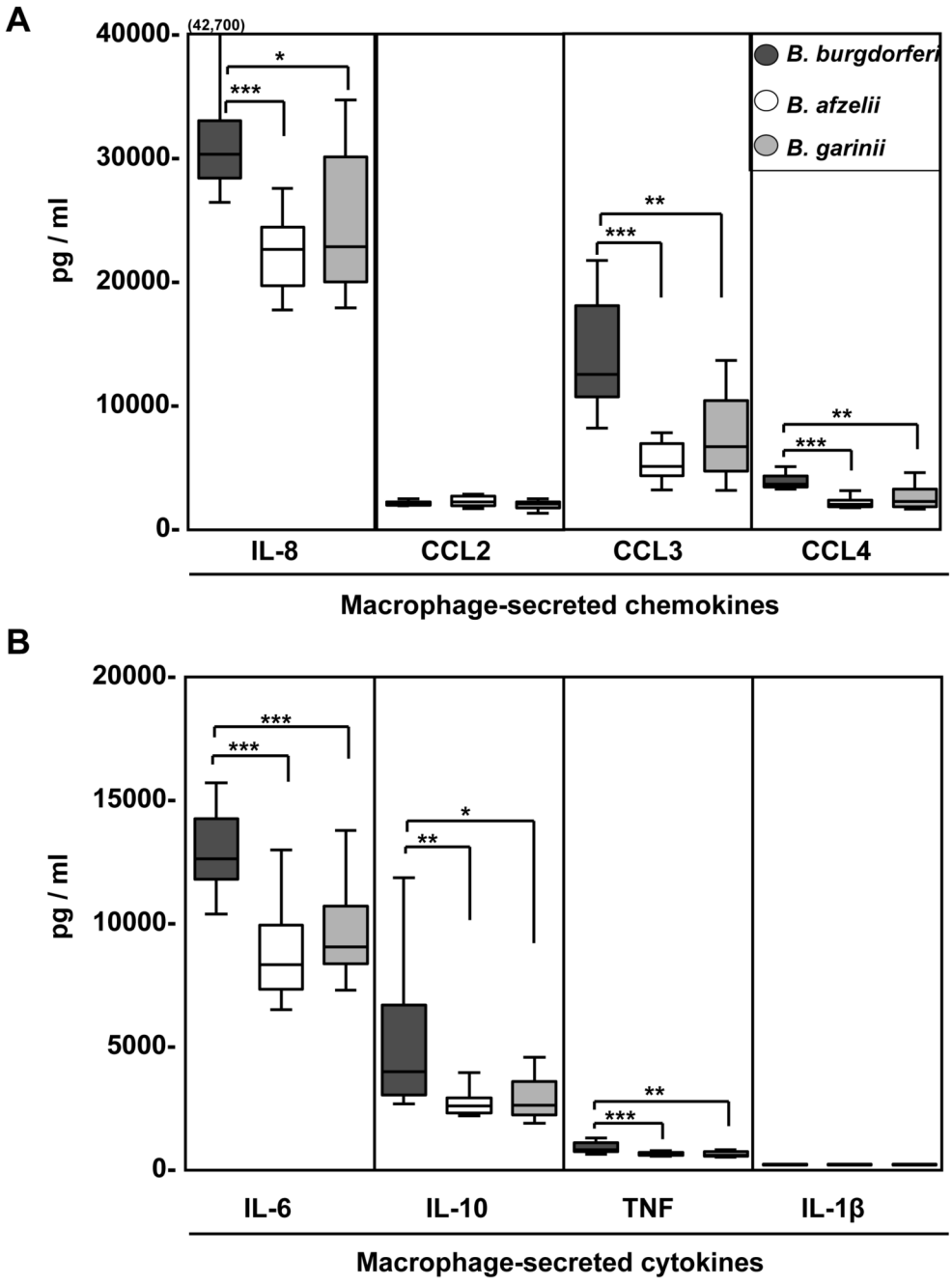


**Figure 3. Comparison of U.S. *B. burgdorferi* isolates and Slovenian *B. afzelii* and *B. garinii* isolates**

Normal human macrophages were stimulated for 48 h with 10 *B. burgdorferi* isolates from EM lesions of U.S. patients, or 10 *B. afzelii* isolates or 10 *B. garinii* isolates from EM lesions of Slovenian patients. *A)* Chemokine and *B)* cytokine levels were determined in culture supernatants using 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. *P ≤ 0.05, **P ≤ 0.01 ***P ≤ 0.001.

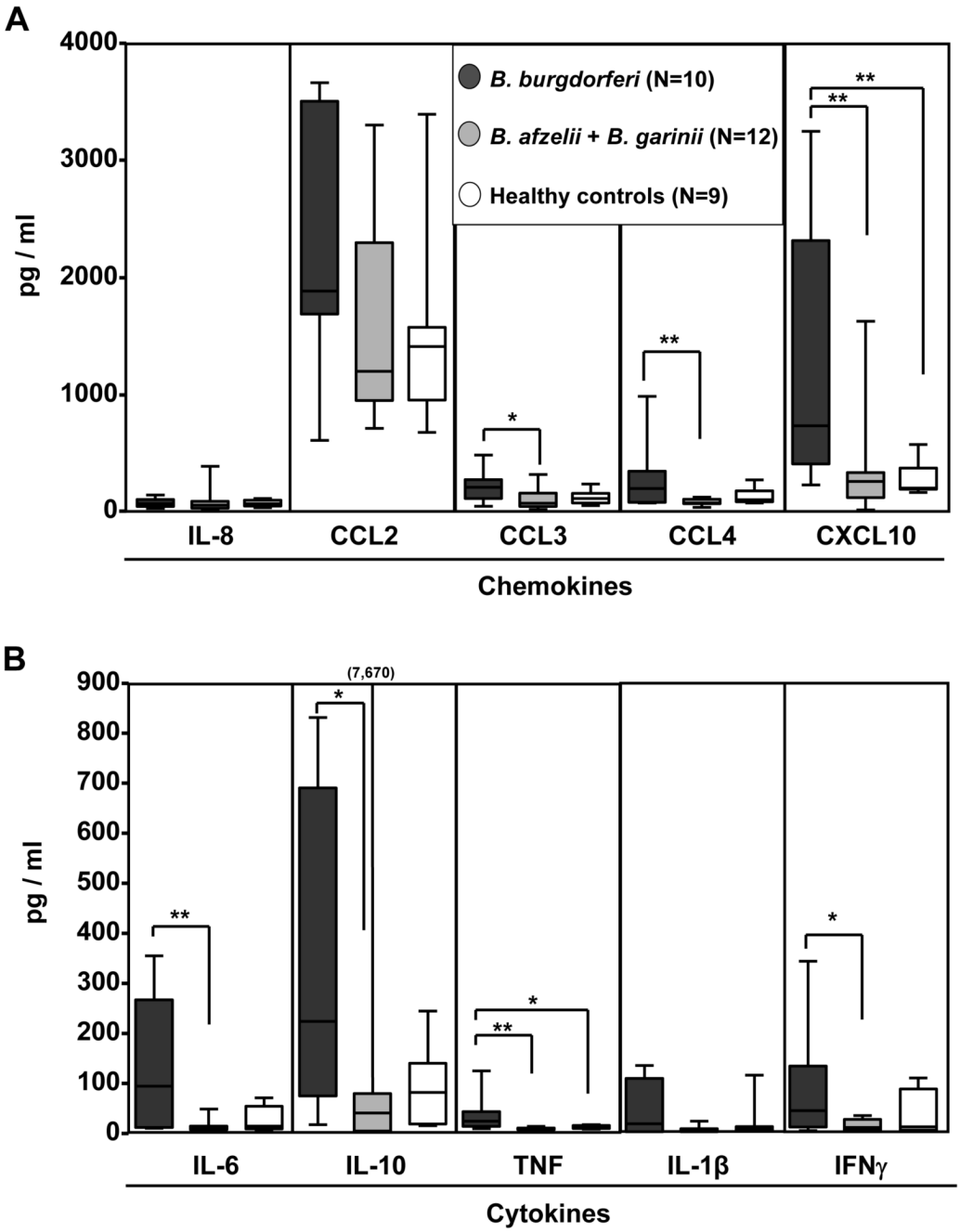


**Figure 4. Serum levels of chemokines and cytokines**

Chemokine and cytokine levels were assessed in serum samples from 22 of the 30 patients from whom the *Borrelia* isolates were obtained and from 9 healthy donors with no history of Lyme borreliosis. Of the 22 patient sera, 10 were from U.S. patients with *B. burgdorferi* infection, and 12 were from Slovenian patients, 8 with *B. afzelii* infection and 4 with *B. garinii* infection. *A)* Chemokine and *B)* cytokine expression was determined using bead-based multiplex assays. The box in the graph represents 25$^{th}$ and 75$^{th}$ percentiles, the horizontal line in the box is the median and the lines outside the box are the 5$^{th}$ and 95$^{th}$ percentiles. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001.