INFECTION AND IMMUNITY, Dec. 2011, p. 4876–4892 Vol. 79, No. 12
0019-9567/11/$12.00 doi:10.1128/IAI.05451-11

Interleukin-10 Alters Effector Functions of Multiple Genes Induced by *Borrelia burgdorferi* in Macrophages To Regulate Lyme Disease Inflammation▽†

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Received 31 May 2011/Returned for modification 18 July 2011/Accepted 18 September 2011

Interleukin-10 (IL-10) modulates inflammatory responses elicited *in vitro* and *in vivo* by *Borrelia burgdorferi*, the Lyme disease spirochete. How IL-10 modulates these inflammatory responses still remains elusive. We hypothesize that IL-10 inhibits effector functions of multiple genes induced by *B. burgdorferi* in macrophages to control concomitantly elicited inflammation. Because macrophages are essential in the initiation of inflammation, we used mouse J774 macrophages and live *B. burgdorferi* spirochetes as the model target cell and stimulant, respectively. First, we employed transcriptome profiling to identify genes that were induced by stimulation of cells with live spirochetes and that were perturbed by addition of IL-10 to spirochete cultures. Spirochetes significantly induced upregulation of 347 genes at both the 4-h and 24-h time points. IL-10 inhibited the expression levels, respectively, of 53 and 65 of the 4-h and 24-h genes, and potentiated, respectively, at 4 h and 24 h, 65 and 50 genes. Prominent among the novel identified IL-10-inhibited genes also validated by quantitative real-time PCR (qRT-PCR) were Toll-like receptor 1 (TLR1), TLR2, IRAK3, TRAF1, IRG1, PTGS2, MMP9, IFI44, IFIT1, and CD40. Proteome analysis using a multiplex enzyme-linked immunosorbent assay (ELISA) revealed the IL-10 modulation/and or potentiation of RANTES/CCL5, macrophage inflammatory protein 2 (MIP-2)/CXCL2, IP-10/CXCL10, MIP-1α/CCL3, granulocyte colony-stimulating factor (G-CSF)/CSF3, CXCL1, CXCL5, CCL2, CCL4, IL-6, tumor necrosis factor alpha (TNF-α), IL-1α, IL-1β, gamma interferon (IFN-γ), and IL-9. Similar results were obtained using sonicated spirochetes or lipoprotein as stimulants. Our data show that IL-10 alters effectors induced by *B. burgdorferi* in macrophages to control concomitantly elicited inflammatory responses. Moreover, for the first time, this study provides global insight into potential mechanisms used by IL-10 to control Lyme disease inflammation.

Lyme disease, the most frequently reported arthropod-borne disease in the United States, results from infection with the spirochete *Borrelia burgdorferi.* The disease is spread to humans and other mammals through the bite of infected *Ixodes* ticks (18). Invasion of the mammalian host with spirochetes results in the activation of inflammatory pathways that lead to the release of inflammatory mediators and an influx of inflammatory cells that result in many of the clinical manifestations of Lyme disease (9, 42–44, 79). Manifestations of the disease include, among others, acute or chronic arthritis, carditis, and neuroborreliosis (23, 31, 75, 79, 93). The inflammatory immune response is of crucial importance for the early containment of infection but at the same time has the potential to result in immunopathology. It is thought that inflammation, either induced by the spirochete or by spirochete antigens left in tissues after bacterial demise, plays a major role in disease pathogenesis. The final outcome of infection, therefore, depends on an intricate balance between the pathogen and the host response.

The anti-inflammatory cytokine interleukin-10 (IL-10) plays a pivotal role in limiting the inflammatory response and preventing tissue damage. This is mainly achieved by downregulating the expression of inflammatory mediators as well as inhibiting effector functions of T cells and mononuclear phagocytes (30). In addition to these activities, IL-10 regulates growth and/or differentiation of T and B cells, NK cells, cytotoxic cells, mast cells, granulocytes, dendritic cells (DCs), keratinocytes, and endothelial cells (63). Different preparations of *B. burgdorferi* spirochetes (live, sonicated, freeze-thawed, and heat inactivated) and lipoproteins induce IL-10 in a variety of cell types. IL-10 production has been detected in joint tissues (24, 61), skin tissues (53) lymph node cells (34), splenocytes (3), glial cells (82), and macrophages (17, 27, 53) in the murine model of Lyme disease. In humans, we have shown that *B. burgdorferi* induces the production of IL-10 *in vitro* in mononuclear cells present in peripheral blood (40). Other investigators have shown production of IL-10 in peripheral blood (20,

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† Supplemental material for this article may be found at http://iai.asm.org/.

▽ Published ahead of print on 26 September 2011.

28, 92), macrophages (94), dendritic cells (92), lymphocytes (49, 73, 76), cerebrospinal fluid (CSF) (20), synovium (98), microglia (19), skin (86), and erythema-migrans skin lesions of *B. burgdorferi*-infected patients (50).

IL-10 has proved to be a key cytokine in regulating inflammatory responses elicited by *B. burgdorferi*. We (35) along with others (17, 53, 54, 96) have reported results of experiments conducted *in vitro* showing that in response to *B. burgdorferi* or its lipoproteins, IL-10 dampens proinflammatory cytokines such as IL-1β, tumor necrosis factor (TNF), IL-6, IL-12, IL-18, and gamma interferon (IFN-γ) of cells that are involved in innate and adaptive immunity in the murine model. Using human monocytic THP-1 cells, we demonstrated that IL-10 down-modulated the production of IL-1β, TNF, IL-12, and IL-6, as elicited by spirochetal lipoproteins (66). Studies by Lisinski and Furie (56) showed that IL-10 decreases production of CXCL8 in *B. burgdorferi*-stimulated endothelial cells. The fact that C57BL/6J (17) and C3H (16) mice deficient in IL-10 and infected with *B. burgdorferi* develop more severe arthritis and harbor fewer spirochetes in joints than wild-type mice suggests an important *in vivo* role for IL-10 as mediator of anti-inflammatory immune responses induced by *B. burgdorferi*. The above *in vitro* and *in vivo* studies suggest that IL-10 may profoundly inhibit a broad spectrum of inflammatory mediators induced by *B. burgdorferi*.

Macrophage function has been proposed to be critical for mice to combat *B. burgdorferi* infection (5). Since monocytes/macrophages are the primary IL-10 responders to pathogen-associated molecular patterns (PAMPS) and since their activation initiates most inflammatory responses (59), we hypothesize that IL-10 inhibits effector functions of multiple genes induced by *B. burgdorferi* in macrophages to control concomitantly elicited inflammation. The viability of this hypothesis was tested by first using murine whole-genome microarray to identify inflammatory mediators that were induced in cultured J774 mouse macrophages in response to stimulation with live *B. burgdorferi* spirochetes and that were further perturbed by the addition of IL-10 to these cultures. Next, alternative approaches such as TaqMan quantitative real-time PCR (qRT-PCR) and proteome analysis (cytokine/chemokine multiplex or single enzyme-linked immunosorbent assays [ELISAs]) were used to confirm the microarray data. Findings from the experiments in which live *B. burgdorferi* spirochetes were used as stimulants were compared to those obtained with macrophages stimulated with sonicated *B. burgdorferi* or with purified recombinant lipidated outer surface protein A (L-OspA) either via microarray, qRT-PCR, or single and multiplex ELISAs. The results of this study are presented and discussed in the global context of IL-10-mediated control of inflammation in Lyme disease.

MATERIALS AND METHODS

Bacteria and lipoprotein. *B. burgdorferi* spirochetes (strain B31, clone 5A19, with the complete plasmid content) were grown *in vitro* in Barbour-Stoenner-Kelly H (BSK-H) medium as previously described (36, 40). Purified recombinant L-OspA was kindly provided by GlaxoSmithKline Biologicals (Rixensart, Belgium). The L-OspA preparation contained less than 0.25 endotoxin units per mg of protein, as assessed by *Limulus* amebocyte assay (Associates of Cape Cod, Woods Hole, MA).

Cell stimulation and culture conditions. The mouse J774 macrophage cell line was obtained from the American Type Culture Collection (Waldorf, MD). We chose this cell line because it has been shown to be phagocytic for *B. burgdorferi* spirochetes (62). In addition, we have shown employing mouse J774 macrophages (27), human THP-1 monocytic cell line (66), and mouse primary lymph node cells (35) that IL-10 alters the expression levels of several cytokines induced by *B. burgdorferi* stimuli irrespective of the cells used. Our published work (27) also indicated the ability of IL-10 to diminish live-spirochete-induced cytokines. Thus, to further understand the extent of IL-10 anti-inflammatory effect on live-spirochete-inducible inflammatory mediators, we selected mouse J774 macrophages as our modeled cell line. Cell culture medium for J774 cells consisted of Dulbecco's medium (Gibco Invitrogen, Carlsbad, CA), 10% heat-inactivated fetal bovine serum, 1 mM HEPES (Gibco Invitrogen), 2 mM L-glutamine (Gibco Invitrogen), and 1 μg/ml antibiotic/antimycotic (Gibco Invitrogen). Cells (3×10^6/ml) were cultured in 24-well plates (Costar, Cambridge, MA) and incubated at 37°C in a humidified atmosphere with 5% CO_2 for various periods of time depending on the experimental procedure. Macrophages were stimulated with live *B. burgdorferi* spirochetes at a 10:1 multiplicity of infection (MOI) in the presence or absence of mouse recombinant IL-10 (10 ng/ml). Live spirochetes were incubated with cells in antibiotic-free medium. Some cells were stimulated also with either L-OspA at 1 μg/ml or sonicated *B. burgdorferi* spirochetes (1×10^7/ml) in the presence or absence of IL-10 (10 ng/ml). Unstimulated cells served as negative controls for all experiments. To study the role of IL-10 alone on constitutive expression of inflammatory mediators, cells incubated with recombinant IL-10 only were included in all real-time PCR and cytokine assays. All cultures were subsequently centrifuged at $400 \times g$ at 4°C for 10 min to collect cell-free supernatants and cell pellets. RNA was extracted from the cell pellets using a Qiagen RNeasy Kit (Qiagen Inc., Valencia, CA), which included a DNase I digestion step. Supernatant and RNA samples were stored at −80°C until they were used.

Mouse whole-genome microarray. Previous experimental designs routinely used in our laboratory and published previously (27) showed no differences in the IL-10 anti-inflammatory effects on inflammatory mediators induced by *B. burgdorferi* stimulants in macrophages when (i) stimulants were cocultured simultaneously with IL-10 and (ii) when IL-10 was added to cells first followed by addition of stimulants or vice versa (27); we therefore decided, in the present study, to focus only on stimulants cocultured simultaneously with IL-10. Thus, to fulfill this objective, total RNA obtained from 4-h and 24-h samples of unstimulated cells, live *B. burgdorferi* spirochetes alone, or live *B. burgdorferi* spirochetes combined with IL-10 were subjected to mouse whole-genome microarray studies. A quantity of 100 ng of total RNA was used to generate Cy-labeled cDNA samples using a low-RNA input linear amplification kit (LRILAK) (Agilent Technologies, Inc., Foster City, CA). Control samples were labeled with Cy3, whereas experimental samples were labeled with Cy5. Labeled cDNA was hybridized overnight to Agilent's Whole Mouse Genome Oligo microarray printed in a 4 by 44,000 format (Agilent Technologies Inc.), which is comprised of 41,534 60-mer oligonucleotide probes representing over 41,000 mouse genes and transcripts at once. Hybridization was performed in a SciGene 4000 HybOven (SciGene Corp., Sunnyvale, CA) at 65°C for 18 h in a rotary chamber at 10 rpm. The slides were then washed using the manufacturer's protocol (Agilent) and scanned in a dual-confocal continuous microarray scanner (GenePix 4000B; Molecular Devices, Sunnyvale, CA), using GenePix Pro, version 6.1, as the image acquisition and extraction software. The microarray data are based on experiments conducted twice with similar RNA samples, and each data set was scanned twice. In addition, we used RNA samples obtained at 24 h from unstimulated cells and from sonicated *B. burgdorferi* spirochetes (1×10^7/ml) alone or combined with IL-10 as stimulants for microarray experiments.

The resulting microarray text data were imported into Spotfire DecisionSite for Functional Genomics (Spotfire Inc., Somerville, MA), filtered, and subjected to statistical analysis. Genes whose expression changed by at least 2-fold or more for upregulated genes and −2-fold or less for downregulated genes (with a corrected one-way analysis of variance, $P < 0.05$) compared with unstimulated cells were considered to be differentially expressed in a statistically significant manner. We then arbitrarily used a cutoff ratio (number of live spirochetes/number of live spirochetes with IL-10) of ≥1.2 to determine genes considered to be inhibited by IL-10. To determine genes that were potentiated by IL-10, we used a corresponding cutoff ratio of ≤0.83, the equivalent of the 1.2 cutoff ratio.

Quantitative real-time PCR. TaqMan PCR was performed to validate the expression level of randomly selected genes from multiple pathways identified by microarray to be upregulated by live *B. burgdorferi* spirochetes and downregulated in macrophages when IL-10 was added to live *B. burgdorferi* cultures. qRT-PCR was carried out using a TaqMan RNA-to-C_T one-step kit (where C_T is threshold cycle) in combination with TaqMan gene expression assays on an ABI Prism HT7900 Sequence Detection System (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. FAM (6-carboxyfluores-

cein)-labeled TaqMan gene expression assays (Applied Biosystems) were used to measure the transcription level of the genes: *Ifi44* (Mm00505670_m1; interferon-induced protein 44), *Ifit1* (Mm0051515_m1; interferon-induced protein with tetratricopeptide repeats 1), *Tlr2* (Mm00442346_m1; Toll-like receptor 2), *Tlr1* (Mm00446095_m1; Toll-like receptor 1), *Irg1* (Mm01224529_m1; immunoresponsive gene 1), *Ptgs2* (Mm01307329_m1; prostaglandin-endoperoxide synthase 2 [COX2]), *CD40* (Mm00441891_m1; CD40 antigen), *MMP9* (Mm00442991_m1; matrix metallopeptidase 9), *Traf1* (Mm00493827_m1; (TNF receptor-associated factor 1), and *Irak3* (Mm00518541_m1; interleukin-1 receptor-associated kinase 3). The relative changes in gene expression levels were calculated using the equation $2^{-\Delta\Delta CT}$ (58), where all values were normalized with respect to the housekeeping gene *Gapdh* (glyceraldehyde-3-phosphate dehydrogenase; 4352932E) mRNA levels. Amplification using 50 ng of RNA was performed in duplicate and with a total volume of 20 μl. Each real-time PCR assay was performed two times, and the results are expressed as the means ± standard deviations (SDs).

Measurement of inflammatory mediators. Concentrations of inflammatory mediators were quantified in cell-free supernatants of macrophage cultures using a Milliplex 32-Plex mouse cytokine detection system (Millipore Corporation, Billerica, MA) according to the manufacturer's instructions. Each sample was assayed in duplicate, and cytokine standards and quality controls supplied by the manufacturer were run on each plate. The multiplex assay was performed two times using cell-free culture supernatants from different experiments. Data were acquired on a Luminex 100 system and analyzed using Bio-Plex Manager software, version 4.1 (Bio-Rad Laboratories, Hercules, CA). Multiplex test kits were validated using high-sensitivity ELISA Opti-EIA sets (BD-Pharmingen, San Jose, CA) or Duo sets (R&D Systems, Minneapolis, MN) where the latter were available. In all cases tested, comparable results were observed using the Luminex-based multiplex assays and individual ELISA kits (data not shown).

Statistical analysis. A corrected one-way analysis of variance was used to analyze the microarray data using Spotfire software (Spotfire DecisionSite for Functional Genomics, Spotfire Inc.). Cytokine multiplex and qRT-PCR data were analyzed using a two-tailed unpaired Student's *t* test. $P < 0.05$ was considered significant.

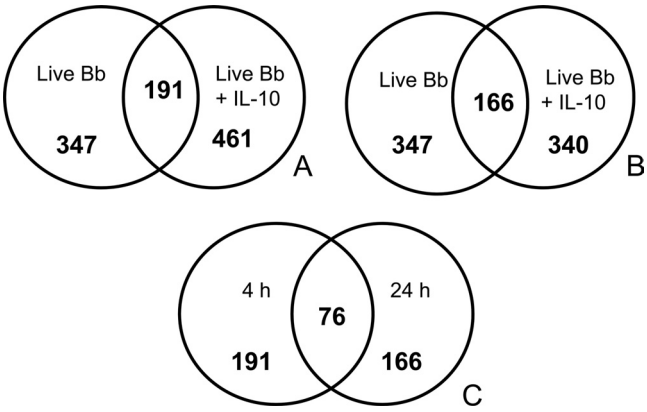

FIG. 1. Distinct gene expression profiles obtained from mouse J774 macrophages stimulated with live *B. burgdorferi* (Bb) spirochetes alone or in combination with IL-10 at 4 h (A) and 24 h (B) and common genes between 4 h and 24 h (C) poststimulation. Macrophages (3×10^6/ml) were incubated with live *B. burgdorferi* spirochetes at an MOI of 10 in the presence or absence of 10 ng/ml of mouse recombinant IL-10. The control culture consisted of cells incubated with medium alone (unstimulated). Microarray analysis was conducted on the cDNA produced from RNA extracted from macrophages at 4 h and 24 h poststimulation. Data are reported as fold change induction relative to the values obtained from unstimulated cells. Genes whose expression changed by at least 2-fold or more for upregulated genes and −2-fold or less for downregulated genes (with a corrected one-way analysis of variance, $P < 0.05$) compared with unstimulated cells were considered to be differentially expressed in a statistically significant manner. The mean fold induction of the expression of each sample was expressed as a ratio of intensities of stimulated to unstimulated cells in two parallel experiments.

RESULTS

Live *B. burgdorferi* spirochetes induce the upregulation of multiple gene transcripts in macrophages. Microarray was employed to first identify genes that are upregulated in macrophages after stimulation with live *B. burgdorferi* spirochetes either alone or combined with IL-10. RNA samples were obtained from unstimulated and stimulated cells at 4 and 24 h poststimulation and subjected to gene expression analyses. Live spirochetes upregulated a total of 347 (fold change of >2.0) macrophage gene transcripts at both the 4-h and 24-h time points, whereas live spirochetes combined with IL-10 induced upregulation of 461 and 340 genes at 4 h and 24 h, respectively (Fig. 1A and B), suggesting the potentiation of these genes by IL-10. Live spirochetes induced 156 (4 h) and 181 (24 h) exclusive gene transcripts while live spirochetes combined with IL-10 elicited 270 (4 h) and 174 (24 h) exclusive genes (see Tables S3 to S6 in the supplemental material). The numbers of overlapping candidate genes identified at 4 h (191 genes) and 24 h (166 genes) between both stimulants are depicted in Fig. 1A and B and listed in Tables 1 to 4) (see also Tables S1 and S2 in the supplemental material). Of the 191 and 166 overlapping genes, 76 of them were common to the 4-h and 24-h time points (Fig. 1C and Tables 1 to 4; see also Tables S1 and S2). We also observed that live spirochetes alone or with added IL-10 downregulated a total of 723 and 698 gene transcripts at 4 h and 24 h, respectively. There were 238 (33% of the total genes) and 312 (45% of the total genes) common genes represented, respectively, at 4 h and 24 h between both stimulants (see Tables S7 and S8). However, the majority of these genes were poorly characterized with regard to immunological functions, and therefore they were not further evaluated in this study.

IL-10 alters the expression levels of multiple genes induced by live *B. burgdorferi* spirochetes in macrophages. As the focus of our study was to identify genes whose expression was induced by live spirochetes and altered by IL-10, we subjected the 191 (4 h) and 166 (24 h) upregulated overlapping genes to further analyses. Two groups of genes were identified within the overlapping gene groups as being induced by live spirochetes but altered by IL-10. These included (i) genes that were induced by live spirochetes and whose expression levels were downregulated by IL-10 and (ii) genes that were induced by live spirochetes and whose expression levels were potentiated by IL-10 (Fig. 2A and B and Tables 1 to 4; see also Tables S1 and S2). IL-10 downregulated the expression levels of 53 and 65 live spirochete-induced genes with a corresponding ratio of >1.2 at 4 h and 24 h, respectively (Tables 1 and 3). Of these 53 and 65 genes whose expression was inhibited by IL-10, 13 of them overlapped at the 4-h and 24-h time points (Fig. 2C and Tables 1 and 3; see also Tables S1 and S2 in the supplemental material). Many of the IL-10-downregulated genes are of known biological functions, encoding protein and membrane transport, receptor signaling, metabolism, cell adhesion, phosphorylation, development, cell cycle, immune responses, signal transduction, proliferation and apoptosis, translation and transcription, and cytokines/chemokines, among many others. Some of the genes on this list that are well characterized and worthy of mention are as follows: chemokine (C-X-C motif) ligand genes *Cxcl2*, *Cxcl3* and *Cxcl10*, immunoresponsive gene

TABLE 1. Selected upregulated gene transcripts in macrophages 4 h after exposure to live *B. burgdorferi* which were downregulated in the presence of added exogenous IL-10[a]

Functional group and description	Gene no.	Annotation	Fold change in expression by culture condition(s)		Fold change ratio (live Bb culture/ live Bb + IL-10 culture)
			Live Bb[b]	Live Bb + IL-10	
Cytokines and Chemokines					
Colony stimulating factor 1 (macrophage)	NM_007778	Csf1	8.14	3.13	2.6
Chemokine (C-X-C motif) ligand 2	NM_009140	Cxcl2	98.28	38.22	2.57
Chemokine (C-X-C motif) ligand 10	NM_021274	Cxcl10	6.53	3.75	1.74
Chemokine (C-C motif) ligand 2	NM_011333	Ccl2	4.6	2.75	1.67
Chemokine (C-C motif) ligand 5	NM_013653	Ccl5	4.31	3.11	1.39
Interleukin 6	NM_031168	IL-6	6	4.54	1.32
Tumor necrosis factor	NM_013693	Tnf	12.16	9.36	1.3
Enzymes					
Cytochrome *c* oxidase, subunit XVII assembly protein homolog (yeast)	BU555670	Cox17	14.34	5.7	2.52
Guanylate nucleotide binding protein 2	NM_010260	Gbp2	5.23	3.31	1.58
Cytochrome *b*-245, beta polypeptide	NM_007807	Cybb	5.49	3.5	1.57
Acyl-coenzyme A synthetase long-chain family member 1	NM_007981	Acsl1	6.45	4.52	1.43
G-protein coupled receptor					
G protein-coupled receptor 109A	NM_030701	Gpr109a	4.61	3.62	1.27
Growth factor					
Jagged 1	NM_013822	Jag1	5.61	2.84	1.98
Kinases					
Polo-like kinase 2 (*Drosophila*)	NM_152804	Plk2	8.65	5.91	1.46
Interleukin-1 receptor-associated kinase 2 ligand-dependent nuclear receptor	NM_172161	Irak2	4.36	3.06	1.42
Nuclear receptor subfamily 4, group A, member 1	NM_010444	Nr4a1	12.56	6.9	1.82
Peptidases					
Mucosa-associated lymphoid tissue lymphoma translocation gene 1	NM_172833	Malt1	6.83	3.1	2.21
Complement component 3	NM_009778	C3	15.32	12.12	1.26
Phosphatases					
Protein tyrosine phosphatase, receptor-type, F interacting protein, binding protein 2	NM_008905	Ppfibp2	3.86	2.22	1.74
Protein tyrosine phosphatase, receptor type, J	NM_008982	Ptpri	4.63	2.85	1.63
Transcription regulators					
Human immunodeficiency virus type I enhancer binding protein 3	AK038070	Hivep3	5.92	2.99	1.98
Early growth response 2	NM_010118	Egr2	4.03	2.43	1.66
V-maf musculoaponeurotic fibrosarcoma oncogene family, protein F (avian)	NM_010755	Maff	7.13	4.52	1.58
Nuclear factor of kappa light chain gene enhancer in B-cells 1, p105	BC050841	Nfkb1	3.26	2.27	1.44
Nuclear factor of kappa light chain polypeptide gene enhancer in B-cell inhibitor, epsilon	NM_008690	Nfkbie	2.85	2.28	1.25
Jun-B oncogene	NM_008416	Junb	5.35	4.43	1.21
Transmembrane receptors					
Macrophage receptor with collagenous structure	NM_010766	Marco	29.32	9.46	3.1
Toll-like receptor 2	NM_011905	Tlr2	8.65	6.55	1.32
Poliovirus receptor	NM_027514	Pvr	2.82	2.27	1.24
Macrophage scavenger receptor 1	NM_031195	Msr1	3.09	2.52	1.23
Transporters					
Solute carrier family 11 (proton-coupled divalent metal ion transporters), member 2	AK148276	Slc11a2	7.73	4.82	1.61
Syntaxin 11	AK017897	Stx11	3.91	3.26	1.2
Other					
Unknown	NM_173363	Eif5	8.91	3.5	2.54
Cytokine-inducible SH2-containing protein	NM_009895	Cish	4.93	2.49	1.98

Continued on following page

TABLE 1—*Continued*

Functional group and description	Gene no.	Annotation	Fold change in expression by culture condition(s)		Fold change ratio (live Bb culture/ live Bb + IL-10 culture)
			Live Bb[b]	Live Bb + IL-10	
TNF receptor-associated factor 1	NM_009421	Traf1	10.75	6.17	1.74
Septin 11	AK028475		3.46	2.17	1.59
Intercellular adhesion molecule	BC008626	Icam1	10.21	6.63	1.54
CD83 antigen	NM_009856	Cd83	3.84	2.56	1.5
Rho guanine nucleotide exchange factor (GEF) 3	NM_027871	Arhgef3	6.15	4.12	1.49
ADP-ribosylation factor-like 5C	NM_207231	Arl5c	7.04	5.03	1.4
CASP8 and FADD-like apoptosis regulator	NM_207653	Cflar	3.64	2.62	1.39
RIKEN cDNA 1190003J15 gene	AK004470	1190003J15Rik	5.94	4.27	1.39
Zinc finger CCCH type containing 12A	NM_153159	Zc3h12a	4.04	3.09	1.3
Density-regulated protein	NM_026603	Denr	2.76	2.13	1.3
Nucleotide-binding oligomerization domain containing 2	NM_145857	Card15	3.94	3.09	1.28
Proline-rich nuclear receptor coactivator 1	NM_001033225	Pnrc1	3.67	2.91	1.26
Syndecan 4	NM_011521	Sdc4	3.82	3.07	1.24
Unknown	XM_134209	BC053440	2.93	2.37	1.24
Interferon-related developmental regulator 1	NM_013562	Ifrd1	2.88	2.33	1.23
CDC42 effector protein (Rho GTPase binding) 2	NM_026772	Cdc42ep2	4.7	3.84	1.22
Growth arrest and DNA-damage-inducible 45 beta	NM_008655	Gadd45b	11.09	9.07	1.22
Deltex 4 homolog (Drosophila)	NM_172442	Dtx4	3.24	2.68	1.21
Poly (ADP-ribose) polymerase family, member 14	NM_001039530	Parp14	2.83	2.35	1.21

[a] A corrected one-way analysis of variance was used to analyze the microarray data. Genes whose expression levels were upregulated 2-fold or more ($P < 0.05$) compared to unstimulated cells were considered to be differentially expressed in a statistically significant manner. The underlined genes are common to both the 4- and 24-h time points.

[b] Bb, *B. burgdorferi* spirochetes.

Irg1; genes that encode monocyte-derived chemokines, *Ccl3* and *Ccl5* (67); proinflammatory cytokine *Tnf* and *Il-6* (66, 85, 86); Toll-like receptors, *Tlr1* and *Tlr2* (1, 2, 14, 47, 97); and *Ptgs2*, which codes for prostaglandins-endoperoxide synthase 2, COX2 (1). Other genes downregulated by IL-10 include the following: IFN response genes such as interferon regulatory factor, *Irf7*; activating transcription factor 3, *Atf3*; interferon-activated gene 202B, *Ifi202b*; interleukin receptor-associated protein kinase 2 and kinase 3, *Irak2* and *Irak3*; TNF receptor-associated factor 1 and factor 2, *Traf1* and *Traf2*; Fas ligand (TNF superfamily, member 6), *Fas*; tumor necrosis factor alpha-induced protein 3, *Tnfaip3*; intracellular adhesion molecule 1, *Icam1*; matrix metallopeptidases, *Mmp9* (37, 38, 85), complement 3, *C3*; and *Cd40* and *Cd47* transcripts (77).

The second group of overlapping genes induced by live spirochetes that were potentiated by IL-10 are shown in Fig. 2D. There were 65 and 50 live-spirochete-induced gene transcripts at 4 h and 24 h that were enhanced by IL-10, as determined by a reduced ratio of <0.83 (Tables 2 and 4). There were 12 IL-10-potentiated genes common to the 4-h and 24-h time points, of which 11 were functionally recognizable (*Bcl3*, *Ccl4*, *Saa1*, *Ccl9*, *Rnf149*, *Il-1B*, *Rgl1*, *Mmp13*, *Pde4b*, *Mrp*, and *Ccrn4*) (Fig. 2D; Tables 2 and 4). Interestingly, some genes, such as *Ptgs2*, *Cd40*, *Tnfaip3*, and *Saa3*, which were enhanced by IL-10 at 4 h subsequently were downregulated by IL-10 at 24 h, suggesting early and late regulation by IL-10. Overall, many of the live-spirochete-induced genes altered (inhibited or potentiated) by IL-10 were similarly perturbed when sonicated *B. burgdorferi* spirochetes were used as the stimulant in microarray studies (data not shown).

We also observed several genes to be induced by live spirochetes that were not detected in the live spirochete cultures to which IL-10 was added at either the 4-h or 24-h time points, which may suggest the complete downregulation of these genes by IL-10. A snapshot of these genes include cytokines (*Il-20*), chemokines (*Cxcl4*), signaling (*Nos2*), apoptosis (*Casp1*), and multiple interferon-induced protein transcripts (*Ifit1* [*Ifi204*, *Ifi27* and *Ifi44*] (see Tables S3 to S5 in the supplemental material). Many of these genes represent newly identified genes inducible by live spirochetes that are unperturbed or downregulated by IL-10.

Validation of *B. burgdorferi*-inducible genes that are downregulated by IL-10 by real-time PCR. The reliability of the gene array analysis was validated by randomly selecting 10 of the spirochete-induced, IL-10-inhibited noncytokine and nonchemokine genes (as these were assessed at the protein level) and subjecting them to TaqMan qRT-PCR. For this study, we used RNA samples collected at 24 h from cells stimulated with live spirochetes and also from cells stimulated with sonicated *B. burgdorferi* or with the lipoprotein outer surface protein A (L-OspA) in the presence and absence of IL-10. To study the effect of IL-10 on the constitutive expression of inflammatory mediators in J774 cells, RNA collected from cells exposed to IL-10 alone was used in all real-time PCR studies. As shown in Fig. 3A to E all selected genes (*Ifit1*, *Ifi44*, *Tlr1*, *Tlr2*, *Irg1*, *Ptgs2*, *Mmp9*, *Cd40*, *Traf1*, and *Irak3*) were significantly ($P < 0.05$) downregulated by IL-10 in response to all stimulants. Worthy of note was the ability of IL-10 by itself to alter the marginal constitutive mRNA expression levels of *Ifit1*, *Ifi44*, *Tlr1*, *Tlr2*, *Ptgs2*, *Mmp9*, *Cd40*, and *Irak3* genes, indicating its inherent capacity to modulate selective genes in J774 cells. Live spirochetes overall significantly ($P <$ 0.05 to 0.0001) induced levels of expression of all genes higher than those induced by sonicated spirochetes and L-OspA.

TABLE 2. Selected upregulated gene transcripts in macrophages 4 h after exposure to live *B. burgdorferi* which were potentiated in the presence of added exogenous IL-10[a]

Functional group and description	Gene no.	Annotation	Fold change in expression by culture condition(s)		Fold change ratio (live Bb culture/ live Bb + IL-10 culture)
			Live Bb[b]	Live Bb + IL-10	
Cytokines					
Interleukin 1 beta	NM_008361	Il1b	146.37	180.93	0.81
Interleukin 10	NM_010548	Il10	3.81	4.74	0.8
Chemokine (C-C motif) ligand 9	NM_011338	Ccl9	4.18	5.62	0.74
Chemokine (C-C motif) ligand 4	NM_013652	Ccl4	11.32	16.23	0.7
Interleukin 1 receptor antagonist	NM_031167	Il1rn	3.91	6.79	0.57
Enzymes					
RAB12, member RAS oncogene family	NM_024448	Rab12	2.26	2.75	0.82
Superoxide dismutase 2, mitochondrial	NM_013671	Sod2	6.41	8.33	0.77
Diacylglycerol *O*-acyltransferase 2	NM_026384	Dgat2	3.15	4.47	0.71
CTAGE family, member 5	AK164018	Mgea6	2.08	3.05	0.68
Carbonic anhydrase 13	NM_024495	Car13	5.5	8.82	0.62
UDP-glucose ceramide glucosyltransferase	NM_011673	Ugcg	3.37	5.5	0.61
Sphingomyelin synthase 1	NM_144792	Tmem23	3.91	6.49	0.6
Prostaglandin-endoperoxide synthase 2	NM_011198	Ptgs2	15.95	27.45	0.58
G-protein coupled receptor					
Adenosine A2b receptor	NM_007413	Adora2b	2.98	6.85	0.44
Ion channel					
Mucolipin 2	NM_026656	Mcoln2	2.96	4.05	0.73
Kinases					
Proviral integration site 3	NM_145478	Pim3	2.85	3.6	0.79
Phosphoinositide-3-kinase, regulatory subunit 5, p101	NM_177320	Pik3r5	3.1	4.03	0.77
Inhibitor of κB kinase epsilon	NM_019777	Ikbke	2.93	3.95	0.74
Proviral integration site 1	NM_008842	Pim1	2.18	2.99	0.73
Mitogen-activated protein kinase kinase kinase kinase 4	AK020498	9430080K19Rik	2.4	3.49	0.69
Hemopoietic cell kinase	NM_010407	Hck	2.6	4.19	0.62
Unknown	NM_010884	Ndrg1	2.68	5.9	0.46
Peptidases					
Caspase 1	NM_009807	Casp1	2.58	3.48	0.74
Matrix metallopeptidase 13	NM_008607	Mmp13	8.11	11.74	0.69
A disintegrin-like and metallopeptidase (reprolysin type) with thrombospondin type 1 motif, 1	NM_009621	Adamts1	2.73	6.27	0.44
Phosphatase					
Dual specificity phosphatase 1	NM_013642	Dusp1	3.51	5.71	0.61
Transcription regulators					
B-cell leukemia/lymphoma 3	NM_033601	Bcl3	4.52	5.47	0.83
E2F transcription factor 5	X86925	E2f5	2.54	3.49	0.73
Hypoxia inducible factor 1, alpha subunit	NM_010431	Hif1a	3.44	4.78	0.72
Kruppel-like factor 7 (ubiquitous)	NM_033563	Klf7	2.22	3.09	0.72
Activating transcription factor 3	NM_007498	Atf3	6.87	9.65	0.71
Microphthalmia-associated transcription factor	NM_008601	Mitf	2.86	4.4	0.65
Nuclear factor, interleukin 3, regulated	NM_017373	Nfil3	2.81	4.78	0.59
MAX dimerization protein 1	AK137548	Mxd1	2.99	5.53	0.54
Signal transducer and activator of transcription 3	NM_213659	Stat3	2.91	5.89	0.49
CCR4 carbon catabolite repression 4-like (*S. cerevisiae*)	NM_009834	Ccrn4l	4.74	10.29	0.46
Transmembrane receptors					
CD40 antigen	NM_170701	Cd40	3.26	4.18	0.78
Fc receptor, IgG, low-affinity IIb	NM_010187	Fcgr2b	7.83	15.52	0.5
Fc receptor, IgG, low-affinity III	NM_010188	Fcgr3	3.3	6.63	0.5
Tumor necrosis factor receptor superfamily, member 9	NM_011612	Tnfrsf9	3.09	5.42	0.57
Transporters					
Solute carrier family 16 (monocarboxylic acid transporters), member 1	NM_009196	Slc16a1	2.22	3.01	0.74

Continued on following page

TABLE 2—*Continued*

Functional group and description	Gene no.	Annotation	Fold change in expression by culture condition(s)		Fold change ratio (live Bb culture/ live Bb + IL-10 culture)
			Live Bb[b]	Live Bb + IL-10	
Synaptotagmin X	NM_018803	Syt10	2.47	3.89	0.64
Serum amyloid A 1	NM_009117	Saa1	20.57	64.77	0.32
Serum amyloid A 3	NM_011315	Saa3	34	66.07	0.51
Other					
TNFAIP3 interacting protein 3	NM_001001495	TNIP3	4.19	14.90	0.28
Tumor necrosis factor, alpha-induced protein 3	NM_009397	Tnfaip3	19.96	58.62	0.34
SAM domain, SH3 domain and nuclear localization signals, 1	NM_023380	Samsn1	3.04	6.97	0.44
RIKEN cDNA 4933426M11 gene	NM_178682	4933426M11Rik	2.45	2.97	0.82
Unknown	AK031731	Nfe2l2	2.21	2.71	0.82
Zinc finger, AN1-type domain 5	NM_009551	Zfand5	2.15	2.67	0.81
Phosphodiesterase 4B, cAMP specific	NM_019840/AK171700	Pde4b	6.83	9.07	0.75
RIKEN cDNA 1810022K09 gene	BC045157	1810022K09Rik	2.64	3.65	0.72
Unknown	AT_ssM_RR_3	AT_ssM_RR_3	2.46	3.47	0.71
RIKEN cDNA 1810029B16 gene	NM_025465	1810029B16Rik	3.35	4.75	0.71
Mesoderm development candidate 1	NM_030705	Mesdc1	2.15	3.09	0.7
Pleckstrin	NM_019549	Plek	2.64	3.81	0.69
Ral guanine nucleotide dissociation stimulator, -like 1	NM_016846	Rgl1	3.09	4.59	0.67
Immediate-early response 3	NM_133662	Ier3	3.72	5.71	0.65
CDNA sequence BC031781	NM_145943	BC031781	2.43	3.82	0.64
Ring finger protein 149	NM_001033135	Rnf149	2.68	4.3	0.62
RIKEN cDNA E130014J05 gene	NM_001040400	E130014J05Rik	2.33	3.75	0.62
RIKEN cDNA 5730508B09 gene	AK162420/NM_027482	5730508B09Rik	4.07	9.32	0.44
Unknown	AK035396	1200016E24Rik	3.71	8.91	0.42
Mitochondrial ribosomal protein L52	AK081551	Mrpl52	3.20	6.20	0.52
Activity regulated cytoskeletal-associated protein	NM_018790	Arc	3.83	6.67	0.57

[a] A corrected one-way analysis of variance was used to analyze the microarray data. Genes whose expression levels were upregulated by at least 2-fold or more ($P < 0.05$) compared to unstimulated cells were considered to be differentially expressed in a statistically significant manner. The underlined genes are common to both the 4- and 24-h time points.

[b] Bb, *B. burgdorferi* spirochetes.

IL-10 regulates the protein expression levels of cytokines and chemokines in macrophages stimulated by live *B. burgdorferi* spirochetes. Cytokine/chemokine multiplex assays were performed to validate at the protein level live-spirochete-induced cytokine and chemokine mRNA gene transcripts that were altered by IL-10. These experiments were performed using 24-h culture supernatants from live and sonicated spirochetes or L-OspA-stimulated cells in the presence and absence of IL-10. As the microarray data showed the potentiating effect of IL-10 on select cytokine/chemokine genes, cultured supernatants collected from cells stimulated with IL-10 alone were also analyzed to assess whether or not IL-10 similarly potentiates selected genes at the protein level. By using this multiplex approach, 18 cytokines/chemokines were observed to be significantly induced by the three stimulants compared to unstimulated or IL-10-modulated cells. The production levels of the prototypic IL-6 and TNF cytokines were significantly ($P < 0.05$ to 0.008) downregulated by IL-10 in macrophages in response to live spirochetes (data not shown). IL-1β elicited by live spirochetes was markedly downregulated by IL-10 (Fig. 4A) although its mRNA transcript was enhanced by IL-10 (Tables 1 and 2), suggesting that IL-10 may regulate its expression at the translational and not transcriptional level. Similar significant ($P < 0.05$) results were obtained when cells were stimulated with sonicated spirochetes or with L-OspA (Fig. 4A), as already reported by us (27) and others (17). IL-10 also significantly ($P < 0.03$ to 0.01) inhibited the production of IL-1α, IFN-γ, and IL-9 in macrophages as induced by all stimulants (Fig. 4A) even though their gene transcripts were not observed by microarray. Of significance was the enhanced production of IL-1β, IL-1α, and IFN-γ by live spirochetes compared to that of sonicated spirochetes and L-OspA.

The chemokines, RANTES/CCL5, IP-10/CXCL10, and MIP-2/CXCL2, were all significantly downregulated by IL-10 in macrophages in response to all stimulants (Fig. 4B and C). MIP-1α/CCL3, whose gene transcript level was moderately downregulated by IL-10 (ratio of 1.15) was moderately but significantly ($P < 0.01$) downregulated at the protein level (Fig. 4B). The production levels of the chemokines granulocyte colony-stimulating factor (G-CSF)/CSF3 and LIX/CXCL5 were markedly decreased ($P < 0.02$ to 0.007) by IL-10 in stimulated macrophages, as shown in Fig. 2C. These highly expressed chemokines were not detected or did not reach a level of significance in the live or sonicated spirochete microarray results. The reason for this discrepancy is not clear. The selective upregulation of MCP-1/CCL2 and MIP-1β/CCL4 by IL-10 in live-spirochete cultures was also confirmed at the protein level (Fig. 4D). IL-10 by itself also significantly ($P < 0.05$ to 0.004) upregulated both of these chemokines compared to unstimulated cells (Fig. 4D), indicating the independent ability of IL-10 to stimulate their pro-

TABLE 3. Selected upregulated gene transcripts in macrophages 24 h after exposure to live *Borrelia burgdorferi* which were downregulated in the presence of added exogenous IL-10[a]

Functional group and description	Gene no.	Annotation	Fold change in expression by culture condition(s)		Fold change ratio (live Bb culture/ live Bb + IL-10 culture)
			Live Bb[b]	Live Bb + IL-10	
Cytokines					
Chemokine (C-X-C motif) ligand 2	NM_009140	Cxcl2	37.17	3.31	11.23
Chemokine (C-C motif) ligand 5	BC033508	Ccl5	13.51	5.54	2.44
Tumor necrosis factor	NM_013693	Tnf	6.77	3.39	2.0
Enzymes					
Diacylglycerol *O*-acyltransferase 2	NM_026384	Dgat2	6.96	2.68	2.6
Ceruloplasmin	NM_007752	Cp	21.69	9.26	2.34
Guanylate nucleotide binding protein 3	NM_018734	Gbp4	6.89	3.62	1.9
Cytochrome *b*-245, beta polypeptide	NM_007807	Cybb	8.66	5.44	1.59
2′-5′ Oligoadenylate synthetase 1A	NM_145211	Oas1a	3.7	2.56	1.45
Prostaglandin-endoperoxide synthase 2	NM_011198	Ptgs2	7.51	5.47	1.37
Carbonic anhydrase 13	NM_024495	Car13	5.17	3.95	1.31
DEXH (Asp-Glu-X-His) box polypeptide 58	NM_030150	D11Lgp2e	4.3	3.35	1.28
Baculoviral IAP repeat-containing 3	NM_007464	Birc3	4.81	3.79	1.27
Superoxide dismutase 2, mitochondrial	NM_013671	Sod2	10.41	8.17	1.27
2′-5′ Oligoadenylate synthetase 2	NM_145227	Oas2	3.23	2.6	1.24
Crystallin, mu	NM_016669	Crym	3.01	2.46	1.22
Cytochrome b_5 type B	NM_025558	Cyb5b	2.68	2.23	1.2
G-protein coupled receptors					
EGF-like module containing, mucin-like, hormone receptor-like sequence 1	NM_010130	Emr1	5.86	4.1	1.43
G protein-coupled receptor 84	NM_030720	Gpr84	5.09	4.15	1.22
Kinases					
Interleukin-1 receptor-associated kinase 3	NM_028679	Irak3	9.68	4.27	2.27
Spleen tyrosine kinase	NM_011518	Syk	3.13	2.31	1.35
Ligand-dependent nuclear receptor					
Nuclear receptor subfamily 4, group A, member 1	NM_010444	Nr4a1	3.98	3.08	1.29
Peptidases					
Ubiquitin specific peptidase 18	NM_011909	Usp18	9.44	4.28	2.2
Matrix metallopeptidase 9	NM_013599	Mmp9	6.38	4.1	1.55
Complement component 3	NM_009778	C3	28.86	22.15	1.3
Phosphatase					
Protein tyrosine phosphatase, receptor-type, F interacting protein, binding protein 2	NM_008905	Ppfibp2	5.3	2.25	2.36
Transcription regulators					
Interferon regulatory factor 7	NM_016850	Irf7	7.44	3.47	2.15
Nuclear antigen Sp100	BC069183	Sp100	4.51	2.59	1.74
Elongation factor RNA polymerase II 2	BC006925	Ell2	5.26	3.54	1.48
Transmembrane receptors					
Toll-like receptor 1	NM_030682	Tlr1	8.63	4.89	1.77
Toll-like receptor 2	NM_011905	Tlr2	5.17	3.08	1.68
Fas (TNF receptor superfamily member 6)	NM_007987	Fas	9.25	7.1	1.3
Macrophage receptor with collagenous structure	NM_010766	Marco	67.71	52.45	1.29
CD40 antigen	NM_170701	Cd40	3.34	2.77	1.2
Transporters					
Solute carrier family 11 (proton-coupled divalent metal ion transporters), member 2	AK148276	Slc11a2	4.99	2.63	1.9
Solute carrier family 31, member 2	NM_025286	Slc31a2	5.94	3.73	1.59
Fatty acid binding protein 4, adipocyte	NM_024406	Fabp4	3.96	2.94	1.35
Serum amyloid A 3	NM_011315	Saa3	39.27	30.33	1.29
Other					
Immunoresponsive gene 1	L38281/AK1521	Irg1	196.02	24.55	7.99

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TABLE 3—*Continued*

Functional group and description	Gene no.	Annotation	Fold change in expression by culture condition(s)		Fold change ratio (live Bb culture/ live Bb + IL-10 culture)
			Live Bb[b]	Live Bb + IL-10	
TNF receptor-associated factor 1	NM_009421	Traf1	23.29	5	4.66
Bone marrow stromal cell antigen 2	NM_198095	Bst2	6.82	3.04	2.24
ISG15 ubiquitin-like modifier	NM_015783	Isg15	7.85	3.52	2.23
C-type lectin domain family 4, member e	NM_019948	Clec4e	4.79	2.23	2.15
Tumor necrosis factor, alpha-induced protein 3	NM_009397	Tnfaip3	6.79	3.47	1.95
ADP-ribosylation factor-like 5C	NM_207231	Arl5c	4.58	2.43	1.88
Argininosuccinate synthetase 1	NM_007494/M3	Ass1	5.25	2.82	1.86
Interferon-activated gene 202B	NM_011940	Ifi202b	3.93	2.41	1.63
Unknown	ENSMUST00000073378	ENSMUST00000073378	4.22	2.87	1.47
RAS p21 protein activator 4	NM_133914	Rasa4	3.03	2.06	1.47
EH-domain containing 1	NM_010119	Ehd1	4.2	2.89	1.45
Unknown	NM_022431	Ms4a11	7.42	5.19	1.43
Unknown	XM_484397	Dgkh	4.75	3.33	1.42
Wingless-related MMTV integration site 6	NM_009526	Wnt6	2.97	2.12	1.4
Zinc finger CCCH type containing 12C	XM_146893	Zc3h12c	5.6	4.03	1.39
Membrane-spanning 4-domains, subfamily A, member 6B	NM_027209	Ms4a6b	8.13	5.95	1.37
Immediate early response 3	NM_133662	Ier3	3.85	2.99	1.29
CD47 antigen (Rh-related antigen, integrin-associated signal transducer)	NM_010581	Cd47	2.66	2.11	1.26
Lymphocyte cytosolic protein 2	NM_010696	Lcp2	3.1	2.46	1.26
Membrane-spanning 4-domains, subfamily A, member 6C	NM_028595	Ms4a6c	7.91	6.33	1.25
Secretory leukocyte peptidase inhibitor	NM_011414	Slpi	14.21	11.82	1.2
Coiled-coil domain containing 50	NM_026202	Ccdc50	3.21	2.37	1.36
SH3-domain binding protein 5 (BTK-associated)	NM_011894	Sh3bp5	2.99	2.24	1.34
Ras association (RalGDS/AF-6) domain family 4	NM_178045	Rassf4	3.28	2.46	1.33
RAB32, member RAS oncogene family	NM_026405	Rab32	2.83	2.14	1.32
CDC42 effector protein (Rho GTPase binding) 2	NM_026772	*Cdc42ep2*	2.83	2.15	1.32
Membrane-spanning 4-domains, subfamily A, member 6D	NM_026835	Ms4a6d	8.57	6.65	1.29

[a] A corrected one-way analysis of variance was used to analyze the microarray data. Genes whose expression levels were upregulated at least 2-fold ($P < 0.05$) compared to unstimulated cells were considered to be differentially expressed in a statistically significant manner. The underlined genes are common to both 4- and 24-h time points.

[b] Bb, *B. burgdorferi* spirochetes.

duction. CCL2 levels were similarly upregulated ($P < 0.001$ to 0.009) in macrophages stimulated with sonicated spirochetes and L-OspA in the presence of IL-10. CCL4 enhancement by IL-10 reached a level of significance only when live *B. burgdorferi* spirochetes were used as stimulant but not in the presence of either sonicated spirochetes or L-OspA (Fig. 4D). IL-5, which induces proliferation and activation of cells, was enhanced by IL-10 in the presence of each and all of the stimulants that were used (data not shown). Live spirochetes induced levels of G-CSF, CXCL2, CXCL5, CCL3, CCL5, and CXCL10 in macrophages higher than those induced by sonicated spirochetes and L-OspA, suggesting differences in stimulation of these mediators by live organisms and their lipoproteins or lysates.

DISCUSSION

Lyme disease is thought to ensue largely as a consequence of both acute and chronic inflammatory responses, induced by either the spirochete or spirochetal antigens left in tissues after bacterial death. Excessive, unchecked inflammation of any origin can be deleterious to the host, and, consequently, several regulatory mechanisms have evolved to control its magnitude and duration. One such pivotal regulator of inflammation is the anti-inflammatory cytokine IL-10. This cytokine is produced in response to stimulation by *B. burgdorferi* in different cells and tissue types, as reported in mice (17, 24, 27, 53, 61), rhesus macaques (40), and human patients (50, 94). Moreover, IL-10 has been shown to control Lyme disease inflammation *in vitro* (17, 35, 53, 54, 96) and *in vivo* (16, 17). In this paper, we focused on understanding the mechanism(s) by which IL-10 controls *B. burgdorferi*-induced inflammatory responses in macrophages. Our results show the following: (i) IL-10 simultaneously altered numerous effector genes induced by live spirochetes in macrophages, and many of these genes encoded mediators from multiple inflammatory pathways; (ii) IL-10-mediated alteration of effector genes correlated with its ability to affect the expression of spirochete-induced macrophage inflammatory mediators both at the mRNA and/or protein levels.

B. burgdorferi triggers the production of inflammatory mediators in macrophages via recognition of both Toll-like receptors (TLRs) and non-TLRs (8, 12, 22, 71, 78, 95, 97). In the present study, gene array analysis identified candidate genes from multiple pathways induced by live spirochetes that were

TABLE 4. Selected upregulated gene transcripts in macrophages 24 h after exposure to live *Borrelia burgdorferi* which were potentiated in the presence of added exogenous IL-10[a]

Functional group and description	Gene no.	Annotation	Fold change in expression by culture condition(s)		Fold change ratio (live Bb culture/ live Bb + IL-10 culture)
			Live Bb[b]	Live Bb + IL-10	
Cytokines					
Chemokine (C-C motif) ligand 4	NM_013652	Ccl4	4.52	5.44	0.83
Chemokine (C-C motif) ligand 2	NM_011333	Ccl2	13.33	16.69	0.8
Chemokine (C-C motif) ligand 9	NM_011338	Ccl9	3.03	3.92	0.77
Interleukin 1 beta	NM_008361	Il1b	52.97	83.67	0.63
Chemokine (C-C motif) ligand 12	NM_011331	Ccl12	2.59	5.99	0.43
Chemokine (C-C motif) ligand 7	NM_013654	Ccl7	8.24	34.01	0.24
Enzymes					
Prolyl 4-hydroxylase, beta polypeptide	NM_011032	P4hb	2.7	3.23	0.83
Ras homolog gene family, member Q	NM_145491	Rhoq	2.31	2.82	0.82
Phosphodiesterase 4B, cAMP specific	NM_019840	Pde4b	3.94	5.02	0.79
Glutaredoxin	NM_053108	Glrx	2.95	3.98	0.74
DNA segment, Chr 1, Brigham and Women's Genetics 1363 expressed	NM_001001566	D1Bwg1363e	2.39	3.31	0.72
Bone marrow stromal cell antigen 1	NM_009763	BstI	2.69	6.54	0.41
G-protein coupled receptor					
Formyl peptide receptor 1	NM_013521	Fpr1	2.84	5.15	0.55
Peptidases					
HtrA serine peptidase 1	NM_019564	Htra1	5.1	9.65	0.53
Matrix metallopeptidase 13	NM_008607	Mmp13	2.62	12.5	0.21
Phosphatases					
Dual specificity phosphatase 2	NM_010090	Dusp2	2.4	5.85	0.41
Protein tyrosine phosphatase, receptor type, J	NM_008982	Ptprj	2.11	7.22	0.29
Transcription regulators					
Zinc finger protein 36	NM_011756	Zfp36	2.25	3.45	0.65
CCR4 carbon catabolite repression 4-like (S. cerevisiae)	NM_009834	Ccrn4l	2.95	4.66	0.63
B-cell leukemia/lymphoma 3	NM_033601	Bcl3	3.9	7.12	0.55
FBJ osteosarcoma oncogene	NM_010234	Fos	2.53	4.77	0.53
Transmembrane receptors					
C-type lectin domain family 4, member a2	NM_011999	Clec4a2	3.29	5.28	0.62
Colony stimulating factor 2 receptor, beta, low-affinity (granulocyte-macrophage)	AK154286	Csf2rb1	2.29	4.05	0.57
Fc receptor, IgG, high affinity I	BC025535	Fcgr1	2.25	5.82	0.39
Interleukin 4 receptor, alpha	NM_001008700	Il4ra	2.91	11.12	0.26
Transporters					
Solute carrier family 28 (sodium-coupled nucleoside transporter), member 2	NM_172980	Slc28a2	3.53	5.44	0.65
Lipocalin 2	NM_008491	Lcn2	12.13	20.55	0.59
Other					
Nuclear factor of kappa light chain gene enhancer in B-cells inhibitor, alpha	NM_010907	Nfkbia	5.76	6.9	0.83
Mitochondrial ribosomal protein L52	NM_026851	Mrpl52	3.35	4.04	0.83
DNA segment, Chr 3, University of California at Los Angeles 1	NM_030685	D3Ucla1	2.6	3.12	0.83
Ring finger protein 149	NM_001033135	Rnf149	3.01	3.72	0.81
Transmembrane protein 176A	NM_025326	0610011I04Rik	2.75	3.4	0.81
RIKEN cDNA 1200002N14 gene	NM_027878	1200002N14Rik	2.67	3.3	0.81
RIKEN cDNA 1190003J15 gene	AK004470/BC051545	1190003J15Rik	93.75	123.62	0.76
Syndecan 4	NM_011521	Sdc4	2.23	3.02	0.74
Serum amyloid A 1	NM_009117	Saa1	90.62	123.61	0.73
Interferon induced transmembrane protein 2	NM_030694	Ifitm2	2.19	2.98	0.73
Rho guanine nucleotide exchange factor (GEF) 3	NM_027871	Arhgef3	2.84	4	0.71
Myristoylated alanine rich protein kinase C substrate	NM_008538	Marcks	2.72	3.89	0.7

Continued on following page

TABLE 4—*Continued*

Functional group and description	Gene no.	Annotation	Fold change in expression by culture condition(s)		Fold change ratio (live Bb culture/ live Bb + IL-10 culture)
			Live Bb[b]	Live Bb + IL-10	
FGF receptor activating protein 1	NM_145583/AK152420	Frag1	2.33	3.35	0.7
RIKEN cDNA 5730508B09 gene	AK162420/NM_027482	5730508B09Rik	2.71	4.07	0.67
Ral guanine nucleotide dissociation stimulator, -like 1	NM_016846	Rgl1	5.28	7.92	0.67
Leucine rich repeat containing 25	NM_153074	Lrrc25	2.92	4.6	0.64
Transmembrane protein 176B	NM_023056	1810009M01Rik	3.26	5.18	0.63
Unknown	ENSMUST00000094405	ENSMUST00000094405	4.02	6.52	0.62
Transformed mouse 3T3 cell double minute 4	NM_008575	Mdm4	3.24	5.49	0.59
C-type lectin domain family 4, member a3	AK156040	Clec4a3	2.92	6.68	0.44
Predicted gene, OTTMUSG00000000971	BC089618	BC089618	2.92	7.86	0.37
CD244 natural killer cell receptor 2B4	NM_018729	Cd244	8.25	25.88	0.32
C-type lectin domain family 4, member b1	NM_027218	Clec4b1	3.06	9.47	0.32

[a] A corrected one-way analysis of variance was used to analyze the microarray data. Genes whose expression levels were upregulated at least 2-fold ($P < 0.05$) compared to unstimulated cells were considered to be differentially expressed in a statistically significant manner. The underlined genes are common to both the 4- and 24-h time points.

[b] Bb, *B. burgdorferi* spirochetes.

significantly inhibited by IL-10. Classification of these genes showed many of them encoding mediators from several inflammatory pathways. Notable among the *B. burgdorferi*-induced genes subjected to IL-10-mediated suppression were those encoding the TLR pathway. Although several members of the TLR family contribute to the host inflammatory response to *B. burgdorferi* (74, 90), our study revealed that IL-10 specifically inhibits the *B. burgdorferi* transcriptional activation of TLR1 and TLR2, along with several of their downstream signaling components, such as interleukin-1 receptor-associated kinase 2 (IRAK2), IRAK3, TRAF1, and TNFAIP3. Binding of IRAKs to the downstream molecule TRAF6 leads to activation of NF-κB, an important regulator of cellular events (13) and mitogen-activated protein kinase (MAPK) kinase signaling pathways (52). Even though all IRAKs bind to TRAF6, only IRAK2 and IRAK3, both of which were inhibited by IL-10, are known to activate NF-κB signaling in cells (52). Interestingly, in the absence of added *B. burgdorferi* stimuli, the marginal constitutive expression of TLR1, TLR2, and IRAK3 were inhibited by IL-10, suggesting the inherent capacity of IL-10 to modulate these select genes. The magnitude of the anti-inflammatory effect of IL-10 on these genes, as induced by *B. burgdorferi* stimuli, varied and may indicate a limitation on its inhibitory capacity for them. Alternatively, it may be that an IL-10 concentration greater than 10 ng/ml is necessary to control their heightened expression levels. Combined, these findings additionally suggest that the pathway IL-10 uses to inhibit constitutive expression of select genes is similar to that used when these genes are induced by *B. burgdorferi*. To our knowledge, our study provides the first documentation of the IL-10 hijacking of the TLR pathway at multiple levels to regulate inflammation in Lyme disease. The inhibition by IL-10 of key genes in the TLR pathway suggests its multifaceted approach to control *B. burgdorferi*-induced inflammatory responses in macrophages.

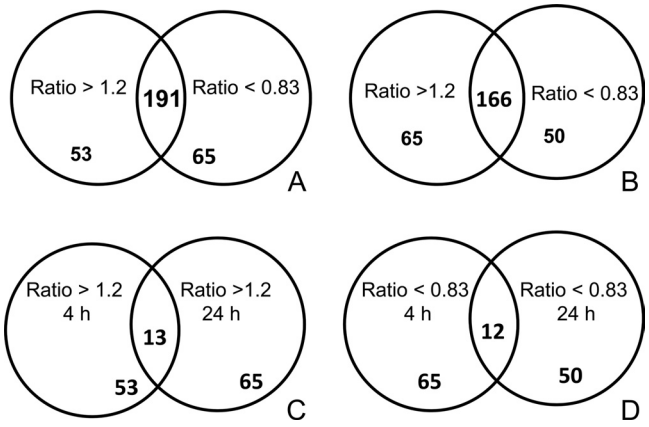

FIG. 2. IL-10 alters the gene expression pattern induced by live *B. burgdorferi* spirochetes in macrophages. Diagrams of the 191 (4 h) and 166 (24 h) overlapping genes (Fig. 1) that were induced by live spirochetes and whose expression levels were inhibited by IL-10 (ratio of ≥1.2) and genes that were induced by live spirochetes and whose expression levels were potentiated by IL-10 (ratio of ≤0.83) are shown for the 4-h (A) and 24-h (B) time points. The common IL-10-inhibited genes between the 4-h and 24-h (C) and the common IL-10-potentiated genes between the 4-h and 24-h (D) time points are also represented.

Many of the IL-10-regulated mediators identified in the present study have been recognized as components of *B. burgdorferi*-induced inflammation in cells or tissues (9, 15–17, 45, 65, 67, 78, 80, 85, 90). However, only the prototypic TNF, IL-6, IL-12, and IL-1β cytokines have been previously shown to be downregulated by IL-10 *in vitro* in mouse or human macrophages (17, 65, 66, 84). Paradoxically, in the present study, IL-1β was inhibited by IL-10 at the protein and not the transcriptional level, contrasting with our previous observation where IL-1β gene transcript was significantly diminished by IL-10 in mouse macrophages stimulated with freeze-thawed *B. burgdorferi* (JD1 strain) or with L-OspA (27). This discrepancy may be due to differences in spirochete strains or preparation and or/techniques used. Alternatively, it may be that live spirochetes do not stimulate IL-1β via a pathway by which IL-10 inhibits its transcriptional expression level. This might possibly explain why adenoviral delivery of IL-10 to *B. burgdorferi*-

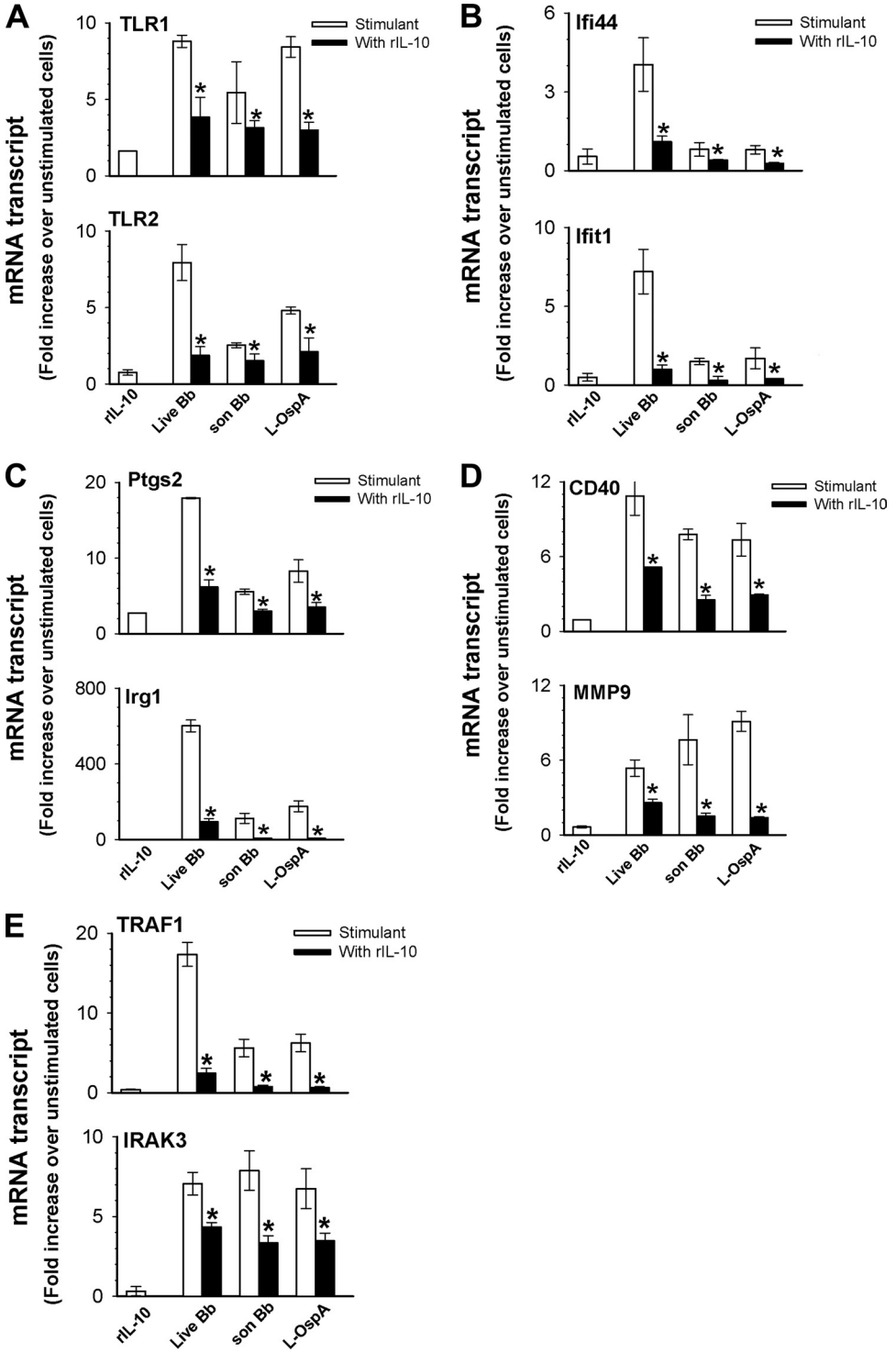

FIG. 3. Confirmation of selected genes by qRT-PCR. Macrophages (3×10^6/ml) were incubated with live *B. burgdorferi* (Bb) spirochetes at an MOI of 10, sonicated (son) spirochetes (1×10^7/ml), or L-OspA (1 μg/ml) in the presence (black bars) or absence (stimulant; white bars) of 10 ng/ml of mouse recombinant IL-10 (rIL-10). Controls consisted of cells incubated with rIL-10 or medium alone (unstimulated cells). RNA samples were collected after 24 h of incubation, and gene transcripts were quantified by TaqMan qRT-PCR. All values were normalized with respect to the housekeeping gene *Gapdh* mRNA levels. Results are presented as fold increase over control (the level in unstimulated cells). Asterisks indicate significant differences from cells incubated with stimulants alone ($P < 0.05$). *P* values were calculated by an unpaired Student's *t* test. Results are representative of one of two experiments. Each bar represents the mean ± SD from samples run in duplicates.

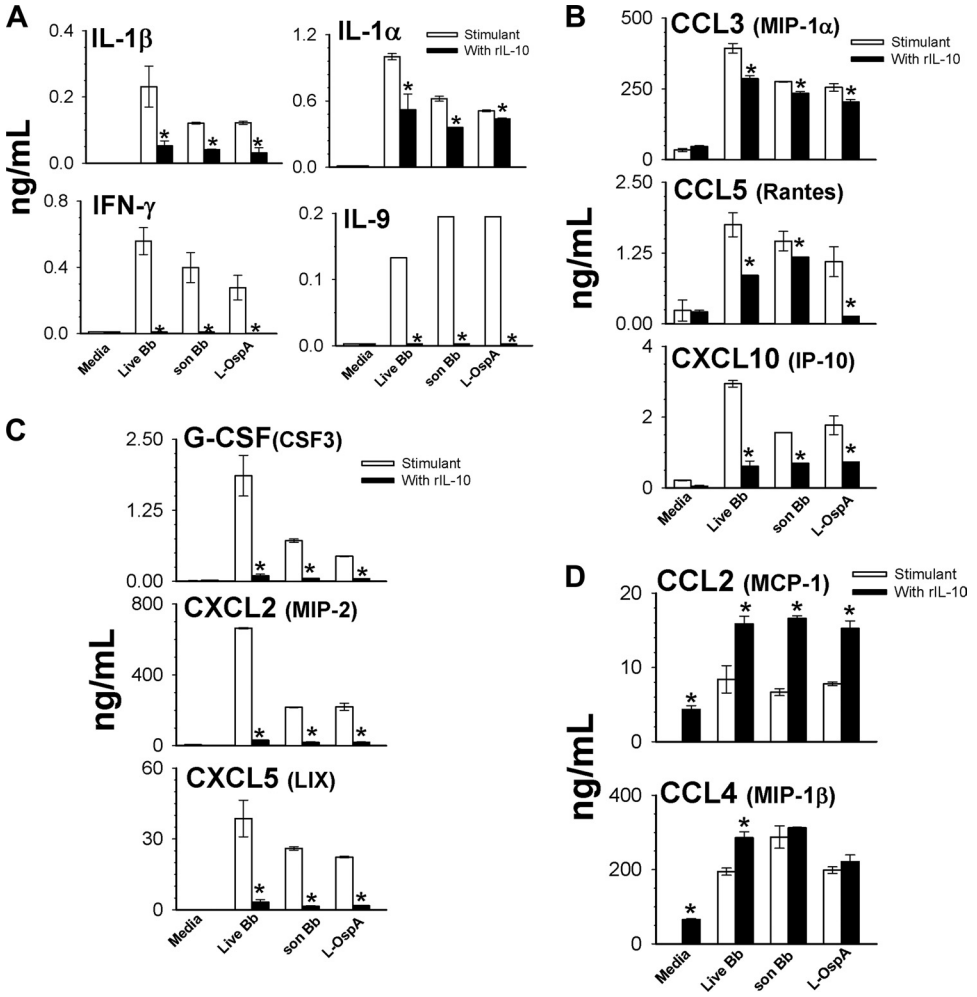

FIG. 4. IL-10-mediated regulation of cytokine (A) and chemokine (B to D) production by macrophages exposed *in vitro* to live or sonicated *B. burgdorferi* spirochetes or with L-OspA. Macrophages (3×10^6/ml) were stimulated as described in the legend of Fig. 3. Cell-free supernatants were harvested from cultures at 24 h, and protein determinations were made by multiplex ELISA. The lower limit of detection of the multiplex ELISA was 3.2 pg/ml. Cytokine and chemokine production levels are shown in ng/ml. Asterisks indicate significant differences from cells incubated with stimulants alone ($P < 0.05$ to $P < 0.0000001$). *P* values were calculated by use of an unpaired Student's *t* test. Each bar represents the mean ± SD of duplicate cultures.

infected C3H mice failed to alter the transcriptional expression of IL-1β in infected joints (16). *B. burgdorferi* infection of C3H IL-10 knockout mice also resulted in reduced pathogen load and decreased *in vivo* expression of IL-1β mRNA gene transcripts. Indeed, as demonstrated in the present study (see Table S5 in the supplemental material) and by other investigators (71), *B. burgdorferi* can activate caspase-1 to induce IL-1β in mouse macrophages, indicating a caspase-1-dependent pathway for production of this cytokine. Studies by Liu and colleagues (57) provide further evidence to suggest that IL-1β also may be induced by *B. burgdorferi* in mouse macrophages via caspase-1-dependent and -independent pathways, which adds complexity to the regulation of this cytokine.

Our present findings have relevance to our recent observations that silencing of the *Tlr1* and *Tlr2* genes by RNA interference (RNAi) in human monocytes stimulated with live spirochetes diminished production of inflammatory mediators elicited by the spirochetes (26). In agreement with our results, there are also those of studies where immune cell activation by live spirochetes or lipoprotein has generally been ascribed to TLR1/TLR2-mediated inflammatory responses (2, 14, 26, 55, 69, 70, 86, 87, 97). Studies have also shown that the mechanism(s) of IL-10-mediated inhibition of lipopolysaccharide (LPS)-induced proinflammatory gene expression involves inhibition of NF-κB or P38 MAPK pathways, as well as destabilization of RNA message (29, 51). We have now provided a global perspective of novel mediators that are regulated by IL-10 in addition to other previously reported prototypical mediators (15, 17, 27). To our knowledge, this is the first documentation of IL-10-mediated inhibition of effectors of the TLR pathway, thus providing some insight into the role of this cytokine in the control of Lyme disease inflammation.

We also confirmed by TaqMan analysis the significant inhibition by IL-10 of the inflammatory mediators IRG1, MMP9, and PTGS2 as induced by live spirochetes, as well as by spirochetal sonicate and L-OspA. IRG1 is worthy of mention be-

cause of the robust expression induced in macrophages by live *B. burgdorferi* spirochetes and the subsequent marked (6-fold) inhibitory effect of IL-10. Other pathogens known to induce the IRG1 gene are *Mycobacterium tuberculosis* (89) and *Mycobacterium paratuberculosis* (6) in addition to LPS (48). The significance of the upregulation of IRG1 is unclear at this time, given that it has not been functionally characterized in macrophages. Its upregulation in *B. burgdorferi*-stimulated macrophages is probably worthy of further investigation. MMP9, a granulocyte-secreted type IV collagenase (88), has been shown previously to be induced by *B. burgdorferi* in both human and murine monocytes in a TLR2-dependent manner (37, 38). MMP9 is also upregulated in erythema migrans lesions of Lyme disease patients (99, 100) and in joints of Lyme disease-susceptible mice (7). Recent studies by Heilpern et al. (46) using MMP9 knockout mice infected with *B. burgdorferi* revealed reduced arthritis in these animals, suggesting that MMP9 plays a role in the genesis of this form of Lyme disease. In the present study, *B. burgdorferi*-induced MMP9 was downregulated 2-fold in the presence of IL-10, using TaqMan assays, indicating the ability of this cytokine to target select genes that contribute to the overall *B. burgdorferi*-induced inflammation. The *Ptgs2* gene also known as COX2 (1) was downregulated 3-fold in spirochete cultures with added IL-10. COX2 has been reported to be expressed in murine B cells (10), microglia (81), peripheral blood mononuclear cells (PBMCs) (74), and joints (4) after exposure to *B. burgdorferi*. The expression of COX2 in joints of *B. burgdorferi*-infected mice has been associated with the initiation of arthritis (4) although a recent study showed that COX2 is also essential for resolution of the inflammatory arthritis induced by *B. burgdorferi* (11). Overall, our data show the collective inhibition of these inflammatory mediators by IL-10 in *B. burgdorferi*-stimulated cultures, thus revealing an aspect of IL-10 regulatory function in Lyme disease not previously investigated.

TLR-stimulated macrophages induce effectors of the adaptive immune system, such as CD40, CD80, and CD86, to drive T-cell activation and proliferation, (60) as well as IFN-α/IFN-β-inducible genes. Our study showed the transcriptional activation by *B. burgdorferi* of the *Cd40* gene and many interferon-inducible genes, namely, *Irf7*, *Ifi202b*, *Ifit1*, *Ifi44*, *Ifi27*, *Oas1*, and *oas2*, all of which were inhibited by IL-10. Studies by Qin et al. (77) have indicated that IL-10 inhibits LPS-induced CD40 gene expression through the inhibition of LPS-induced IFN-β gene expression and induction of suppressor of cytokine signaling 3 (SOCS3). SOCS3 was also shown to be significantly upregulated in cultures stimulated with live spirochetes either alone or when combined with IL-10 at both 4 and 24 h in the present study (see Tables S1 and S2 in the supplemental material). A SOCS3 synergistic effect by live spirochetes combined with IL-10 was also seen using TaqMan analysis (data not shown) as previously reported by us (27). We have shown that enhanced SOCS expression correlated with the IL-10-mediated downregulation of inflammatory mediators in macrophages (27). Consistent with a role for SOCS in *B. burgdorferi*-induced inflammation was the recent observation that CD14 recognition by *B. burgdorferi* triggers p38-dependent SOCS and that reduced SOCS expression in cells resulted in greater expression of cytokines through diminished regulation of the TLR2 pathway (83).

The observed *B. burgdorferi* IFN-inducible genes in the present study are consistent with previous reports by other investigators (24, 61, 74, 83, 85). Salazar and coworkers (85) have shown that live spirochetes induced transcription of several type I interferon-associated genes in human PBMCs, such as *Irf7*, *Ifit1*, *Ifi44*, *Oas1*, and *Oas2* seen here. The *Irf7* (83) as well as the *Oas1*, *Ifiti*, *Ifi44*, and *Oas2* (74) genes are also induced by live spirochetes in human immune cells. Other studies have shown marked upregulation of similar IFN-responsive genes in the joints of Lyme arthritis-resistant mice (24) and in mouse bone marrow-derived macrophages stimulated with live spirochetes (61). These findings indicate that the role of type I IFN in arthritis development after a *B. burgdorferi* infection is independent of TLR2, suggesting an alternative pathway for induction of IFN-responsive genes, as also demonstrated employing PBMCs and purified human monocytes (85). Cervantes et al. (21) recently demonstrated a TLR8-mediated induction of IFN-β by live *B. burgdorferi* spirochetes in human monocytes. In contrast, spirochete recognition of TLR7 and TLR9 was necessary for the expression of IFN genes in human plasmacytoid DCs (74). Whether the IFN-inducible genes elicited by *B. burgdorferi* in macrophages that were downregulated by IL-10 in this study are TLR dependent or independent remains to be investigated.

The influx of inflammatory cells in pathogen-induced diseases can be either beneficial or detrimental to the host. An interesting observation made in our study was the ability of IL-10 to selectively potentiate *B. burgdorferi*-induced expression levels of well-characterized CC chemokines, including CCL2 and CCL4, which attract monocytes, and CCL7 and CCL9, which attract T cells. This potentiating effect of IL-10 was due to its ability to distinctly upregulate the mRNA transcripts of these chemokines. Indeed, we previously observed, using PCR array, that IL-10 alone induced the mRNA gene transcripts of the above mentioned chemokines along with several others (CCL11, CCL12, CCL17, and CCL24) and their putative receptors (CCR1, CCR2, CCR6, and CCR9) in mouse J774 macrophages (our unpublished observations). Some of these chemokines have been previously recognized as part of the *B. burgdorferi*-induced inflammatory milieu. These include CCL2 (39, 44, 67, 80, 91, 100), CCL4 (67), and CCL9 (72). The anti-inflammatory effects of IL-10, manifested by this cytokine's ability to control the influx of inflammatory cells in tissues and, ultimately, their level of production of inflammatory mediators in Lyme disease, are very intriguing. This ability of IL-10 to repress the expression of a large fraction of live-spirochete-induced genes and potentiate others suggests its selective regulation of genes to control inflammation in Lyme disease. How IL-10 concomitantly enhances and represses *B. burgdorferi*-inducible inflammatory mediators is not known. However, a recent report that inappropriate proinflammatory responses can be selectively controlled through epigenetic modifications to individual promoters while leaving other responses intact (32) suggests a phenomenon that is worth exploring and that may help explain the IL-10 selective anti-inflammatory effect in Lyme disease.

Finally, our finding of the enhanced amplification of the transcription and secretion of inflammatory mediators as elicited by live spirochetes compared to the effects of L-OspA and sonicated spirochetes in J774 macrophages is worthy of men-

tion since studies have demonstrated that *in vitro* monocyte/macrophage models may not be as representative of the true phagocytic capacity as primary cells (64). Although we did not perform phagocytosis studies here, the mouse J774 macrophages are known to be phagocytic for several bacterial pathogens (33, 41, 68) including *B. burgdorferi*, where degraded spirochetes were observed in intracellular compartments (62). Our findings of heightened responses to live spirochetes corroborated those of other investigators who have noted that live spirochetes induce greater responses in innate immune cells than those of lipoproteins or lysates (25, 64, 74, 85, 90). As suggested, this enhancing effect may be attributed to phagocytosis and degradation of live spirochetes in phagolysosomes, which ultimately leads to synergistic amplification of multiple signaling pathways and enhancement of inflammatory mediators compared to those generated by a single agonist (64, 85). Thus, in all likelihood the greater inflammatory responses elicited by live spirochetes in the present study may have resulted from phagocytosis and degradation of live spirochetes in macrophages.

In conclusion, we have found that IL-10 inhibits *B. burgdorferi*-induced effectors that participate in several pathways and, especially, the TLR pathway. Consequently, multiple inflammatory mediators underwent changes when exposed to IL-10. A number of these mediators are newly identified IL-10-regulated genes potentially in the context of Lyme disease. Our study provides a more global understanding of potential mechanisms used by IL-10 to control Lyme disease inflammation. Functional studies are now necessary to identify specific mediators of IL-10 anti-inflammatory activities. This may allow the development of specific immunotherapeutic approaches for both early- and late-stage Lyme disease.

ACKNOWLEDGMENTS

The project described was supported by grant R21AI073356 from the National Institute of Allergy and Infectious Diseases, grant RR00164 from the National Center for Research Resources, National Institutes of Health, and grant HRD-0734232 from NSF-CREST.

The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institute of Allergy and Infectious Diseases or the National Institutes of Health.

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Editor: S. R. Blanke