Borrelia burgdorferi-Induced Tolerance as a Model of Persistence via Immunosuppression

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If left untreated, infection with Borrelia burgdorferi sensu lato may lead to chronic Lyme borreliosis. It is still unknown how this pathogen manages to persist in the host in the presence of competent immune cells. It was recently reported that Borrelia suppresses the host’s immune response, thus perhaps preventing the elimination of the pathogen (I. Diterich, L. Härter, D. Hassler, A. Wendel, and T. Hartung, Infect. Immun. 69:687-694, 2001). Here, we further characterize Borrelia-induced immunomodulation in order to develop a model of this anergy. We observed that the different Borrelia preparations that we tested, i.e., live, heat-inactivated, and sonicated Borrelia, could desensitize human blood monocytes, as shown by attenuated cytokine release upon restimulation with any of the different preparations. Next, we investigated whether these Borrelia-specific stimuli render monocytes tolerant, i.e., hyporesponsive, towards another Toll-like receptor 2 (TLR2) agonist, such as lipoteichoic acid from gram-positive bacteria, or towards the TLR4 agonist lipopolysaccharide. Cross-tolerance towards all tested stimuli was induced. Furthermore, using primary bone marrow cells from TLR2-deficient mice and from mice with a nonfunctional TLR4 (strain C3H/HeJ), we demonstrated that the TLR2 was required for tolerance induction by Borrelia, and using neutralizing antibodies, we identified interleukin-10 as the key mediator involved. Although peripheral blood mononuclear cells tolerized by Borrelia exhibited reduced TLR2 and TLR4 mRNA levels, the expression of the respective proteins on monocytes was not decreased, ruling out the possibility that tolerance to Borrelia is attributed to a reduced TLR2 expression. In summary, we characterized tolerance induced by B. burgdorferi, describing a model of desensitization which might mirror the immunosuppression recently attributed to the persistence of Borrelia in immunocompetent hosts.

Borrelia burgdorferi sensu lato is the causative agent of Lyme borreliosis (LB), the most common vector-borne disease in the United States (32) and in many European countries (39a). If infection with this pathogen is not treated adequately with antibiotics, it may lead to a chronic multisystemic disorder. Infection with this pathogen is not treated adequately with antibiotics, it may lead to a chronic multisystemic disorder in the United States (32) and in many European countries (39a). If left untreated, infection with Borrelia burgdorferi sensu lato may lead to chronic Lyme borreliosis. It is still unknown how this pathogen manages to persist in the host in the presence of competent immune cells. It was recently reported that Borrelia suppresses the host’s immune response, thus perhaps preventing the elimination of the pathogen (I. Diterich, L. Härter, D. Hassler, A. Wendel, and T. Hartung, Infect. Immun. 69:687-694, 2001). Here, we further characterize Borrelia-induced immunomodulation in order to develop a model of this anergy. We observed that the different Borrelia preparations that we tested, i.e., live, heat-inactivated, and sonicated Borrelia, could desensitize human blood monocytes, as shown by attenuated cytokine release upon restimulation with any of the different preparations. Next, we investigated whether these Borrelia-specific stimuli render monocytes tolerant, i.e., hyporesponsive, towards another Toll-like receptor 2 (TLR2) agonist, such as lipoteichoic acid from gram-positive bacteria, or towards the TLR4 agonist lipopolysaccharide. Cross-tolerance towards all tested stimuli was induced. Furthermore, using primary bone marrow cells from TLR2-deficient mice and from mice with a nonfunctional TLR4 (strain C3H/HeJ), we demonstrated that the TLR2 was required for tolerance induction by Borrelia, and using neutralizing antibodies, we identified interleukin-10 as the key mediator involved. Although peripheral blood mononuclear cells tolerized by Borrelia exhibited reduced TLR2 and TLR4 mRNA levels, the expression of the respective proteins on monocytes was not decreased, ruling out the possibility that tolerance to Borrelia is attributed to a reduced TLR2 expression. In summary, we characterized tolerance induced by B. burgdorferi, describing a model of desensitization which might mirror the immunosuppression recently attributed to the persistence of Borrelia in immunocompetent hosts.
investigated extensively in vitro and in vivo (for a review, see reference 21). A status of macrophage hyporesponsiveness to a high or lethal LPS dose after preexposure to low LPS doses has been described (13, 40). Recently, similar desensitization experiments were reported which demonstrated that stimuli other than LPS, e.g., highly purified lipoteichoic acid (LTA) (22) and macrophage-activating lipopeptide 2 (MALP-2) from mycoplasma (36), can also render macrophages tolerant to subsequent restimulation. Furthermore, it was shown in these same studies that tolerance can also be induced by two heterologous stimuli, independent of the receptor involved in their recognition and signaling. In this case, the appropriate term is cross-tolerance or heterotolerance. To our knowledge it has not yet been investigated whether B. burgdorferi also has the capacity to desensitize macrophages. However, Borrelia-induced hyporesponsiveness could represent a mechanism enabling the survival of this pathogen in the host despite the presence of immune cells. We tested this hypothesis in desensitization experiments with Borrelia-specific stimuli and with other well-characterized TLR2 and TLR4 agonists. Additionally, the involvement of the TLR2 and the TLR4, as well as of endogenous IL-10 formation, in tolerance and cross-tolerance induction was addressed.

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MATERIALS AND METHODS

Borrelia cultivation and preparation of Borrelia-specific stimuli. B. burgdorferi sensu stricto (strain N40, kindly provided by T. Kamradt, Berlin, Germany) was cultivated as described previously (8). Borrelia cultures passed fewer than eight times after isolation from mice were grown to log phase (≥10^7 cells/ml) and differentially prepared for stimulation experiments. For experiments with live Borrelia, B. burgdorferi cell counts were determined by microscopy using a Thoma counting chamber with a modified depth of 0.02 mm. The culture was adjusted to a concentration of 5 × 10^5 cells/ml. Due to the low replication rate of Borrelia and its complex requirements with regard to culture conditions, an increase in bacterial numbers during the 24 h of subsequent incubation is improbable.

Before heat inactivation or sonication, Borrelia cultures were washed twice. Briefly, cultures were centrifuged at 10,000 × g for 30 min, the supernatant was removed, and the pellet was resuspended with pyrogen-free saline solution. Subsequently, the bacterial numbers were determined as described above. Finally, washed Borrelia was either incubated for 30 min at 56°C for heat inactivation or sonicated, yielding Borrelia lysate as described elsewhere (8). Protein concentration of the lysate was adjusted to a final concentration of 1 mg/ml. Remaining viable spirochetes were excluded visually under the microscope after 1 week of incubation under standard cultivation conditions.

The numbers of endotoxin units (EU) contained in 10 μg of protein from sonicated Borrelia cultures and from 10^7 heat-inactivated Borrelia cells were below the detection limit (0.1 and 0.05 EU, respectively), as assessed by a Limulus amoebocyte lysate assay (QCL-1000; Charles River Endosafe, Charleson, S.C.). The spike recoveries (0.5 EU) were 107 and 103%, respectively. Live Borrelia was used only for the first set of experiments because it constitutes a highly variable stimulus which is difficult to standardize, as the pathogen changes its surface protein expression depending on cultivation conditions (5). All the other experiments were carried out with the same batch of heat-inactivated or sonicated Borrelia.

Isolation of PBMC. Peripheral blood mononuclear cells (PBMC) were isolated with cell preparation tubes (Vacutainer CPT, sodium citrate; Becton Dickinson Biosciences [BD Biosciences], Heidelberg, Germany) according to the manufacturer’s instructions. Briefly, blood from healthy donors (differential blood cell counts were performed with a Pentra60 [ABX Technologien, Montpellier, France] to rule out acute infections) was centrifuged in the tubes for 20 min at 600 × g. PBMC, separated from erythrocytes and neutrophils by the gel procedures and transferred into Steriflip tubes (Millipore, Eschborn, Germany) and washed twice with RPMI 1640 (Bio Whittaker, Apen, Germany) supplemented with 2.5 IU of heparin (Liquemine; Hofmann LaRoche, Grenzach-Wyhlen, Germany)/ml. The overall PBMC count (i.e., monocytes and lymphocytes) was determined with a Pentra60. PBMC were adjusted to a final concentration of 10^6 cells/ml. In case the PBMC were stimulated with LPS from Salmonella enterica serovar Abortus equi; Sigma-Aldrich, Seelze, Germany) or left untreated to serve as controls. The volume was adjusted to 220 μl with medium (RPMI 1640 supplemented with 100 IU of penicillin-streptomycin/ml containing 10% fetal calf serum for the primary murine bone marrow cells or 2.5 IU of heparin/ml for the PBMC). After incubation for 24 h at 37°C in the presence of 5% CO2, the supernatants were transferred to 96-well round-bottom plates (Greiner) and stored at −70°C until cytokine amounts were measured. The remaining adherent, tolerated monocytes in the plate were washed twice with RPMI 1640 and subsequently restimulated with 1 ng of LPS/ml, 10 μg of LPS/ml, 10 μg of Borrelia lysate/ml, or 10^6 heat-inactivated B. burgdorferi sensu stricto cells/ml. Autologous plasma (10%) was added to the PBMC. The final volume of the incubation was adjusted to 220 μl of RPMI 1640 (supplemented as described above). After 24 h of incubation at 37°C and 5% CO2, the supernatants were transferred to 96-well round-bottom plates (Greiner) and stored at −70°C until cytokine amounts were measured. To study the involvement of mediators in tolerance induction, neutralizing antibodies (anti–IL-10 [Endogen, Eching, Germany], pan-specific anti-transforming growth factor β [anti-TGF-β; R&D Systems, Wiesbaden, Germany], and polyclonal anti-mu-galactosylcolony-stimulating factor [G-CSF]–sheep immunoglobulin G raised in our laboratory [2]) or IL-10 (a kind gift from S. Narula, Schering Plough, Kenilworth, N.J.) was added for the first 24 h incubation period.

MTT assay. MTT (3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyl-2H-tetrazolium bromide; Sigma) stock solution (5 mg/ml in phosphate-buffered saline) was diluted 1:5 in RPMI 1640. Two hundred microliters of this working solution was added to the adherent cells in a 96-well culture plate for 2 h at 37°C and 5% CO2.

Cells with mitochondrial activity convert dissolved MTT to insoluble purple formazan. After the incubation, the supernatant was removed and the cells were lysed for 10 min with 95% isopropanol–5% formic acid. Absorbance of converted dye was measured at 555 nm, with 690 nm as the reference wavelength.

Cytokine measurement in culture supernatant by ELISA. The concentrations of IL-6, IL-10, tumor necrosis factor α (TNF-α), and IL-12 were determined in cell culture supernatants by using commercially available antibody pairs and recombinant standards. Monoclonal antibody pairs

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against human TNF-α and IL-10 were purchased from Endogen (Perbio Science, Bonn, Germany) and Pharmingen (BD Biosciences), respectively. Recombinant human TNF-α was a gift from S. Poole (National Institute for Biological Standards and Controls, Herts, Great Britain) and was used as a standard. For IL-10, the standard was purchased from BD Biosciences. For the measurement of murine TNF-α, polyclonal antibodies from R&D Systems and a standard from Pharmingen were used. Assays were carried out in flat-bottom, Maxisorb 96-well plates (Nunc, Wiesbaden, Germany). Binding of secondary biotinylated antibodies was detected with horseradish peroxidase-conjugated streptavidin (Bio-source, Camarillo, Calif.), and 3′,3′,5′-tetramethylbenzidine solution (Sigma) was used as a substrate.

RNA extraction and TLR2 mRNA quantification. PBMC (5 × 10⁶/ml) were pipetted into 24-well culture plates in 1 ml of medium. After 24 h of incubation at 37°C and 5% CO₂ with 10 μg of lysate/ml or without a stimulus, the supernatant was removed and cells were either lysed for RNA extraction or washed and restimulated with lysate (10 μg/ml) or with LPS (1 ng/ml) for another 3 h. After the removal of the supernatant, RNA was prepared from the adherent cells with a QIAamp RNA blood mini kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions, including DNA digestion with the RNase-free DNase-Set (Qiagen).

Six microliters of RNA was reverse transcribed in a sample volume of 20 μl containing 2.5 μM oligonucleotide dT16 (custom primer; Gibco BRL), MgCl₂ (5 mM), deoxynucleoside triphosphates (1 mM each), RNase inhibitor (1 U/μl), and murine leukemia virus reverse transcriptase (2.5 U/μl) in PCR buffer (all from PE Applied Biosystems, Weiterstadt, Germany). Samples were incubated at 21°C for 10 min, 42°C for 15 min, 94°C for 5 min, and 5°C for 5 min in a GeneAmp PCR System 2400 (PE Applied Biosystems).

For relative quantification, real-time PCR was performed by using a LightCycler rapid thermal cycler system (Roche Diagnostics GmbH, Mannheim, Germany). The cDNA for TLR2, TLR4, and GAPDH were amplified by using LightCycler-FastStart DNA Master SYB-BGreen (Roche Diagnostics) according to the manufacturer’s protocol. The sequences of the primers were GGC CAG CAA ATT ACC TG7 GTG (forward) and AGG CGG ACA GCC ACT (reverse) and TGG TGG AAC TTG AAC GAA TGG (forward) and AGG ACC GAC ACA CCA ATG (reverse) for TLR2 and TLR4, respectively. For GAPDH the primers were AGA AAG TTG AAC GAA TGG (forward) and AGG ACC GAC ACA CCA ATG (reverse) for TLR2 and TLR4, respectively. For GAPDH the primers were GAA GGT GAA GGT CGG AGT C (forward) and GAA GAT GGT GAT GGAT ATT TC (reverse). The MgCl₂ concentrations were adjusted to 2 mM for TLR2, 3 mM for TLR4, and 4 mM for GAPDH. The thermal cycling was performed according to the manufacturer’s protocol (50 cycles) with annealing temperatures of 58, 55, and 65°C and elongation times of 3, 6, and 11 s for TLR2, TLR4, and GAPDH, respectively. The amplification was followed by a melting curve analysis in which the temperature was increased from 65°C to 95°C and then increased to 95°C at 0.1°C/s. The specific melting temperatures for TLR2, TLR4, and GAPDH products were 86, 84, and 86.5°C, respectively. The size of the amplification product was checked initially on an agarose gel stained with ethidium bromide. The TLR2 and TLR4 results were normalized to the GAPDH signal.

Flow cytometry analysis of TLR expression. PBMC were stained after 24 h of preincubation and restimulation for 4 and 6 h as described above. Cells were detached from the 24-well plate by a 30-min incubation with Accutase (PAA Laboratories) at 37°C, split into three micronic tubes (Apogent, Wehrheim, Germany), and stained with anti-CD45-peridinin chlorophyll protein, anti-CD14-fluorescein isothiocyanate (BD Biosciences), and anti-CD14-phycocerythrin, anti-TLR4-phycocerythrin, or isotype control (eBioscience, San Diego, Calif.) at room temperature for 30 min. After the cells were washed, fluorescence was measured in a FACSCalibur system (BD Biosciences). The median fluorescence of the isotype control in arbitrary units was subtracted from the TLR-specific median. Monocytes were identified by the height of their CD14 expression.

Statistics. Data are shown as means ± standard errors of the mean (SEM) of results for either four different blood donors or primary cells from four to seven individual mice. To take into account the varying viabilities of the cells on day 2, TNF-α concentrations were divided by the mitochondrial activity assessed with the MTT assay. Repeated-measure analysis of variance (ANOVA) followed by Dunnett’s multiple comparison test was performed by using GraphPad Prism 3.00 (GraphPad Software, San Diego, Calif.). A P value of <0.05 was considered significant.

RESULTS

Borrelia induced tolerance in human PBMC. We tested whether Borrelia is able to induce a state of hyporesponsive-
investigated whether concentrations were used in the subsequent restimulation experiment with the nontolerized (con) cells. * represents a significance is for comparison.

Data are expressed as the means ± SEM divided by the OD for comparison with the nontolerized (con) cells. * represents a P value of <0.05 and *** represents a P value of <0.001 based on ANOVA followed by Dunnett’s multiple comparison test.

FIG. 2. Induction of Borrelia cross-tolerance to LTA or LPS in human PBMC. Human PBMC (10^5/well) from four different donors were incubated with culture medium (con, black bars); 0.1, 1, or 10 µg of Borrelia lysate/ml (white bars); or 10^2 or 10^3 heat-inactivated Borrelia cells (heat-inact. Bb; striped bars) for 24 h and then washed and incubated for another 24 h in the presence of 10 µg of LTA/ml (a) or 10 ng of LPS/ml (b). The concentrations of TNF-α were measured by ELISA. Data are expressed as the means ± SEM divided by the OD assessed by the MTT test. The statistical significance is for comparison with the nontolerized (con) cells. * represents a P value of <0.05 and *** represents a P value of <0.001 based on ANOVA followed by Dunnett’s multiple comparison test.

lysate/ml, respectively). In summary, all Borrelia-specific stimuli tested could desensitize macrophages to either the same or another Borrelia-specific stimulus. This desensitized state is termed Borrelia tolerance.

Comparison of TNF-α-inducing potencies of different bacterial stimuli. We next tested the TNF-α-inducing capacities of different bacterial stimuli during 24 h of incubation in vitro. Treatment of human PBMC with LPS, LTA, or two Borrelia-specific stimuli (heat-inactivated Borrelia and Borrelia lysate) induced dose-dependent TNF-α release. LPS (1 ng/ml), LTA (10 µg/ml), 10^6 heat-inactivated B. burgdorferi cells/ml, and Borrelia lysate (10 µg/ml) induced comparable amounts of TNF-α in the range of 200 to 500 pg/ml. Therefore, these concentrations were used in the subsequent restimulation experiments.

Borrelia-induced cross-tolerance to LTA and LPS. Next, we investigated whether Borrelia-specific stimuli—which according to the literature (1, 3, 16, 23) act via TLR2—render macrophages tolerant towards another TLR2 agonist such as LTA from gram-positive Staphylococcus aureus or towards a TLR4 agonist, e.g., LPS from Salmonella enterica serovar Abortus equi. PBMC were first treated overnight with either heat-inactivated Borrelia or Borrelia lysate. Then, the cells were re-stimulated with either 10 µg of LTA/ml or 1 ng of LPS/ml. Figure 2 demonstrates that prestimulation with Borrelia-specific stimuli led to reduced responsiveness of the PBMC to restimulation with LTA as well as with LPS, as shown by TNF-α release. Tolerance induction was concentration dependent. The stronger the stimulus during the preincubation period, the lower the TNF-α response to the second stimulus. Thus, Borrelia induced cross-tolerance towards two different heterologous stimuli, one also signaling via TLR2, the other TLR4 mediated.

LPS- and LTA-induced cross-tolerance to Borrelia-specific stimuli. Stimulation of macrophages with LPS renders the cells tolerant to a subsequent LPS stimulation. This kind of tolerance has been known since the 1960s and 1970s (4, 14, 30). Recently, we reported tolerance induction by LTA, including cross-tolerance to LPS inducible by LTA and vice versa, indicating that cross-tolerance between TLR2 and TLR4 agonists is possible (22). Therefore, our results prompted us to test whether cells rendered tolerant by LPS or LTA were also hyporesponsive to restimulation with Borrelia lysate.

In the experiments whose results are shown in Fig. 3, LPS or LTA was used for prestimulation. For subsequent restimulation, Borrelia lysate was added to the cells. Similar to that in the previous experiments, cytokine release in response to the second stimulus was decreased in a dose-dependent fashion depending on the concentration of the first stimulus. The same results were obtained when heat-inactivated Borrelia was used for restimulation instead of Borrelia lysate (data not shown).

IL-10 is involved in tolerance induction by Borrelia and LPS. Next we were interested in the mechanism of Borrelia-induced tolerance. We first checked whether soluble mediators are involved. As reported previously (8), Borrelia is a relatively strong inducer of IL-10; e.g., 10 µg of lysate/ml induces 1 to 1.5 ng of IL-10/ml in human PBMC after 24 h of incubation. Furthermore, it has been shown that endogenous anti-inflammatory factors such as IL-10 and TGF-β mediate the phenomenon of LPS tolerance in human monocytes in vitro (35). In order to examine this mechanism, neutralizing antibodies were incubated simultaneously with the Borrelia lysate during the preincubation period. Pretreatment of PBMC with Borrelia and specific neutralizing antibodies against the anti-inflammatory cytokines IL-10, TGF-β, and G-CSF showed that neither
antibodies against TGF-β or G-CSF alone nor the combination of both types of antibodies could block Borrelia-induced tolerance (Fig. 4). The neutralizing activity of the antibodies was assessed in control experiments by blocking the immuno-suppressive effect of recombinant cytokines on LPS-inducible cytokine release (data not shown). Anti-IL-10 antibodies alone partially prevented tolerance induction by Borrelia. Addition of anti-TGF-β or anti-G-CSF antibodies or both did not augment the inhibitory effect of anti-IL-10 antibodies.

We further examined the role of IL-10 in Borrelia-induced tolerance and checked whether the cells could be rendered hyperresponsive to Borrelia by levels of IL-10 induced by Borrelia lysate. Preincubation of PBMC for 24 h with 1 ng of biologically active IL-10 led to a reduced TNF-α release when cells were restimulated with 10 μg of lysate/ml (control, 2 ± 0.9 ng/ml; IL-10-tolerant cells, 0.5 ± 0.4 ng/ml). The partial reduction of TNF-α by IL-10 only might indicate that further factors contribute to tolerance induction.

These findings suggest that IL-10, not G-CSF or TGF-β, is involved in tolerance induction by Borrelia in human PBMC but that other mediators are needed additionally to completely prevent the process.

**TLR2 and TLR4 mRNA down-regulation by Borrelia-induced tolerance.** Recently published results of experiments by Wang et al. suggest that TLR2 is down-regulated in the case of synthetic bacterial lipopeptide tolerance (41). We therefore measured the TLR2 mRNA expression of human PBMC with Borrelia-induced tolerance. Stimulation of PBMC for 24 h in the presence of Borrelia lysate (10 μg/ml) or LPS (1 ng/ml) led to significantly reduced TLR2 mRNA expression, which remained attenuated after restimulation of the tolerized cells (Fig. 5a).

We further determined the expression of TLR4 mRNA in the same samples and similarly observed a significant down-regulation compared to baseline levels by stimulation with Borrelia lysate or LPS for 24 h. TLR4 mRNA levels remained reduced after a second stimulation of tolerized cells. These data suggested that in the case of Borrelia-induced tolerance, down-regulation of the TLR2 and TLR4 contributes to suppression of TNF-α formation upon restimulation with either a TLR2 or a TLR4 agonist.

**TLR2 and TLR4 surface expression in Borrelia-induced tolerance.** Since mRNA levels may not necessarily reflect protein levels, we additionally assessed TLR2 and TLR4 expression on the surface of monocytes, which are the TNF-α-producing cells in our test system, by flow cytometry. Stimulation of PBMC overnight with Borrelia lysate led to an increase of TLR2 expression on the surface of monocytes (median fluorescence of the control in arbitrary units was 101 ± 18; that of lysate-stimulated cells was 208 ± 52 [not significant]). When the tolerant cells were restimulated for another 4 h with the same stimulus, the TLR2 expression returned to control levels (108 ± 44). With the use of LPS as the second stimulus, i.e., in the case of cross-tolerance, TLR2 expression was down-regulated below the expression level of the control cells (75 ± 17). No major differences in TLR4 expression were seen, neither with stimulation with LPS or lysate nor with lysate-induced tolerance and cross-tolerance. The median fluorescence ranged between 31 ± 9 on unstimulated controls and 34 ± 4 on lysate-stimulated tolerized or nontolerized PBMC.
TLR2 but not TLR4 is required for tolerance and cross-tolerance induction by *Borrelia*. To further investigate the role of TLR2 and TLR4 in *Borrelia*-induced tolerance, we carried out desensitization experiments using primary murine bone marrow cells from TLR2 knockout mice and C3H/HeJ mice (with a nonfunctional TLR4) and corresponding wild-type cells. As expected, there was no measurable TNF-α or IL-10 release in TLR2−/− bone marrow macrophages after stimulation with either heat-inactivated *Borrelia* or *Borrelia* lysate after 8 or 24 h (data not shown). Hereby, we confirmed data from others (1, 3, 16, 23) showing that *Borrelia*-specific stimuli employ TLR2. Cells from TLR2−/− mice responded to LPS to the same extent as did cells from wild-type mice, reflecting the normal responsivity to a TLR4 agonist (data not shown). Cells from TLR2- and TLR4-defective mice, obtained by crossing both strains, did not release TNF-α upon stimulation with either *Borrelia* or LPS or LTA (data not shown). We checked that the cells were still responsive by activating them with other stimuli such as CpG oligonucleotides, a TLR9-mediated stimulus modeling bacterial DNA, and the phorbol ester phorbol myristate acetate, a receptor-independent stimulus (data not shown).

Preincubation of cells from TLR2−/− mice with a *Borrelia*-specific stimulus had no significant effect on the responsiveness of the cells to LPS (Fig. 6b). Their behavior was similar to that of saline-pretreated cells. This result indicates that the cells had not been desensitized by heat-inactivated *Borrelia* and demonstrates that TLR2 is required for *Borrelia*-induced tolerance. Furthermore, as expected, no TNF-α release could be measured upon restimulation of the TLR2-deficient cells with either lysate (Fig. 6d) or heat-inactivated *Borrelia* (data not shown), independent of the preincubation stimulus. In contrast, restimulating wild-type and TLR2−/− cells with LPS after LPS pretreatment resulted in strongly reduced TNF-α release, showing that the cells had been rendered tolerant (Fig. 6a). Thus, the TLR2 was not required for LPS-induced tolerance. In line with our PBMC results (Fig. 1 and 3), this experiment shows that prestimulation of primary murine wild-type cells with LPS or *Borrelia* lysate tolerized the cells to *Borrelia* lysate.

The same experiments were also conducted with primary murine bone marrow cells from C3H/HeJ mice, which lack a functional TLR4, and cells from the corresponding wild-type mice. Cells from both mouse strains responded similarly to *Borrelia* lysate after LPS (data not shown) could be induced by LPS pretreatment showing that the cells had been rendered tolerant (Fig. 6a). Thus, the TLR2 was not required for LPS-induced tolerance. In line with our PBMC results (Fig. 1 and 3), this experiment shows that prestimulation of primary murine wild-type cells with LPS or *Borrelia* lysate tolerized the cells to *Borrelia* lysate.

Hence, in the absence of TLR2, no cytokine release could be induced by *Borrelia* and no state of hyporesponsiveness for restimulation with LPS was achieved. In line with these findings, no desensitization to *Borrelia* stimuli could be induced by LPS in the absence of a functional TLR4.

**DISCUSSION**

Understanding the immunopathology of LB is still a major challenge. Although it induces strong immune activation, e.g., in phases of arthritis, the causative agent of LB persists and...
leads to a chronic pathology in the immunocompetent host. Of note, the inflammatory episodes associated with LB are typically self-limiting and the site of manifestation often changes, e.g., between different joints. These phenomena suggest counter-regulatory anti-inflammatory mechanisms, and the long phases of latency indicate phases of immune evasion.

A possible mechanism for survival of \textit{Borrelia} in the immunologically competent host was recently proposed: since ex vivo experiments with whole blood from patients with LB revealed a significantly reduced capacity to release TNF-\alpha and gamma interferon in comparison to that of blood from healthy controls, it was postulated that \textit{Borrelia} modulates the host’s immune system in order to evade immune clearance (8). There are a few reports about \textit{Borrelia} acting as an immunomodulator (28, 33, 39). Further evidence was found for \textit{Borrelia}-induced immunomodulation by demonstrating that \textit{Borrelia} induces a stronger anti-inflammatory cytokine response than do endotoxins from various other gram-negative bacteria (8). These data are in line with findings from other authors indicating that \textit{B. burgdorferi} is a potent inducer of the anti-inflammatory cytokine IL-10 (9-11). In addition, the modulatory and regulatory capacity of IL-10 with regard to \textit{Borrelia}-induced cytokine release has been found in different in vitro models (24, 29).

In the present study, we examined whether tolerance could represent a possible model for \textit{Borrelia}-induced immunomodulation. First, we checked whether \textit{Borrelia} can render cells hyporesponsive, and second, we investigated some of the underlying mechanisms involved in this immunomodulation. \textit{Borrelia} could indeed render human PBMC tolerant, i.e., unable to react to a second stimulation with \textit{Borrelia}, as shown by a reduced capacity to release TNF-\alpha and IL-10. All the settings tested led to a significantly reduced TNF-\alpha release as a result of preincubation with \textit{Borrelia} compared to that of saline controls. As no major differences between live and killed \textit{Borrelia} with respect to the pathogen’s ability to render the monocyte hyporesponsive could be observed, we selected the more standardized stimuli (heat-inactivated \textit{Borrelia} and \textit{Borrelia} lysate) for the subsequent experiments. Based on these experiments, we demonstrated that \textit{Borrelia} is able to modulate the monocyte immune response. Next, assuming that \textit{Borrelia} can induce a more general desensitization in the cell, we investigated whether a monocyte rendered tolerant by \textit{Borrelia} was also hyporesponsive to a different stimulus. Differences in the potency of TNF-\alpha induction among the bacterial stimuli (\textit{Borrelia}, LPS, and LTA) were excluded by choosing equipotent concentrations, i.e., concentrations which induced comparable amounts of TNF-\alpha release during 24 h of incubation.

According to the literature, \textit{Borrelia} signal via the TLR2 (1, 3, 16, 23). This notion could be confirmed in our experiments with primary murine bone marrow cells from mice lacking either a functional TLR2 or a TLR4 or both. The findings shown in Fig. 2 demonstrate that \textit{Borrelia} desensitizes monocytes in a more general manner, because it also rendered PBMC hyporesponsive to subsequent stimulation with heterologous stimuli such as the TLR2 agonist LTA or the TLR4 agonist LPS. The extent of desensitization was concentration dependent. Using LPS or LTA to tolerance the cells similarly led to hyporesponsiveness to \textit{Borrelia} (Fig. 3). We did not observe any difference in levels of cross-tolerance induction of TNF-\alpha release by the tested stimuli. These findings confirm our data regarding cross-tolerance between LTA and LPS, showing that the degree of hyporesponsiveness induced by the TLR2 or the TLR4 agonist does not vary (22). Opposing results have been published by others, who postulate that LPS pretreatment is less effective in other cross-tolerance models. Sato et al., for example, describe poor tolerance induction by LPS in response to MALP restimulation (36).

Results of tolerance experiments presented here suggest that the underlying mechanisms which lead to \textit{Borrelia} tolerance seem to be very similar to those involved in tolerance induced by cell wall components from other bacteria, since no difference could be observed between the levels of tolerance and cross-tolerance induced by the tested combinations. Furthermore, cross-tolerance data indicate that signal pathways shared by LPS and TLR2 agonists seem to be impaired. Similar heterologous tolerance phenomena have recently also been described for MALPs from mycoplasma (36), bacterial DNA (CpG) (37), \textit{Staphylococcus aureus} (20), LTA (22), arabinose-capped lipoarabinomannan (26), and LPS. There is accumulating evidence that tolerance induced by different TLR2 and TLR4 agonists shares common intracellular signal transduction pathways (36, 41). However, recently Jacinto et al. suggested the involvement of unique TLR2 signaling components downstream of TLR2 and upstream of MyD88/IL-1 receptor-associated kinase (IRAK) in LTA tolerance by demonstrating that IRAK expression and IRAK activity differ with LPS and LTA tolerance (17). Controversial data are reported regarding the involvement of the TLR in LPS and non-LPS tolerance and cross-tolerance. Our findings with primary murine bone marrow cells show that in the absence of the \textit{Borrelia} recognition

![FIG. 7. Induction of tolerance and cross-tolerance in primary bone marrow cells from C3H/HeN and C3H/HeJ mice. Bone marrow cells (5 x 10^5/well) from C3H/HeN mice (black bars) and C3H/HeJ mice (striped bars) were incubated with culture medium (con), 1 or 10 ng of LPS/ml, or 10^5, 3 x 10^5, or 10^6 heat-inactivated \textit{Borrelia} cells (heat-inact. Bb) for 24 h and then washed and incubated for another 24 h in -induced immunomodulation by demonstrating that lia\textit{Borrelia}

inflammatory episodes associated with LB are typi-

leads to a chronic pathology in the immunocompetent host. Of note, the inflammatory episodes associated with LB are typically self-limiting and the site of manifestation often changes, e.g., between different joints. These phenomena suggest counter-regulatory anti-inflammatory mechanisms, and the long phases of latency indicate phases of immune evasion.

A possible mechanism for survival of \textit{Borrelia} in the immunologically competent host was recently proposed: since ex vivo experiments with whole blood from patients with LB revealed a significantly reduced capacity to release TNF-\alpha and gamma interferon in comparison to that of blood from healthy controls, it was postulated that \textit{Borrelia} modulates the host’s immune system in order to evade immune clearance (8). There are a few reports about \textit{Borrelia} acting as an immunomodulator (28, 33, 39). Further evidence was found for \textit{Borrelia}-induced immunomodulation by demonstrating that \textit{Borrelia} induces a stronger anti-inflammatory cytokine response than do endotoxins from various other gram-negative bacteria (8). These data are in line with findings from other authors indicating that \textit{B. burgdorferi} is a potent inducer of the anti-inflammatory cytokine IL-10 (9-11). In addition, the modulatory and regulatory capacity of IL-10 with regard to \textit{Borrelia}-induced cytokine release has been found in different in vitro models (24, 29).

In the present study, we examined whether tolerance could represent a possible model for \textit{Borrelia}-induced immunomodulation. First, we checked whether \textit{Borrelia} can render cells hyporesponsive, and second, we investigated some of the underlying mechanisms involved in this immunomodulation. \textit{Borrelia} could indeed render human PBMC tolerant, i.e., unable to react to a second stimulation with \textit{Borrelia}, as shown by a reduced capacity to release TNF-\alpha and IL-10. All the settings tested led to a significantly reduced TNF-\alpha release as a result of preincubation with \textit{Borrelia} compared to that of saline controls. As no major differences between live and killed \textit{Borrelia} with respect to the pathogen’s ability to render the monocyte hyporesponsive could be observed, we selected the more standardized stimuli (heat-inactivated \textit{Borrelia} and \textit{Borrelia} lysate) for the subsequent experiments. Based on these experiments, we demonstrated that \textit{Borrelia} is able to modulate the monocyte immune response. Next, assuming that \textit{Borrelia} can induce a more general desensitization in the cell, we investigated whether a monocyte rendered tolerant by \textit{Borrelia} was also hyporesponsive to a different stimulus. Differences in the potency of TNF-\alpha induction among the bacterial stimuli (\textit{Borrelia}, LPS, and LTA) were excluded by choosing equipotent concentrations, i.e., concentrations which induced comparable amounts of TNF-\alpha release during 24 h of incubation.

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