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Borrelia miyamotoi activates human dendritic cells and elicits T cell responses

Lauren M. Mason[a], Joris Koetsveld[a], Alex Wagemakers[a], Michelle M. Fikrig[a], Jasmin I. Ersoz[a], Anneke Oei[b], Teunis B.H. Geijtenbeek[c], Joppe W. Hovius[a,d]

[a] Amsterdam UMC, University of Amsterdam, Center for Experimental and Molecular Medicine, Amsterdam Infection & Immunity, Meibergdreef 9, 1105AZ Amsterdam, Netherlands

[b] Amsterdam UMC, University of Amsterdam, Department of Medical Microbiology, Amsterdam Infection & Immunity, Meibergdreef 9, 1105AZ Amsterdam, Netherlands

[c] Amsterdam UMC, University of Amsterdam, Department of Experimental Immunology, Amsterdam Infection & Immunity, Meibergdreef 9, 1105AZ Amsterdam, Netherlands

[d] Amsterdam UMC, University of Amsterdam, Division of infectious diseases, Meibergdreef 9, 1105AZ Amsterdam, Netherlands

Abstract

The spirochete *Borrelia miyamotoi* has recently been shown to cause relapsing fever. Like the Lyme disease agent, *Borrelia burgdorferi, B. miyamotoi* is transmitted through the bite of infected ticks, however, little is known about the response of the immune system upon infection. Dendritic cells (DCs) play a central role in the early immune response against *B. burgdorferi*. We investigated the response of DCs to two different strains of *B. miyamotoi* using *in vitro* and *ex vivo* models and compared this to the response elicited by *B. burgdorferi.* Our findings show that *B. miyamotoi* is phagocytosed by monocyte derived-DCs, causing upregulation of activation markers and production of proinflammatory cytokines, in a similar manner to *B. burgdorferi.* Recognition of *B. miyamotoi* was demonstrated to be partially mediated by TLR2. DCs migrated out of human skin explants upon inoculation of the skin with *B. miyamotoi.* Finally, we showed that *B. miyamotoi*-stimulated DCs induced proliferation of naïve CD4+ and CD8+ T cells, to a larger extent than *B. burgdorferi.* In conclusion, we show here that DCs respond to and mount a immune response against *B. miyamotoi* that is similar to the response to *B. burgdorferi,* and are able to induce T cell proliferation.

Introduction

The tick-borne relapsing fever spirochete *Borrelia miyamotoi* was recently shown to be pathogenic (1-3). Similar to the Lyme disease agent, *Borrelia burgdorferi* sensu lato, *B. miyamotoi* infects *Ixodes* ticks in Europe, North America and Asia, however, the clinical presentation is more similar to other relapsing fever *Borrelia,* as symptoms are more systemic and viral-like and include high fever, headache, myalgia, arthralgia and malaise (3-5). The development of chronic meningoencephalitis has been described in three immunocompromised patients infected with *B. miyamotoi* (1, 2, 6). As yet, it is not clear what the public health burden of this disease is (7). Furthermore, as it has only recently become possible to cultivate *B. miyamotoi in vitro* (8), up to now very few *in vitro* studies have been performed and little is known about the immunopathogenesis of *B. miyamotoi*.

In contrast, *B. burgdorferi* has been studied extensively *in vitro* and much of the immunopathogenesis has been elucidated. Upon entry into the skin via tick bite, *B. burgdorferi* encounters dendritic cells (DCs); professional antigen presenting cells which orchestrate adaptive immune responses by presenting phagocytosed antigen to CD4+ T cells which they encounter upon migration into draining lymph nodes (9). DCs prime CD4+ T cells to become effector T helper (Th) cells, of which various subsets exist with each a different specialization, depending on the type of response needed to clear the pathogen (10). DCs have been shown to phagocytose and respond to *B. burgdorferi in vitro*, producing cytokines and upregulating activation markers (11). DCs recognize pathogens through pattern recognition receptors (PRR), which can recognize pathogen-associated molecular patterns expressed by pathogens. The

central PRR responsible for recognition of *B. burgdorferi* is Toll like receptor 2 (TLR2) (12, 13) that recognizes lipoproteins expressed on the outer surface of the spirochete. We have shown previously that, in an *ex vivo* human skin model, DCs migrated out of the skin in response to inoculation with *B. burgdorferi* in a TLR-2 dependent manner (14). In *in vitro* studies, DCs primed with *B. burgdorferi* were able to induce Th cells, and elicited a protective immune response *in vivo* in mice (15). *B. burgdorferi* induced mixed Th1/Th2 responses in mice infected through syringe inoculation (16).

It is not known whether *B. miyamotoi* is recognized by DCs and whether the spirochete elicits DC responses. Furthermore, the adaptive T cell responses that arise from DC-*B. miyamotoi* interactions have not been previously studied. In this study, using two laboratory strains of *B. miyamotoi* and *in vitro* (monocyte derived (mo)DC) and *ex vivo* (human skin) models, we demonstrated that human DCs phagocytose *B. miyamotoi* and become activated, migrate out of the skin and produce cytokines in response, in a manner similar to *B. burgdorferi*. *B. miyamotoi*-stimulated DCs were able to induce T cell proliferation. Unraveling the ways in which the immune system responds to and fights *B. miyamotoi* is crucial to our understanding of the disease and may help to explain why the clinical presentation of infection with *B. miyamotoi* differs from that of Lyme disease.

Materials and methods

Borrelia *culture*

Borrelia miyamotoi strains HT31 (Asian isolate) and LB-2001 (American isolate) were cultured in MKP-F medium (AMC, the Netherlands) (8) and *Borrelia burgdorferi* sensu stricto strain B31 was cultured in MKP medium (AMC, Amsterdam, The Netherlands) at 33°C until cultures reached the mid to late log growth phrase. Spirochetes were assessed for motility, quantified by dark-field microscopy and recovered by centrifugation at 4000 x g for 8 minutes. Subsequently, spirochetes were resuspended in Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco, Paisley, UK) supplemented with 10% foetal calf serum (FCS, Lonza, Verviers, Belgium) and L-glutamine (Gibco) (for *in vitro* experiments) or phosphate buffered saline (PBS) (Fresenius Kabi, Graz, Austria) (for *ex vivo* experiments). Spirochetes were passaged no more than 5 times prior to experimentation.

Monocyte derived dendritic cell (moDC) generation and stimulation

MoDCs were generated from peripheral blood mononuclear cells (PBMCs) from fresh heparinized blood donated by consenting, healthy volunteers as described previously (17, 18). A total of 1 x 10^5 moDCs in 100µl RPMI 1640 medium supplemented with 10% FCS and L-glutamine were stimulated in a 96-wells flat-bottom plate (Corning®, Sigma-Aldrich) for 48 hours at 37°C with 5% CO_2 with various multiplicities of infection (MOIs) of *Borrelia* strains, 10 ng/ml ultrapure Lipopolysaccharide (LPS) of *Escherichia coli* (Invitrogen, Paisley, UK) or 50 ng/ml Pam3CSK4 (Invivogen, San Diego, CA, USA). For lysate stimulation experiments, lysates of LB-2001 strains expressing vsp1 or vlpC2 were prepared as previously reported (19). In selected experiments 10 µg/ml TLR2-blocking αTLR2.5 (Ebioscience, San Diego, CA, USA) was used. Cells were washed and resuspended in FACS buffer (0.5% bovine serum albumin (BSA), 0.01% NaN_3

and 0.35 mM EDTA in PBS) and stained with fluorophore-conjugated antibodies against HLA-DR, CD83 or CD86 (BD Biosciences, Franklin Lakes, NJ) and flow cytometry was performed using a FACS-CANTO II (BD Biosciences). The results were analysed using Flow-Jo (Treestar, Ashland, OR) and Prism 7 (GraphPad Software, La Jolla, CA).

Phagocytosis

Phagocytosis experiments were performed in essence as previously described (17). Viable CFSE-stained *B. burgdorferi or B. miyamotoi* were incubated with moDC at 37°C at an MOI of 5 or 50 for various time points. Phagocytosis was stopped by adding ice-cold FACS buffer (0.5% bovine serum albumin (BSA), 0.01% NaN_3 and 0,35 mM EDTA in PBS) and putting the samples at 4°C. The cells were then washed and analysed with flow cytometry. The phagocytosis index was defined as the mean fluorescence intensity × fraction CFSE-positive cells, minus the 4 ◦C binding control.

Lymphocyte proliferation

PBLs were isolated from fresh heparinised blood donated by consenting, healthy volunteers by density centrifugation on Ficoll and Percoll (both GE healthcare, Chicago, IL), washed in PBS and labelled with 1 µM CFSE in PBS. Labelled PBLs were then co-cultured together with washed allogenic *Borrelia*-stimulated moDCs at DC:PBL ratios of 1:1, 1:2 and 1:5 in 96-wells flat bottom plates (Corning®, Sigma-Aldrich) in Iscove's Modified Dulbecco's Medium (IMDM, Gibco) with 10% FCS. After four days, supernatant was harvested and PBLs were stained with fluorophore-conjugated αCD4 and αCD8 antibodies (BD Biosciences) and analyzed by flow cytometry. For

each DC:PBL donor combination, the DC:PBL ratio which produced optimal proliferation results was selected and is shown in the results and analysis. *Borrelia*-stimulated moDCs were confirmed to have similar expression of maturation markers and cytokine production to earlier results prior to co-culture. All donors were seronegative, as measured by a commercially available *B. burgdorferi* C6 ELISA (Immunectis, XX, USA).

Ex vivo experiments

Ex vivo skin experiments were conducted as previously described (14, 18). Briefly, human surplus skin from breast or abdomen was obtained from healthy donors undergoing corrective surgery at the Kennemer Gasthuis Haarlem, the Netherlands. A total of 10^4 or 10^6 spirochetes or 100 ng/ml ultrapure LPS of *Escherichia coli* were injected intradermally in 50 ul PBS. Eight mm biopsies were taken immediately from the injection site and then washed in Iscove's Modified Dulbecco's Medium (IMDM) (Life Technologies, Paisley, UK) with 2% FCS, before being transferred into 1 ml IMDM with 10% FCS and L-glutamine - without antibiotics - and incubated for 48 hours at 37°C with 5% CO_2. Cells were harvested, washed twice, counted and resuspended in FACS buffer for staining with fluorophore-conjugated antibodies against HLA-DR, CD11c, CD83 and CD1a (BD Biosciences). Flow cytometry was performed as for moDCs. The number of DCs migrated per condition was calculated from the percentage of total migrated cells that were HLA-DR+/CD11c+. The supernatant and migrated cells from three separate biopsies, taken from random locations of the skin, were pooled together to constitute one replicate of each condition.

Cytokine measurement

Concentrations of the cytokines IL-1β, IL-6, IL-8, TNFα, IL-12p70 and IL-10 were measured in supernatant of 48h stimulated moDCs (1 x 10^5 in 100µl) or the supernatant of migrated DCs using a human inflammatory cytometric bead array (CBA) kit (BD Biosciences), according to the manufacturer's instructions. Results were analyzed using Flow-Jo and GraphPad Prism 7 software.

Statistics

Statistical analysis was performed with GraphPad Prism 7. Data was considered significant at $p < 0.05$, with one asterisk (*) representing $0.01 < p < 0.05$, two asterisks (**) representing $0.001 < p < 0.01$ and three asterisks (***) representing $0.0001 < p < 0.001$. One-way ANOVA and Tukey's post-hoc tests were used to analyze normally distributed data and Kruskal-Wallis and Dunn's post-hoc tests were used to analyse data that was not normally distributed.

Ethics statement

In vitro experiments were performed using surplus human healthy volunteer blood collected for the purpose of a separate study. Written informed consent was obtained from human blood donors in accordance with protocols approved by the Medical Ethics Committee of the Academic Medical Center (Approval number NL34294.018.10) and the declaration of Helsinki. As for the human skin, the Medical Ethical Committee of the Academic Medical Center, Amsterdam, deemed that no approval was necessary for the use of (otherwise) discarded tissue.

Results

B. miyamotoi is phagocytosed by DCs

To determine whether DCs are capable of phagocytosing *B. miyamotoi,* monocyte-derived DCs were incubated with CFSE-labelled strains HT31 and LB-2001 for various time points at 37°C. Uptake by DCs was analyzed by flow cytometry and compared to that of *B. burgdorferi* strain B31. The CFSE signal (fig 1a) and phagocytosis index (fig 1b) of DCs incubated with both *B. miyamotoi* strains increased over time, as similarly observed for B31, indicating that *B. miyamotoi* is phagocytosed by DCs, in a comparable manner to *B. burgdorferi*.

B. miyamotoi activates DCs to produce cytokines

The activation of DCs and production of cytokines are crucial for their function. DCs were stimulated with viable *B. miyamotoi* strains HT31 and LB-2001 and *B. burgdorferi* strain B31 for 48 hours at an MOI of 5 and 50 and maturation marker expression was measured by flow cytometry (fig 2a,b). Notably, expression of the maturation markers CD83 and CD86 was significantly upregulated by stimulation with *B. miyamotoi* strains at both MOIs, compared to unstimulated DCs, similarly to stimulation with LPS. The *B. burgdorferi* strain B31 induced CD83 expression at both MOIs, whereas CD86 was only significantly upregulated at the higher MOI. HLA-DR expression was only significantly induced by HT31 at an MOI of 50. Next we measured cytokine production by *B. miyamotoi*-stimulated DCs (fig 2c). Both *B. miyamotoi* strains as well as *B. burgdorferi* strain B31 induced IL-8, IL-6 and TNFα production at an MOI of 50. DCs stimulated with *B. miyamotoi* strains also produced IL-12, at significantly higher levels than B31-stimulated DCs, and HT31-stimulated DCs additionally produced significantly higher levels of IL-

176 1β than unstimulated DCs. These results indicate that *B. miyamotoi* strains are capable of
177 inducing DC activation and cytokine production in a similar manner to *B. burgdorferi.*

178 *B. miyamotoi* expresses variable major proteins (VMPs) – surface lipoproteins that form the
179 basis of the humoral immune response against the spirochete, and populations of spirochetes
180 have different VMP expression by spontaneous full gene switching in an attempt to evade
181 antibody responses. In a prelimary effort to explore whether VMPs affect the activation of DCs
182 by *B. miyamotoi* strains, we measured the maturation markers of DCs stimulated with lysates of
183 LB-2001 containing different VMPs. We selected an LB-2001 that expressed the surface
184 lipoprotein variable small protein 1 (Vsp1), an LB-2001 strain that had switched to express
185 variable large protein C2 (VlpC2) after challenge with αVsp1 antibodies, and an LB-2001 strain
186 challenged with αOVA (i.e. still expressing Vsp1) as a control. Our preliminary data show that all
187 three lysates (at a concentration of 10 ng/ml) induced upregulation of CD83 and CD86 and no
188 differences were observed in the expression of these markers between the different lysates,
189 suggesting that activation of DCs by *B. miyamotoi* is independent of the VMP expressed (data
190 not shown).

191 **DCs migrate out of the skin in response to inoculation of *B. miyamotoi***

192 To investigate whether *B. miyamotoi* activates DCs in a more physiological *ex vivo* model, we
193 inoculated human skin with HT31 or LB-2001, took biopsies of the inoculated skin and cultured
194 the biopsies in medium for 48 hours, and we analysed the number of migrated DCs. Migration
195 of DCs from skin inoculated with HT31 or LB-2001 was approximately 10-fold higher than
196 migration of DCs from PBS-inoculated skin, and similar to (i.e. not statistically different from)

197 the numbers of DCs migrated from B31-inoculated skin (fig 3a). Preliminary data indicate that,
198 as we have previously shown for *B. burgdorferi* [REF], that LCs consisted of the minory of
199 migrated cells upon *B. miyamotoi* inoculation, i.e. 0,5-2% (data not shown). In addition,
200 significantly higher levels of the proinflammatory cytokines IL-6 and IL-8, but not IL-1β or IL-10
201 were measured in the supernatants of *Borrelia*-inoculated biopsies compared to PBS-inoculated
202 biopsies (fig 3b). IL-12 and TNFα levels were under the detection limit. These results strongly
203 suggest that *B. miyamotoi* induces DC migration from the skin.

204 **DC responses to *B. miyamotoi* are partially mediated by TLR2**

205 To determine whether TLR2, the main PRR responsible for recognition of *B. burgdorferi,* is
206 involved in the recognition of *B. miyamotoi,* DCs were preincubated with or without a blocking
207 TLR2 antibody and stimulated with HT31 or LB-2001 for 48 hours. Notably, the antibody against
208 TLR2 strongly diminished the expression of CD83 induced by both strains (fig 4b). Moreover, the
209 TLR2 antibody suppressed CD83 upregulation induced by TLR2-agonist Pam3CSK4, but not that
210 induced by the TLR4-agonist LPS, indicating that the antibody is specific for TLR2 (fig 4a). CD86
211 upregulation in response to HT31 was also abrogated by the TLR2 antibody, and a trend was
212 observed towards lower CD86 expression in LB-2001 stimulated DCs, although this was not
213 significant. A trend toward lower production of the cytokines IL-12 and IL-6 (fig 4b) as well as IL-
214 8 and TNFα (data not shown), by *B. miyamotoi*-stimulated DCs preincubated with αTLR2 was
215 also observed, although not statistically significant. These results demonstrate that TLR2 plays a
216 role in DC recognition of *B. miyamotoi.*

B. miyamotoi–stimulated DCs are better inducers of PBL proliferation than *B. burgdorferi*-stimulated DCs

In order to explore the adaptive responses induced by *B. miyamotoi*-stimulated DCs, and compare these to DCs stimulated by *B. burgdorferi,* DCs stimulated with HT31, LB-2001 or B31 for 48 hours were co-cultured with allogenic CFSE-labelled PBLs for 4 days, after which time the CFSE signal of responder cells was measured by flow cytometry. Proliferating cells had a lower CFSE signal, as CFSE was halved between daughter cells during cell division (fig 5a). Significantly higher percentages of total PBLs, CD4+ and CD8+ T cells were induced to proliferate by HT31- and LB-2001-stimulated DCs compared to unstimulated DCs (fig 5b,c,d). The percentage of proliferating PBLs induced by *B. miyamotoi*-stimulated DCs was also significantly higher than that of *B. burgdorferi*-stimulated DCs, which did not induce significant proliferation compared to unstimulated DCs. These results demonstrate that DCs stimulated by *B. miyamotoi* are able to induce T cell proliferation.

Discussion

In this study, we investigated the response of DCs to *B. miyamotoi*, from recognition to induction of T cell responses. Uptake of pathogens and activation of DCs is the first step towards orchestration of adaptive immune responses. We demonstrated that DCs were able to phagocytose two different strains of *B. miyamotoi,* HT31 and LB-2001, in a manner similar to the *B. burgdorferi* strain B31. Most probably due to technical reason we were unable to show

phagocytosis in the ex vivo skin model (data not shown). After 48 hours of stimulation, DCs became activated, upregulating the costimulatory molecule CD86, necessary for T cell activation, and CD83 which characterizes DC activation. HLA-DR, the major histocompatibility complex II molecule which presents antigen to T cells was also upregulated in several experiments although this varied among donors. Proinflammatory cytokines IL-6, IL-8 and TNFα were produced by DCs in response to *B. miyamotoi* strains at high MOIs, as with B31. *B. miyamotoi* strains elicited a greater cytokine response than *B. burgdorferi,* as, unlike stimulation with B31, DCs stimulated with HT31 or LB-2001 additionally produced the Th1-inducing cytokine IL-12 and HT31 induced production of significantly higher concentrations of IL-1β, although a trend was also seen for LB-2001 and B31 and which may be significant at higher MOIs.

B. miyamotoi induced migration of DCs out of human skin explants in a more physiological *ex vivo* model of early *B. miyamotoi* infection. This result was comparable to previous work in which we have shown that DCs migrate out of skin in response to the spirochetes *B. burgdorferi* (14), *Treponema pallidum* and *Leptospira interrogans* (18). Spirochetes were inoculated intradermally in numbers comparable to those found in biopsies of erythema migrans, in order to mimic a tick bite (20). Due to variations in the skin used for experiments, i.e. thickness, region (breast or abdomen), age of donor we observed variability in the migration of DCs in response to stimulation. This variation is in line with our previous observations. As expected, no differences in the expression of activation markers was observed in DCs migrated from *B. miyamotoi* and PBS inoculated skin (data not shown) as DCs that have migrated – also in response to PBS – have a mature and activated phenotype. However, skin explants inoculated

with *B. miyamotoi* produced greater amounts of IL-6 and IL-8, whereas IL-1β and IL-10 production was not significantly induced after inoculation by either *B. miyamotoi* or *B. burgdorferi* compared to PBS, as we have observed in previous experiments with *B. burgdorferi* (14). Although it is likely that DCs are the main producers of these cytokines, alongside DCs, other possible sources for the cytokine production observed in inoculated skin are keratinocytes and fibroblasts (21, 22).

Our data strongly suggest that TLR-2 is involved in the recognition of *B. miyamotoi* by DCs, as blocking the receptor using an antagonistic TLR2 antibody impaired activation and cytokine production in response to *B. miyamotoi* stimulation. TLR2 recognises diacylated and triacylated lipopeptides by forming heterodimers with TLR1 and TLR6 (23, 24). Upon recognition, the Myeloid differentiation primary response 88-dependent activation of nuclear factor κB signaling cascade is induced, which triggers transcription of co-stimulatory molecules required for T cell activation, pro-inflammatory and anti-inflammatory cytokines, matrix metalloproteinases and adhesion molecules, which enable the maturation and migration of DCs (9). We did not observe a complete block of *B. miyamotoi*-induced DC activation and cytokine production upon blocking TLR2. This may be due to incomplete blocking by the antagonistic antibody, as we also observed residual cytokine production by DCs stimulated by the TLR2 agonist Pam3CSK4 in the presence of aTLR2. Another explanation could be that other PRR are involved in the recognition of *B. miyamotoi* alongside TLR2. Since other PRRs such as TLR5, 7,8 and 9 have been reported to recognize *B. burgdorferi* (9), they may be similarly implicated in recognition of *B. miyamotoi*.

As with other tick-borne relapsing fever spirochetes, *B. miyamotoi* expresses variable major proteins (VMPs) including Vlps and Vsps (25, 26), surface proteins that are the basis of the humoral immune response against relapsing fever spirochetes. The *B. miyamotoi* strain LB-2001 has recently been demonstrated to predominantly express Vsp1, with a minority population expressing VlpC2. After infection of a mammalian host, an IgM response is mounted against the predominantly expressed VMP, in this case Vsp1, however the minority populations expressing a different VMP are not affected and are able to evade the antibody response of the host, enabling relapses in spirochetemia to occur (19). Our preliminary data suggest that DCs become activated in response to LB-2001 strains expressing either VMP, indicating that they are capable of recognizing either or that other spirochetal proteins are driving the activation.

DCs stimulated with *B. miyamotoi* were able to elicit a T cell response, as we demonstrated proliferation of the CD4+ and CD8+ PBL fractions, as well as total PBLs using an allogenic MLR. This response was greater than that generated by *B. burgdorferi*-stimulated DCs. The reason remains to be investigated. Regardless, differences in DC-induced adaptive immune responses might explain why *B. miyamotoi* appears to be readily cleared after human infection - and relapses only seldomly occur - and *B. burgdorferi* is able to cause an chronic infection. Indeed, preliminary unpublished data indicate that *B. burgdorferi* was able to inhibit expression of genes involved in antigen presentation in monocytes to a greater extent than *B. miyamotoi* (personal communication Leo Joosten, Radboudumc). *B. miyamotoi*-stimulated DCs released IL-12, a Th1 polarizing cytokine. Of interest, Th1 and Th2 responses have previously been implicated in *B. burgdorferi* infection; IL-4 and IFN-γ were detected in the lymph nodes of mice infected with *B.*

burgdorferi (16), and Th1 responses have been observed in the joints and skin of Lyme borreliosis patients (27-29). Further studies should determine which subsets of Th cells are primed as part of the immune response to *B. miyamotoi.*

In summary, we have shown that *B. miyamotoi* strains HT31 and LB-2001 are phagocytosed by, and activate DCs, leading to upregulation of activation markers and production of proinflammatory cytokines in a manner similar to that of the *B. burgdorferi* strain B31. In addition, *B. miyamotoi* activated DCs to migrate out of human skin explants. Activation of DCs was found only to be partially dependent on TLR2. Interestingly, *B. miyamotoi*-stimulated DCs induced significantly more proliferation of CD4+ and/or CD8+ T cells compared to *B. burgdorferi*-stimulated DCs.

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References

1. Gugliotta, J. L., H. K. Goethert, V. P. Berardi, and S. R. Telford, 3rd. 2013. Meningoencephalitis from Borrelia miyamotoi in an immunocompromised patient. *N Engl J Med* 368: 240-245.
2. Hovius, J. W., B. de Wever, M. Sohne, M. C. Brouwer, J. Coumou, A. Wagemakers, A. Oei, H. Knol, S. Narasimhan, C. J. Hodiamont, S. Jahfari, S. T. Pals, H. M. Horlings, E. Fikrig, H. Sprong, and M. H. van Oers. 2013. A case of meningoencephalitis by the relapsing fever spirochaete Borrelia miyamotoi in Europe. *Lancet* 382: 658.
3. Platonov, A. E., L. S. Karan, N. M. Kolyasnikova, N. A. Makhneva, M. G. Toporkova, V. V. Maleev, D. Fish, and P. J. Krause. 2011. Humans infected with relapsing fever spirochete Borrelia miyamotoi, Russia. *Emerg Infect Dis* 17: 1816-1823.
4. Krause, P. J., S. Narasimhan, G. P. Wormser, L. Rollend, E. Fikrig, T. Lepore, A. Barbour, and D. Fish. 2013. Human Borrelia miyamotoi infection in the United States. *N Engl J Med* 368: 291-293.
5. Molloy, P. J., S. R. Telford, 3rd, H. R. Chowdri, T. J. Lepore, J. L. Gugliotta, K. E. Weeks, M. E. Hewins, H. K. Goethert, and V. P. Berardi. 2015. Borrelia miyamotoi Disease in the Northeastern United States: A Case Series. *Ann Intern Med* 163: 91-98.
6. Boden, K., S. Lobenstein, B. Hermann, G. Margos, and V. Fingerle. 2016. Borrelia miyamotoi-Associated Neuroborreliosis in Immunocompromised Person. *Emerg Infect Dis* 22: 1617-1620.
7. Wagemakers, A., P. J. Staarink, H. Sprong, and J. W. Hovius. 2015. Borrelia miyamotoi: a widespread tick-borne relapsing fever spirochete. *Trends Parasitol* 31: 260-269.
8. Wagemakers, A., A. Oei, M. M. Fikrig, W. R. Miellet, and J. W. Hovius. 2014. The relapsing fever spirochete Borrelia miyamotoi is cultivable in a modified Kelly-Pettenkofer medium, and is resistant to human complement. *Parasit Vectors* 7: 418.
9. Mason, L. M., C. C. Veerman, T. B. Geijtenbeek, and J. W. Hovius. 2014. Menage a trois: Borrelia, dendritic cells, and tick saliva interactions. *Trends Parasitol* 30: 95-103.
10. de Jong, E. C., H. H. Smits, and M. L. Kapsenberg. 2005. Dendritic cell-mediated T cell polarization. *Springer Semin Immunopathol* 26: 289-307.
11. Suhonen, J., J. Komi, J. Soukka, O. Lassila, and M. K. Viljanen. 2003. Interaction between Borrelia burgdorferi and immature human dendritic cells. *Scand J Immunol* 58: 67-75.
12. Hirschfeld, M., C. J. Kirschning, R. Schwandner, H. Wesche, J. H. Weis, R. M. Wooten, and J. J. Weis. 1999. Cutting edge: inflammatory signaling by Borrelia burgdorferi lipoproteins is mediated by toll-like receptor 2. *J Immunol* 163: 2382-2386.
13. Shin, O. S., R. R. Isberg, S. Akira, S. Uematsu, A. K. Behera, and L. T. Hu. 2008. Distinct roles for MyD88 and Toll-like receptors 2, 5, and 9 in phagocytosis of Borrelia burgdorferi and cytokine induction. *Infect Immun* 76: 2341-2351.
14. Mason, L. M., A. Wagemakers, C. van 't Veer, A. Oei, W. J. van der Pot, K. Ahmed, T. van der Poll, T. B. Geijtenbeek, and J. W. Hovius. 2016. Borrelia burgdorferi Induces TLR2-Mediated Migration of Activated Dendritic Cells in an Ex Vivo Human Skin Model. *PLoS One* 11: e0164040.
15. Mbow, M. L., N. Zeidner, N. Panella, R. G. Titus, and J. Piesman. 1997. Borrelia burgdorferi-pulsed dendritic cells induce a protective immune response against tick-transmitted spirochetes. *Infect Immun* 65: 3386-3390.
16. Christe, M., B. Rutti, and M. Brossard. 2000. Cytokines (IL-4 and IFN-gamma) and antibodies (IgE and IgG2a) produced in mice infected with Borrelia burgdorferi sensu stricto via nymphs of Ixodes ricinus ticks or syringe inoculations. *Parasitol Res* 86: 491-496.

17. Mason, L. M., E. A. Herkes, M. A. Krupna-Gaylord, A. Oei, T. van der Poll, G. P. Wormser, I. Schwartz, M. M. Petzke, and J. W. Hovius. 2015. Borrelia burgdorferi clinical isolates induce human innate immune responses that are not dependent on genotype. *Immunobiology* 220: 1141-1150.
18. Mason, L. M. K., and J. W. R. Hovius. 2018. Investigating Human Dendritic Cell Immune Responses to Borrelia burgdorferi. *Methods Mol Biol* 1690: 291-299.
19. Wagemakers, A., J. Koetsveld, S. Narasimhan, M. Wickel, K. Deponte, B. Bleijlevens, S. Jahfari, H. Sprong, L. S. Karan, D. S. Sarksyan, T. van der Poll, L. K. Bockenstedt, A. D. Bins, A. E. Platonov, E. Fikrig, and J. W. Hovius. 2016. Variable Major Proteins as Targets for Specific Antibodies against Borrelia miyamotoi. *J Immunol* 196: 4185-4195.
20. Liveris, D., G. Wang, G. Girao, D. W. Byrne, J. Nowakowski, D. McKenna, R. Nadelman, G. P. Wormser, and I. Schwartz. 2002. Quantitative detection of Borrelia burgdorferi in 2-millimeter skin samples of erythema migrans lesions: correlation of results with clinical and laboratory findings. *J Clin Microbiol* 40: 1249-1253.
21. Marchal, C. M., B. J. Luft, X. Yang, J. Sibilia, B. Jaulhac, and N. M. Boulanger. 2009. Defensin is suppressed by tick salivary gland extract during the in vitro interaction of resident skin cells with Borrelia burgdorferi. *J Invest Dermatol* 129: 2515-2517.
22. Schramm, F., A. Kern, C. Barthel, S. Nadaud, N. Meyer, B. Jaulhac, and N. Boulanger. 2012. Microarray analyses of inflammation response of human dermal fibroblasts to different strains of Borrelia burgdorferi sensu stricto. *PLoS One* 7: e40046.
23. Takeuchi, O., T. Kawai, P. F. Muhlradt, M. Morr, J. D. Radolf, A. Zychlinsky, K. Takeda, and S. Akira. 2001. Discrimination of bacterial lipoproteins by Toll-like receptor 6. *Int Immunol* 13: 933-940.
24. Takeuchi, O., S. Sato, T. Horiuchi, K. Hoshino, K. Takeda, Z. Dong, R. L. Modlin, and S. Akira. 2002. Cutting edge: role of Toll-like receptor 1 in mediating immune response to microbial lipoproteins. *J Immunol* 169: 10-14.
25. Barbour, A. G. 2016. Multiple and Diverse vsp and vlp Sequences in Borrelia miyamotoi, a Hard Tick-Borne Zoonotic Pathogen. *PLoS One* 11: e0146283.
26. Hamase, A., Y. Takahashi, K. Nohgi, and M. Fukunaga. 1996. Homology of variable major protein genes between Borrelia hermsii and Borrelia miyamotoi. *FEMS Microbiol Lett* 140: 131-137.
27. Gross, D. M., A. C. Steere, and B. T. Huber. 1998. T helper 1 response is dominant and localized to the synovial fluid in patients with Lyme arthritis. *J Immunol* 160: 1022-1028.
28. Salazar, J. C., C. D. Pope, T. J. Sellati, H. M. Feder, Jr., T. G. Kiely, K. R. Dardick, R. L. Buckman, M. W. Moore, M. J. Caimano, J. G. Pope, P. J. Krause, J. D. Radolf, and N. Lyme Disease. 2003. Coevolution of markers of innate and adaptive immunity in skin and peripheral blood of patients with erythema migrans. *J Immunol* 171: 2660-2670.
29. Yssel, H., M. C. Shanafelt, C. Soderberg, P. V. Schneider, J. Anzola, and G. Peltz. 1991. Borrelia burgdorferi activates a T helper type 1-like T cell subset in Lyme arthritis. *J Exp Med* 174: 593-601.

Figure legends

Fig. 1: DCs phagocytose *B. miyamotoi*

MoDCs were incubated with CFSE-stained *Borrelia* strains for various time points. a) The CFSE positive DC gate was determined based on the CFSE expression of a positive control of DCs incubated with mixed *Borrelia* compared to DCs alone (left histogram). The CFSE MFI of DCs increased over time for moDCs incubated with all three *Borrelia* strains at an MOI of 50 (right three histograms). b) The phagocytosis index ((% CFSE+ DCs x MFI at 37°C) minus (% CFSE+ DCs x MFI at 4°C)) increased over time for all three *Borrelia* strains, at an MOI of 5 and 50. Graphs show one representative experiment of two independent experiments.

Fig. 2: DCs become mature and produce cytokines in response to *B. miyamotoi*

MoDCs were stimulated with *Borrelia* strains for 48 hours. a) The MFI of maturation markers CD83, CD86 and HLA-DR increased on DCs stimulated with each of the three *Borrelia* strains at an MOI of 5 and 50 compared to unstimulated DCs. Histograms are from one respresentative experiment of eight. b) Fold difference in MFI of CD83, CD86 and HLA-DR compared to the unstimulated control increased on DCs stimulated with each *Borrelia* strain. Graphs show the mean (SEM) of eight independent experiments and donors. Asterisks denote significance of difference compared to unstimulated control. c) DCs stimulated with B31, HT31 or LB-2001 at an MOI of 50 produced higher amounts of IL-8, IL-6 and TNFα than unstimulated DCs. In addition, moDCs stimulated with the *B. miyamotoi* isolates HT31 and LB-2001 produced IL-12, at significantly higher levels than B31-stimulated moDCs. HT31-stimulated moDCs additionally secreted IL-1β. Graphs show the mean (SEM) of 8 independent experiments and donors. Asterisks denote significance of differences compared to unstimulated control.

Fig. 3: Skin DCs migrate out of the skin in response to inoculation with *B. miyamotoi*

Human skin was inoculated with *Borrelia* strains. a) Inoculation of HT31 or LB-2001 spirochetes induced migration of DCs out of the skin comparable to B31 and LPS injection and significantly greater than PBS injection. b) Skin injected with HT31 or LB-2001 produced significantly more IL-6 and IL-8 but not IL-1β or IL-10 than PBS-injected skin, as measured in the supernatant of biopsies. Graphs show one representative experiment of two independent experiments. Bars represent the SEM of triplicate measurements; each triplicate consists of three pooled biopsies. Asterisks denote significance of differences compared to PBS-injected skin.

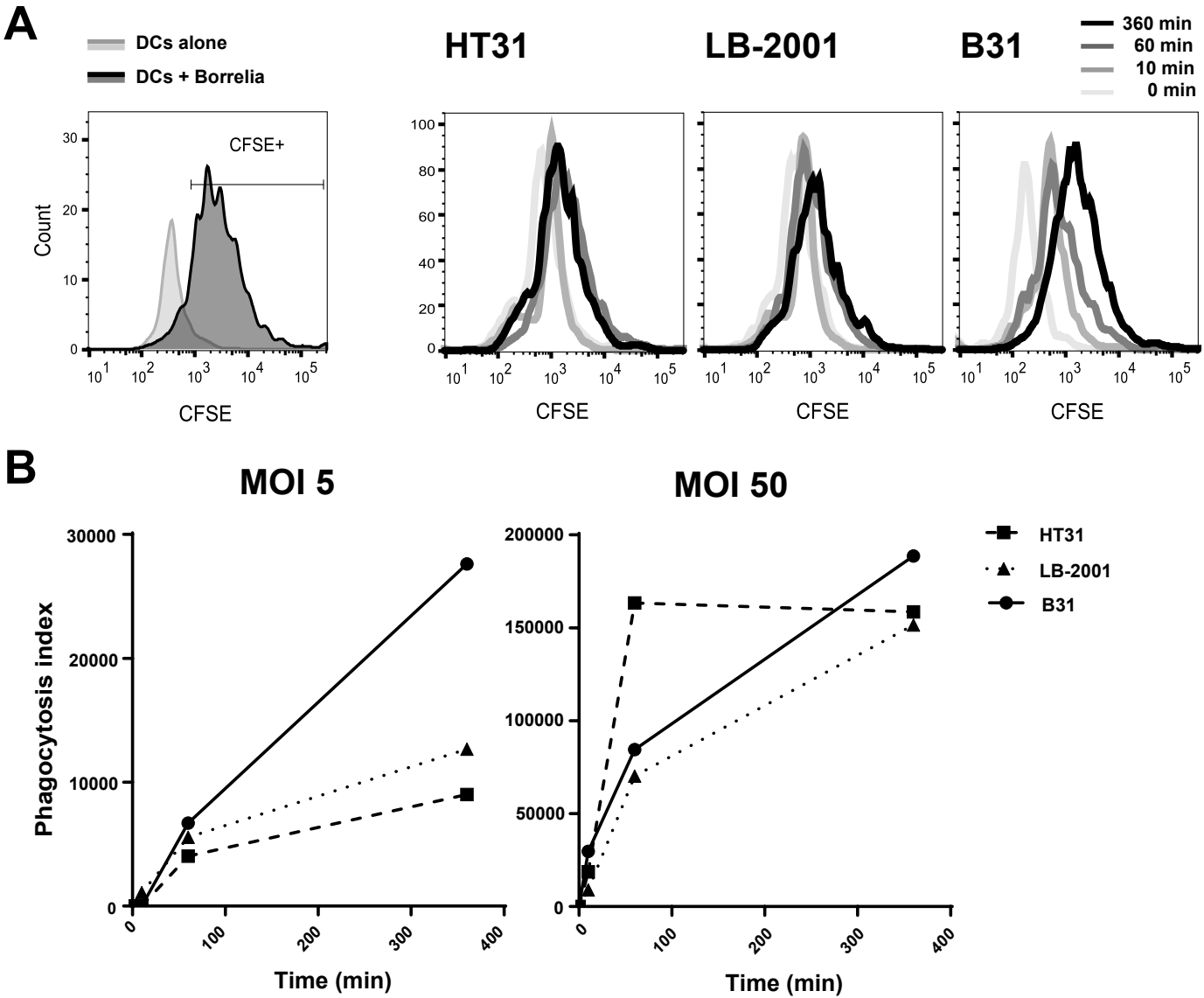
Fig. 4: Blocking TLR2 partially supresses DC responses to B. miyamotoi

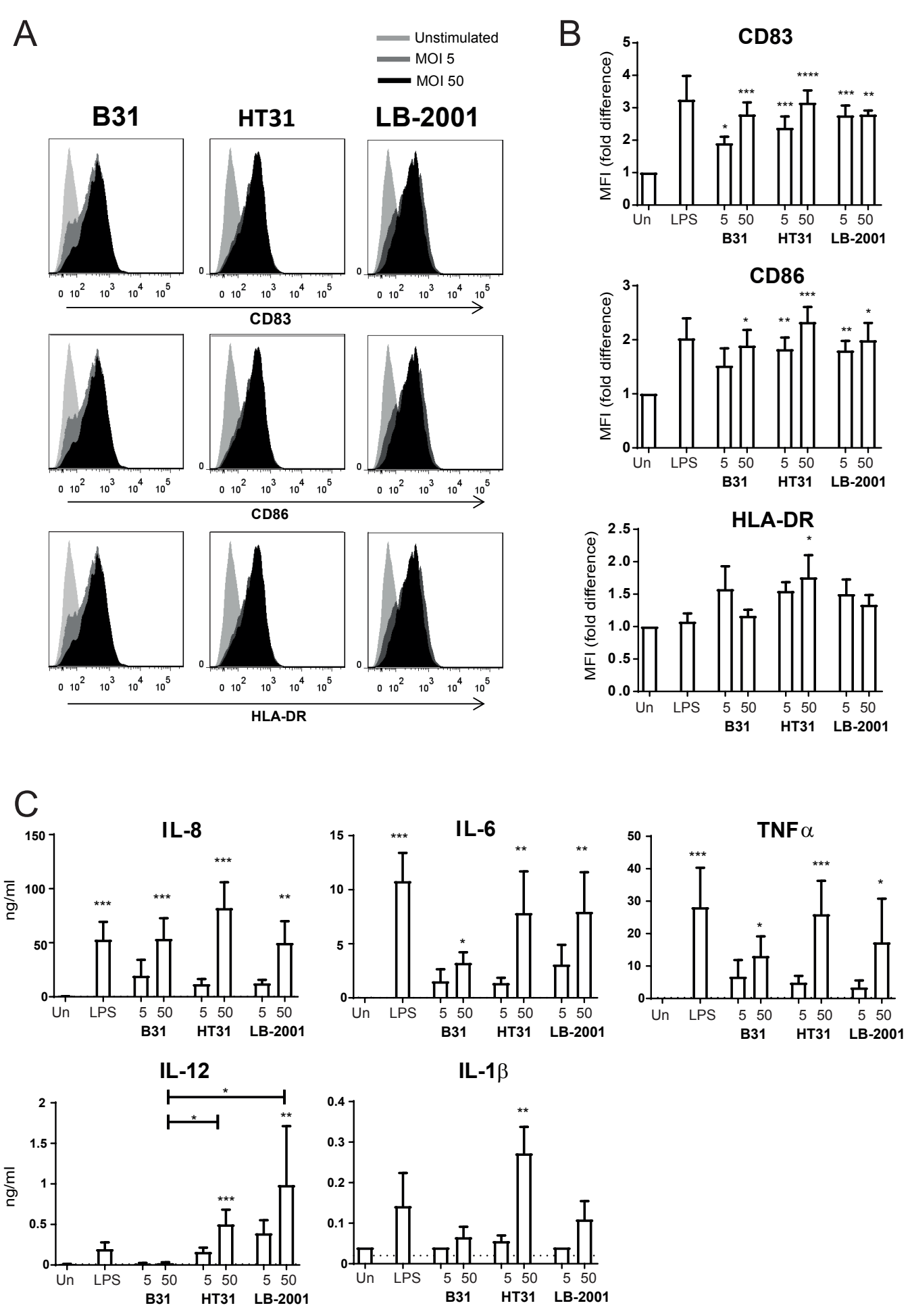
MoDCs were incubated with a blocking αTLR2 antibody prior to stimulation with *B. miyamotoi.* a) The increase in CD83 expression in response to HT31 or LB-2001 stimulation at an MOI of 5 or 50, and the increase in CD86 expression in response to HT31 stimulation, were impaired by preincubation with αTLR2. The response to LPS was unaffected, whereas CD83 upregulation by Pam3CSK4 (Pam) was abrogated by blocking TLR2. b) A trend was observed toward lower

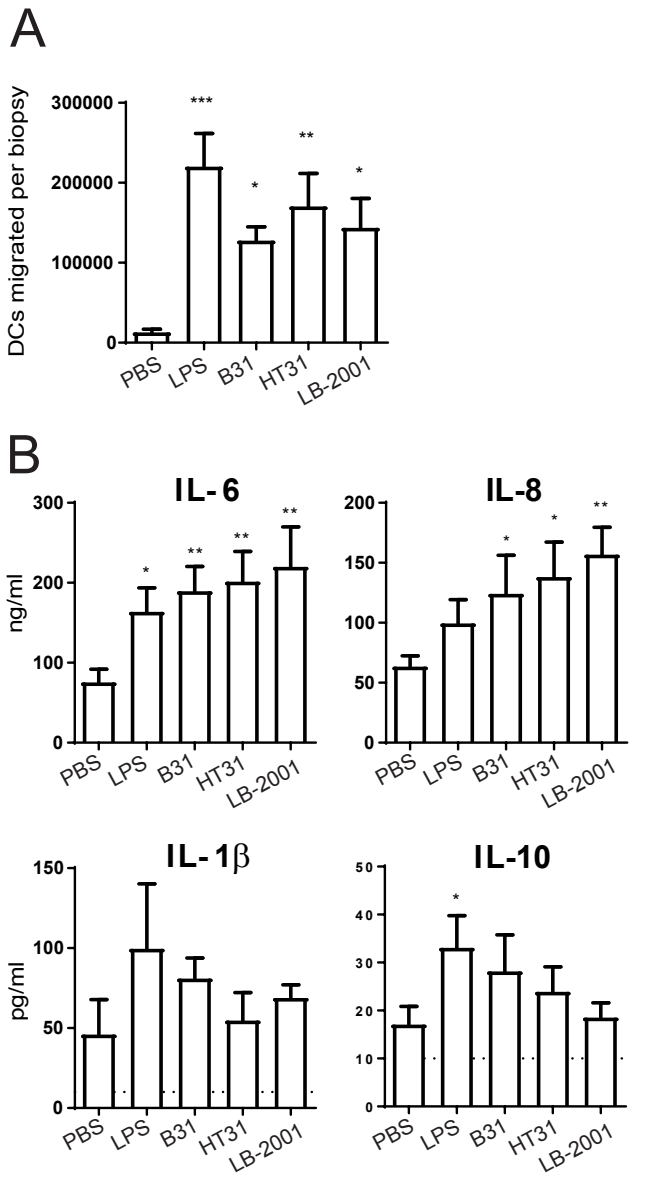
production of cytokines IL-12 and IL-6, as well as TNFα and IL-8 (not shown), in response to stimulation after preincubation with αTLR2. Graphs show one representative experiment of three independent experiments. Error bars represent SEM of triplicate measurements. Asterisks denote significant differences between stimulated moDCs with and without blocking TLR2, for each condition.

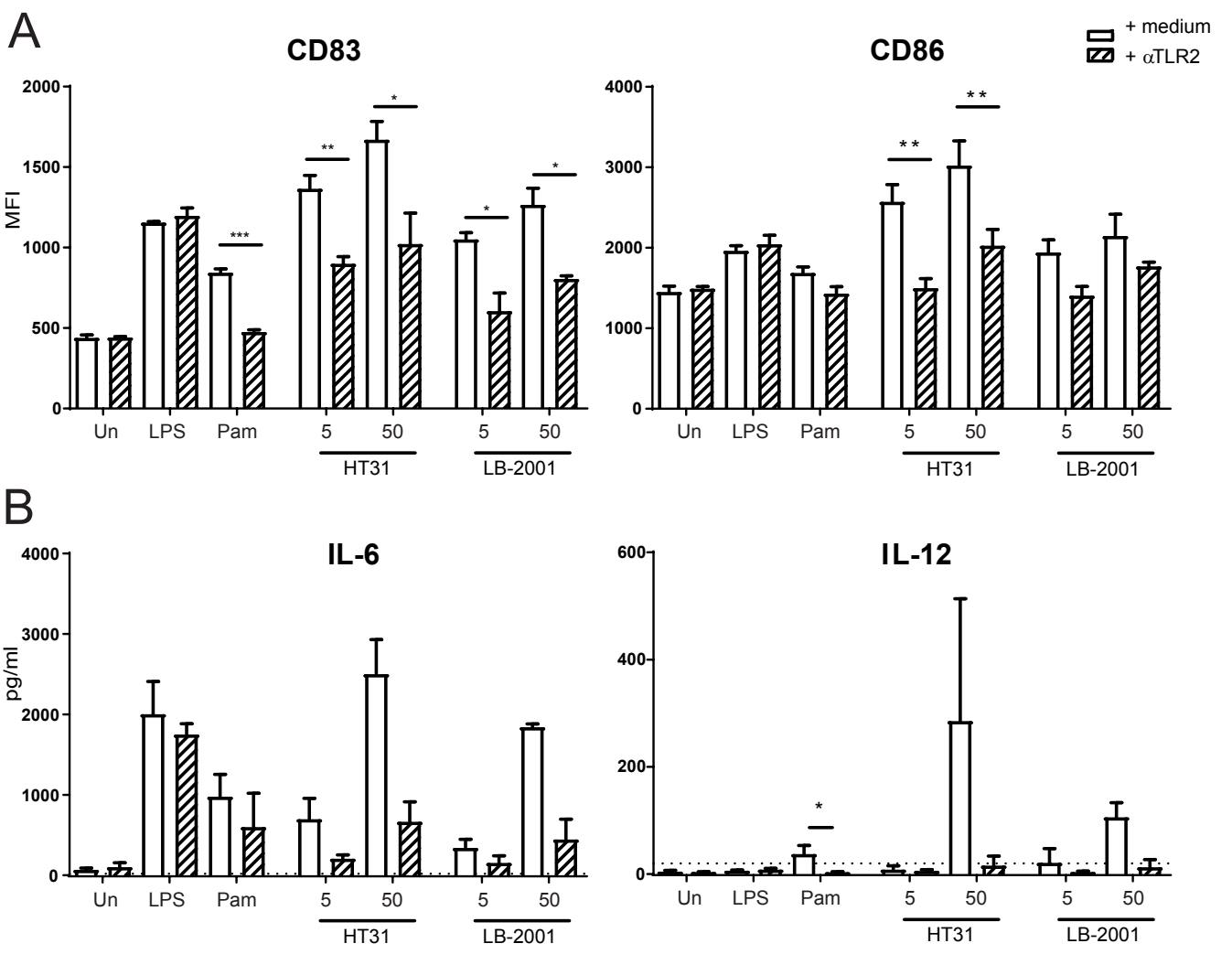
Fig 5: *B. miyamotoi*–stimulated DCs induce greater proliferation of PBLs than *B. burgdorferi*-stimulated DCs

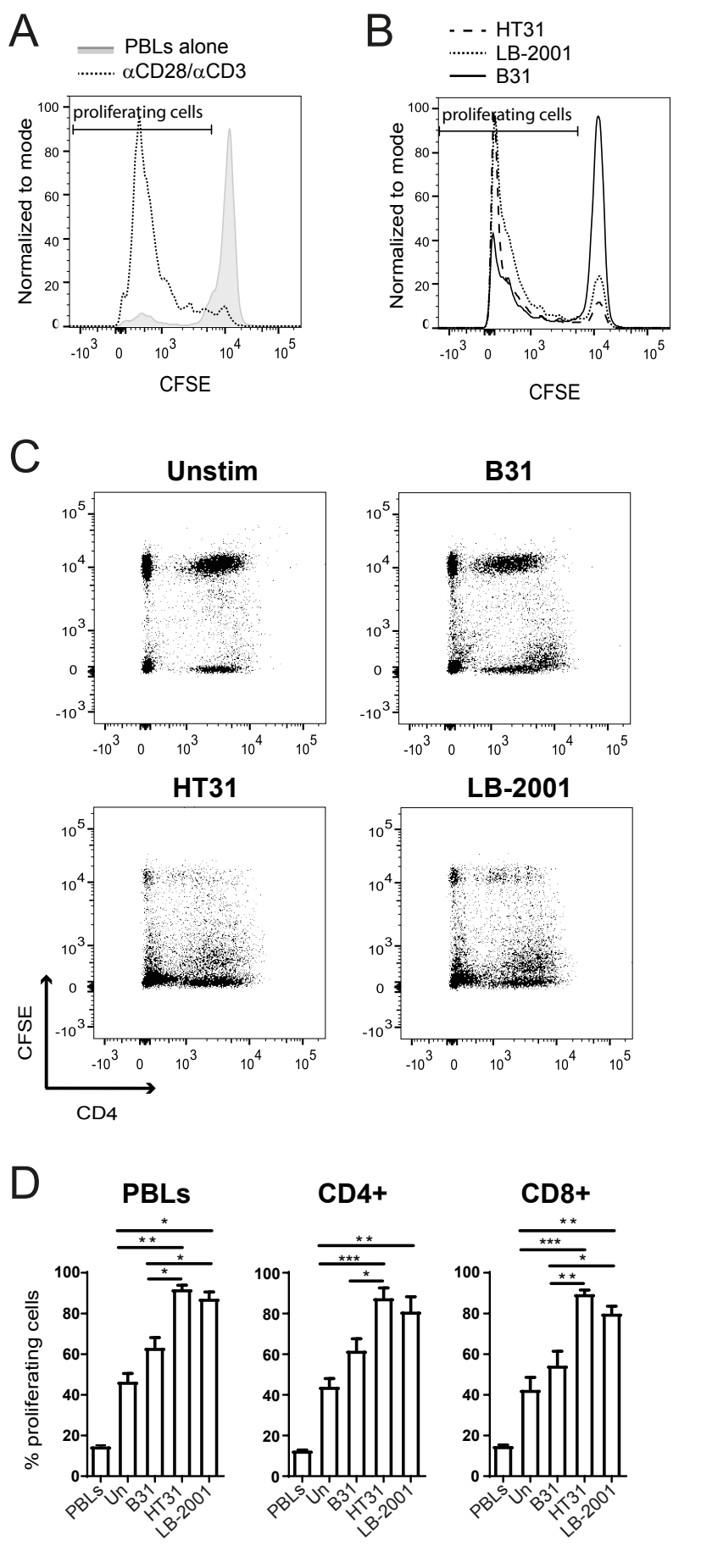
DCs stimulated with B31, HT31 and LB-2001 at an MOI of 50 for 48 hours were co-cultured with allogenic CFSE-stained PBLs for 4 days. a) The lower CFSE signal peak in proliferating PBLs stimulated with αCD28 and αCD3 compared to PBLs alone was used to determine the gate for proliferating cells. b) Higher percentages of PBLs proliferated in response to HT31- and LB-2001-stimulated DCs than B31-stimulated DCs. c) CD4+ T cells had a lower CFSE signal after PBL co-culture with HT31- and LB-2001-stimulated DCs. This was less pronounced after co-culture with B31-stimulated DCs. d) Pooled results of four experiments using different DC:PBL donor combinations revealed that a significantly higher percentage of PBLs were induced to proliferate by HT31- and LB-2001-stimulated DCs compared to unstimulated DCs. The percentage of proliferating PBLs induced by B31-stimulated DCs was not significantly different to unstimulated. This was also true for the CD4+ and CD8+ T cell populations of PBLs. Furthermore, HT31- and LB-2001 stimulated DCs induced significantly higher percentages of proliferating total PBLs and CD8+ T cells compared to B31-stimulated DCs and HT31-stimulated DCs induced a significantly higher percentage of proliferating CD4+ T cells than B31-stimulated DCs. Graphs show the mean (SEM) of pooled results of the best DC:PBL ratio of each of four independent DC:PBL donor combinations. Asterisks denote significance of difference compared to unstimulated control DCs.

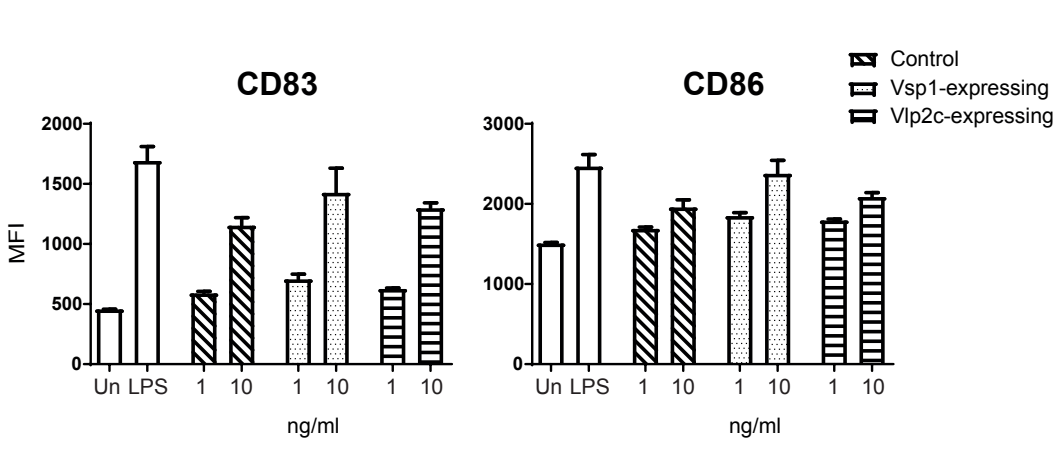

A
DCs alone
DCs + Borrelia
HT31
LB-2001
B31
360 min
60 min
10 min
0 min
CFSE+
Count
CFSE
B
MOI 5
MOI 50
HT31
LB-2001
B31
Phagocytosis index
Time (min)


A
Unstimulated
MOI 5
MOI 50
B31
HT31
LB-2001
CD83
CD86
HLA-DR
B
CD83
CD86
HLA-DR
MFI (fold difference)
Un
LPS
5
50
B31
HT31
LB-2001
C
IL-8
IL-6
TNFα
IL-12
IL-1β
ng/ml


A
DCs migrated per biopsy
300000
200000
100000
0
***
*
**
*
PBS
LPS
B31
HT31
LB-2001
B
IL-6
ng/ml
300
200
100
0
*
**
**
**
PBS
LPS
B31
HT31
LB-2001
IL-8
200
150
100
50
0
*
*
**
PBS
LPS
B31
HT31
LB-2001
IL-1β
pg/ml
150
100
50
0
PBS
LPS
B31
HT31
LB-2001
IL-10
50
40
30
20
10
0
*
PBS
LPS
B31
HT31
LB-2001


A
CD83
CD86
+ medium
+ αTLR2
MFI
2000
1500
1000
500
0
4000
3000
2000
1000
0
Un
LPS
Pam
5
50
HT31
LB-2001
B
IL-6
IL-12
pg/ml
4000
3000
2000
1000
0
600
400
200
0
*
**
***


A
PBLs alone
αCD28/αCD3
proliferating cells
Normalized to mode
CFSE
B
HT31
LB-2001
B31
proliferating cells
Normalized to mode
CFSE
C
Unstim
B31
HT31
LB-2001
CFSE
CD4
D
PBLs
CD4+
CD8+
% proliferating cells
PBLs
Un
B31
HT31
LB-2001


CD83
CD86
Control
Vsp1-expressing
Vlp2c-expressing
MFI
2000
1500
1000
500
0
3000
2000
1000
0
Un
LPS
1
10
1
10
1
10
ng/ml