

MyD88 negatively controls hypergammaglobulinemia with autoantibody production during bacterial infection

Running title: MyD88 controls hypergammaglobulinemia during bacterial infection

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Nonstandard abbreviations : BSK-H medium, Barbour-Stoenner-Kelly medium; CSR, class
20 switch recombination; Ds, day of sacrifice; IL-1R, Interleukine 1 receptor; IL18R,
Interleukine 18 receptor; RF, rheumatoid factor

Key words : B cell, bacterial infection, TLRs, Th1/Th2 cells, autoimmunity

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Abstract

25 A large body of evidence has convincingly shown that Toll like receptors are necessary
sensors for infections with pathogens, but their activation was also suggested to generate
autoimmunity. During experimental infections, the lack of these sensors or of their signaling
molecules should lead to a deficient immune response. We found out that MyD88, the major
adaptator of the Toll/IL1 receptor signaling pathway, ~~can actually acts~~ ~~acts in fact~~ as a
30 negative regulator of B cell function ~~in some settings~~. Indeed, ~~compared to wild type animals,~~
MyD88 deficient mice infected by *Borrelia burgdorferi* developed extreme
hypergammaglobulinemia ~~compared to wild-type animals~~, and high levels of IgM
autoantibodies. *In vivo*, cell transfer experiments and cell blocking assays showed that this
phenotype was not linked to the absence of MyD88 in B cells, but rather to CD4 T cell and
35 likely dendritic cell dysfunctions leading to a Th1 to Th2 cytokine switch. In addition, our
results suggest a relative defect in the Ig class switch recombination process since MyD88 KO
mice developed mostly IgM antibodies. Collectively, these data emphasize the complex role
of the Toll/IL1 receptor pathway in tuning the immune response against infection and
avoiding autoimmunity.

40 Introduction

Polyclonal lymphocyte activation is a general consequence of bacterial, viral and parasitic infections. Generally speaking, three main consequences associated with polyclonal lymphocyte activation have been described depending on the nature of the infectious agent : protection from infection, immunosuppression and autoimmunity. On one hand, this non-specific lymphocyte activation, largely exceeding the level of the specific anti-pathogen response, could control pathogen dissemination (33). On the other hand, it is able to inhibit the host specific response to lymphocytic choriomeningitis virus impairing virus neutralization (35), or to negatively influence the course of murine *Trypanosoma cruzi* infection (28). Finally, in humans, hypergammaglobulinemia and significant levels of autoantibodies, including Rheumatoid Factors (RFs) and antinuclear antibodies, are frequently described during the active phases of infectious states, sometimes leading to tissue damage. Experimentally, the mechanisms which govern pathogen-induced polyclonal B cell activation seem diverse, including direct mitogenic properties of the experimental pathogens (possibly via membrane bound Toll like receptors, but also via non variable regions of the membrane bound Ig) (36, 10, 2), as well as cognate CD4 T cell help induced by B cell presented pathogen derived peptides (14). When analysed, autoantibodies were frequently produced during the course of these experimental infections.

These general considerations could have important implications regarding the occurrence of autoimmune diseases. Indeed, in a scenario of a multistep process leading to overt autoimmunity, infection-induced polyclonal lymphocyte activation is regularly considered as an early candidate event that can drive autoreactive B lymphocytes into an affinity maturation pathogenic process (41). If this scenario is correct, uncontrolled non-specific B cell activation during infection could be harmful. However, to date, very little is known about the

mechanisms which control non-specific B cell activation during infection.

65 In order to understand the mechanisms of the autoreactive B cell tolerance breakdown, during
an experimental bacterial infection by *Borrelia Burgdorferi* (*B.burgdorferi*), we previously
designed experimental infections of self-reactive B cell Tg mice (42). We showed *in vitro* that
Toll like receptors (TLRs) expressed on B cells were implicated not only in the autoantibody
production but also in the non-specific B cell activation (42). We expected that, *in vivo*, TLR
70 deficient mice would have been devoided of such a non-specific B cell activation. However,
to our surprise, mice deficient in the major adaptor protein of the Toll/IL1 receptor signalling
pathway, MyD88, were found to exhibit dramatic non specific B cell activation,
hypergammaglobulinemia and high levels of autoantibodies during the course of
B.burgdorferi infection. Thus, MyD88 appears to control potentially harmful non-specific B
75 cell activation.

Materials and Methods

Mice

C57BL/6 mice were purchased from Harlan (Gannat, France). Four week-old MyD88 and TLR2 KO and heterozygous (+/-) mice, and ICE (Il-1 converting enzyme) KO mice (22) on C57BL/6 background were originally supplied from the CDTA institute (Orleans, France). Some MyD88 KO and MyD88 +/-, TLR2 KO and TLR2 +/- mice were directly used for experiments. MyD88 KO mice were bred and maintained on a C57BL/6 background. All mouse lines were housed in our Institute's animal facility, in isolator cages. MyD88 KO and MyD88 +/- were selected by PCR genotyping as previously described (23). *B.burgdorferi* inoculated mice and the non-infected controls were housed in the bacteriology Institute's animal facility. All animal experiments were performed with the approval of the "Direction départementale des services vétérinaires" (Strasbourg, France).

B. burgdorferi infection

The *B.burgdorferi* sensu stricto cN40 isolate was cultivated at low passage in Barbour-Stoener-Kelly (BSK-H) medium (Sigma) supplemented with 6% normal rabbit serum (Sigma) at 33°C. Four to five week-old mice were infected with 10⁶ spirochetes by intradermal injection of 0.1 ml in the shaved back skin. Control mice were injected with an equal volume of sterile BSK-H medium and housed in the same conditions as infected animals. Mice were sacrificed 3 to 4 weeks after inoculation. In one experiment, mice from 8 to 10 weeks of age were used, with results similar to those for 4 to 5 weeks of age (B and T cell status in MyD88 KO versus MyD88 +/- mice, infected and non infected animals, 3 mice in each group). The infectious status of the animals was evaluated by culture of different specimens (bladder, ear, heart, spleen) in 7 ml-tubes of BSK-H medium for up to 4 weeks at 33°C.

Quantitative PCR

DNA was extracted from the joints and the lymph nodes (LN) of individual mice on the MagNA Pure system (Roche Diagnostics, France) using the MagNA Pure LC DNA isolation large volume kit after external lysis by collagenase A and then proteinase K.

Quantification of *B.burgdorferi*-specific *fla* gene was done on the Light-cycler system (Roche Diagnostics, France). The primers used to amplify *fla* gene were those previously described (16). Quantification of mouse-specific *gapdh* gene was done on the ABI Prism 7000 instrument (Applied Biosystems, Courtaboeuf, France) using a commercial kit (taqMan rodent GAPDH control reagent, Applied Biosystems). External standards for *fla* and *gapdh* genes were developed as previously described (46). The number of *B. burgdorferi* in tissue specimens was calculated by comparing the crossing points of the samples with those of the standards and normalized to 10^4 of *gapdh* DNA.

Flow cytometry

Preparation of LN and spleen cell suspensions, their staining for flow cytometry, and the analysis of the B cell phenotype have been already described (20, 42). The T cells were monitored using double staining with anti-CD3_{FITC}, anti-CD4_{PE}, or anti-CD8_{FITC} (Pharmingen), or triple staining with anti-CD4_{PE}, anti-CD25_{biotin}, and anti-CD45RB_{FITC} Abs (Pharmingen) for regulatory T cells. Dendritic cells were identified by anti-CD11c_{biotin} (Pharmingen) staining. The expression analysis of the activation marker CD86 was performed using anti-CD86_{PE} Ab (Pharmingen). Non-viable cells were excluded and identified by incorporation of propidium iodide (PI, 5 mg/ml). The analysis was performed with a FACSCalibur using the CellQuest software package (BD Biosciences).

ELISA

Mice were bled by retro-orbital puncture under anesthesia. Sera were centrifuged (12', 10000

rpm) and stored at -20°C until ELISA analysis.

125 ELISA assays for serum IgM were done as described (10, 48).

For murine RF, plates were coated with total murine IgG (50 mg/ml, Jackson Immunosearch), and serum RF concentrations were revealed by adding a peroxidase coupled anti-mouse IgM.

For anti-dsDNA antibodies, calf thymus DNA was absorbed at 10 µg/l (Sigma); ssDNA was removed by digestion with S1 nuclease (100 UI/ml, Amersham) and bound antibodies were
130 revealed with an anti-mouse IgM-peroxidase (80 ng/ml, Jackson Immunosearch). For anti-thyroglobulin antibodies, plates were coated with thyroglobulin (50µg/ml, Sigma) and binding of antibodies was revealed by adding anti-mouse IgM peroxidase (80 ng/ml, Jackson Immunosearch). In each test (RF, anti-dsDNA, anti-tyrosine) serum from MRL/lpr mice was used as positive control; sera of MRL/lpr, MyD88 +/- and MyD88KO mice were tested using
135 1/100, 1/250, 1/500 and 1/1000 dilutions.

Mouse sera were tested for murine IgG antibodies to *B.burgdorferi* as previously described (42) Anti-mouse IgG1, IgG2b, and IgG3 (Jackson Immunosearch) were used to reveal the different Ig isotypes (1/2000 dilution).

For murine IgE, plates were coated with anti-murine IgE antibody (1µg/ml, Pharmingen),
140 then a biotin coupled anti-mouse IgE (0.5µg/ml, Pharmingen) was added and serum concentrations of IgE were revealed using a peroxidase coupled streptavidin (1/20000, Jackson Immunosearch).

ELISA assays for IL-12, IFN-γ, IL-4 and IL-10 were done using commercial kits (Bender Medsystems) as described by the manufacturer, on diluted sera (1/5, and 1/10 dilutions) and
145 culture supernatants.

Cell purification

Spleen or draining LN were harvested from 8-12 week-old mice.

Cells were isolated with a magnetic cell separation (MACS) depletion protocol (Miltenyi Biotec). Single cell suspensions ~~was~~ were depleted of non-B cells with anti-CD43 magnetic beads (Miltenyi Biotec). Purity of B cells was confirmed by staining with anti-CD19 Ab (more than 90% purity) and ~~staining~~ with anti-B220 Ab (more than 98% purity). T cells were purified ~~by removing non-T cells from~~ using a pan T cell isolation kit (anti-CD11b, anti-CD45R, anti-DX5 and anti-Ter119 negative sorting, Miltenyi Biotec). In all the experiments, more than 95% of recovered cells were CD3 positive.

B cell proliferation assays

Spleens or LN were removed from MyD88 KO or MyD88 +/- mice, or control C57BL/6 and teased apart. The proliferation assays were done as previously described (42). Cells (10^6) were incubated in 24-well BD Falcon plate (final volume of 1 ml) with one of the following reagents : 10 μ g/ml of LPS from *Salmonella typhosa* (Sigma), 10 μ g/ml of a F(ab')₂ goat anti-mouse IgM (Jackson ImmunoSearch), 10 μ g/ml of sonicated *B.burgdorferi*. After 60 hours of culture at 37°C, the phenotype of the cells was determined by flow cytometry analysis.

Preparation of sonicated *B.burgdorferi*

Sonicated *B.burgdorferi* was prepared as previously described (42). The protein content of the preparation was determined by Lowry assay.

B cell-depleted LN cells culture

LN cells from infected MyD88 KO or MyD88 +/- mice were depleted of B cells (20 days after infection) using anti-CD19 magnetic beads (Miltenyi Biotec). In each well, $7 \cdot 10^5$ of either MyD88KO or MyD88 +/- B-cell depleted LN cells were cultured in the presence of 10 μ g/ml sonicated *B.burgdorferi*. Unstimulated cultures were used as controls.

B and T cell transfer

μ MT mice were transferred intravenously with $5 \cdot 10^7$ MACS purified B cells 3 days before

B.burgdorferi infection. CD3εKO mice were transferred as μMT mice were, but with 2.10^7 MACS purified T cells.

Anti-CD4 treatment (8, 21, 42, 48)

175 Nondepleting mouse anti-CD4 antibody (YTS177.9.6.1) in ascite form diluted in PBS (50 μl ascite + 50 μl PBS per mouse per injection) was administered intraperitoneally 2 days before *B.burgdorferi* infection and then twice a week until sacrifice. Control animals were injected with PBS. CD4-T cell blockade was objectivized with a LN CD4-CD8 stain as previously described (48).

180 Dendritic cell differentiation from bone marrow cells

To obtain dendritic cells we used a modified version of an already described method (26). Bone marrow cells were cultured in bacterial Petri dishes (100 mm diameter, Falcon, No.1029) in RPMI 1640 supplemented with gentamicine (40 μg/ml, GibcoBRL), 10% FCS (GibcoBRL), β-mercaptoethanol (50 μM, Sigma), and GMCSF (20 ng/ml, Peprotech) for 9
185 days. Medium was changed on days 3 and 8. At day 6, half of the supernatant containing non-adherent cell was removed and centrifuged. The pellet was then resuspended in 10 ml culture medium and put back in a new Petri dish. After 9 days, 70% of bone marrow cultured cells expressed CD11c as dendritic cells do.

Dendritic cell stimulation

190 Dendritic cells ($0.5.10^6$ cells per culture well) were cultured for 24 hours with anti-CD40 antibody (10 μg/ml, Pharmingen), LPS (1 μg/ml, Sigma) or *B.burgdorferi* (2.5 and 1 μg/ml) without any GMCSF. Cells were then removed, centrifuged and stained for flow cytometry analysis.

B cell stimulation with IL-4

195 Purified B cells (anti-CD43 negative sorting, $0.5.10^6$ B cells per well) were cultured for 60

hours at 37°C in the presence of murine IL-4 (0.1 ng/ml, Roche Diagnostics) with or without sonicated *B.burgdorferi* (10 µg/ml). Unstimulated cells were used as controls.

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Results

200 *B.burgdorferi* infection in MyD88 knock-out (KO) mice

B.burgdorferi induces a chronic infection in many inbred mouse strains, including C57BL/6. This infection is characterized by chronic bacteremia, invading many tissues. The highest concentrations of spirochetes are found in ankle joints, heart and skin. Inflammation peaks about 2 to 4 weeks after infection and then regresses in the presence of *B.burgdorferi* specific
205 adaptive immune responses (6). As *B.burgdorferi* lipoproteins activate innate immune cells via TLRs, two recent studies have checked the importance of the adaptor molecule MyD88 in *B.burgdorferi* induced inflammation and infection resolution. MyD88 deficient mice developed carditis and arthritis as wild type mice did but with higher pathogen burden (7, 24). In the following experiments, 4 weeks-aged to 5 week old MyD88 KO mice on C57BL/6
210 background and MyD88 +/- littermates were injected with *B.burgdorferi*, or culture medium (BSK-H), and analysed mostly 3 to 4 weeks after infection. Two weeks after *B.burgdorferi* infection, MyD88 KO mice developed severe arthritis as frequently as MyD88 +/- mice, indicating that MyD88-dependent signalling is not required for the onset of *B.burgdorferi* induced disease. All the infected mice had positive cultures from at least one tissue sample
215 (heart, ear, bladder) at 4 wk. All the KO mice mounted an anti-*B.burgdorferi* IgG response as well as MyD88 +/- animals (table 1), as already described (7, 24).

Surprisingly, *B.burgdorferi* infection induces considerable B cell activation

B.burgdorferi spirochete and its lipoproteins have been shown *in vitro* to possess mitogenic
220 activity on mouse WT B lymphocytes (45, 27). *In vivo*, *B.burgdorferi* induces polyclonal hypergammaglobulinemia in which *B.burgdorferi* specific Ig represent only a minor fraction

(42, 7). Previous work suggests that TLRs, particularly TLR2 and TLR1, present on the B cell surface, could be involved (4, 13, 49). As most known mammalian TLRs, including TLRs1/2 complexes, signal through the adaptor protein MyD88, one could expect that non-specific B cell activation would be abolished or seriously impaired in *B.burgdorferi* infected MyD88 KO mice. Spleen and lymph nodes (LN) B cells from infected and non-infected MyD88 KO and MyD88 +/- mice were analysed by flow cytometry. Infection resulted in at least a three fold increase in LN B cell numbers in MyD88 +/- mice as compared to control mice injected with culture medium alone. However, LN B cell numbers of MyD88 KO infected mice reached at least 10 times the number of B cells in the non-infected animals (figure 1A, 1B, table 2). This increase concerned mostly IgM+/IgD+ mature B cells (table 2), as confirmed by IgM/IgD/CD21/CD23 staining profile (not shown), with an abnormal activation state (CD86 staining intensity, figure 1C). LN follicular organisation (follicles, mantle zones, germinal centers) appeared normal (not shown). By contrast, we found no significant modification in the splenic B cell populations in both MyD88 KO and +/- mice possibly reflecting the fact that the spleen retains only sporadically spirochetes (our culture results, not shown and Wooten et al. PCR results, 49).

We detected a strong IgM hypergammaglobulinemia in MyD88 KO animals. Consistent with previous work, *B.burgdorferi* infection resulted in a two to fourfold increase in the serum concentration of total IgM in MyD88 +/- infected animals as compared with MyD88 +/- uninfected controls. In MyD88 KO mice, this increase was dramatic, reaching 20 times the IgM levels of MyD88 KO non infected mice (figure 1D, non infected MyD88 +/- or MyD88 KO mice, n = 13, mean IgM level : 0.200 +/- 0.084 mg/l ; infected MyD88 +/- mice, n = 5, mean IgM level : 0.536 +/- 0.276 mg/l ; infected MyD88 KO mice, n = 8, mean IgM level : 3.946 +/- 2.302 mg/l). Contrasting with IgM production, the increase in IgG levels were

comparable in MyD88 KO and MyD88 +/- infected animals (figure 1E) even if the relative contribution of the IgG sub-classes was modified, suggesting that both T-dependent (IgG1, IgE) and to a higher degree T-independent (IgG3) responses were activated in the absence of MyD88 during *B.burgdorferi* infection (figure 2A, 2B). Accordingly, both IgG1 and IgG3 anti-*B.burgdorferi* responses were enhanced in infected MyD88 KO mice (figure 2C).

Recent evidence has demonstrated that MyD88 plays a significant role in the host defense to *B.burgdorferi*, with MyD88 deficient mice harbouring higher numbers of spirochetes in ankle joints, heart, and ears, 2, 4 and 8 weeks after infection compared to WT animals (7, 24). Thus, we wondered whether our results could be the consequence of a higher bacterial burden in MyD88 KO than in MyD88 +/- infected mice. We first used real time PCR to quantify the *B.burgdorferi* flagellin DNA (*fla*) in joints and LN of the infected mice. After 20 days of infection, the mean number of flagellin copies was indeed 100 times higher in MyD88 KO mice joints than in MyD88 +/- mice but the amount of *B.burgdorferi* in MyD88 KO and MyD88 +/- infected LN were moderate and similar (not shown). One could argue that B cells circulate and could encounter antigens, and/or could be sensitive to growth factors that are due to the high level of bacteria. However, *B.burgdorferi* infected TLR2 deficient mice have also been shown to harbour up to 20-fold more spirochetes in tissues than TLR2 +/- littermates (7, 49 and our own unpublished results) and IgM production in infected TLR2 KO animals was comparable to that seen in infected MyD88 +/- (WT) mice (figure 1D). Finally, we have injected increasing amounts of *B.burgdorferi* (10^3 to 10^7 spirochetes) in C57BL/6, TLR2 and MyD88 KO mice in order to get various bacterial levels by the end of the 20 day experiment. There was no strict correlation between the IgM production and *B.burgdorferi* bacterial burden (figure 3) but we could not rule out that the high pathogen burden in MyD88 infected KO mice, contribute to B cell hyperactivation.

***B.burgdorferi*-induced B cell activation in MyD88 KO mice includes autoreactive B cells**

Clinical observations and different experimental models show that autoreactive B cells can be activated during microbial infections. *B.burgdorferi* infected MyD88 KO mice produced high levels of several IgM autoantibodies like RF, anti-ds DNA and anti-thyroglobulin antibodies, with levels similar to those found in the lupus prone autoimmune strain MRL/lpr (figure 4). By contrast no IgG autoantibodies could be detected in their serum. MyD88 KO mice appeared healthy 4 weeks after infection, except for the induced arthritis. Likewise, 3 months after *B.burgdorferi* infection, MyD88 KO mice still showed B cell activation, IgM hypergammaglobulinemia and autoantibody production, with no sign of auto-immune disease, in particular no proteinuria (not shown). Together, these data suggest that these autoantibodies are natural autoantibodies.

In summary, although previous work strongly suggested the ability of *B.burgdorferi* to directly stimulate B cells via their TLRs, our results clearly indicate that the deficiency of the main adaptor of the TLR signalling leads to B cell expansion, IgM overproduction and essentially no class switch after *B.burgdorferi* infection. In order to explain this phenotype, we explored *in vivo* and *in vitro* different mechanistic hypotheses : intrinsic B cell defect, T cell and dendritic cell dysfunctions.

B cell hyperactivation in MyD88 KO mice during *B.burgdorferi* infection is not directly linked to an intrinsic B cell defect

Purified lymph node B cells were stimulated *in vitro* with sonicated *B.burgdorferi*. In keeping with previous results (42), *B.burgdorferi* directly stimulated MyD88 +/- B cells, as assessed by cell size, CD86 expression and CFSE staining intensity (figure 5A, B and C). However,

MyD88 KO B cells did not present any sign of activation in presence of *B.burgdorferi*,

295 although they did respond to BCR stimulation by an anti-IgM antibody.

In order to elucidate this apparent paradox, we tested B cell activation *in vivo* by cellular transfers. Purified B cells from MyD88 KO or MyD88 +/- animals were transferred in μ MT mice that lack mature B cells, on a C57BL/6 background. Figure 5D summarizes the results of the transfer experiments. After *B.burgdorferi* infection, MyD88 +/- B cells and MyD88 KO

300 B cells were similarly represented in this C57BL/6 non-MyD88 deficient environment. More importantly, we did not observe any increase in IgM production in animals transferred with MyD88 KO B cells, compared to animals transferred with MyD88 +/- B cells. On the contrary, MyD88 KO B cells were even less able to produce IgM than MyD88 +/- B cells suggesting that these cells are less responsive to *B. burgdorferi* when MyD88 is missing.

305 Taken together, *in vitro* and *in vivo* results show that MyD88 deficient non-B cell partner(s) is (are) necessary to drive the B cell hyperactivation in response to *B.burgdorferi* infection.

MyD88 KO T cells

Do MyD88 deficient T cells drive B cell hyperactivation ? As shown in table 2 and figure 6A,

310 *B.burgdorferi* infection induces a significant increase of LN CD4 and CD8 T cells, although much less important than the B cell increase, in MyD88 KO mice compared to MyD88 +/- mice. To study the role of CD4 T lymphocytes, we treated MyD88 KO mice with a non-depleting anti-CD4 mAb under conditions known to completely block CD4+ cells for 4 weeks (8, 21, 42, 48). As indicated in figure 6B, this treatment slightly reduced the number of LN B

315 cells in MyD88 KO infected mice, although not significantly. However, the IgM levels were drastically reduced in anti-CD4-treated MyD88 deficient mice (figure 6C). Thus, CD4 T cells strongly influence the hyperIgM production in MyD88 KO mice infected with *B.burgdorferi*.

We next wondered if the absence of MyD88 in T cells was responsible of the observed B cell phenotype in MyD88 KO infected mice ? Purified T cells from MyD88 KO or MyD88 +/- mice were transferred in T cell deficient animals (CD3 epsilon deficient mice) on a C57BL/6 background. Figure 6D summarizes the results of these transfer experiments. The absence or the presence of MyD88 in T cells did not influence the total number of B cells, nor the IgM levels in infected recipients. We conclude from these experiments that the hyperIgM observed in MyD88 KO infected mice is CD4 T cell-dependent, but independent of MyD88 signalling in T cells.

Recently, numbers of reports have suggested that CD4⁺ regulatory T cells could be involved in suppressing B cell functions (17, 37). However, our data do not support a role for defective MyD88 KO regulatory T cells in the activated B cell phenotype. Numbers of regulatory T cells, assessed by CD4⁺ CD25^{hi} CD45RB^{low} staining were similar in MyD88 KO and MyD88 +/- LN and increased in similar proportions with Bb infection (figure 6E and not shown). In addition, transfers systems of MyD88 KO or MyD88 +/- T cells included similar numbers of regulatory T cells with no influence on B cell activation.

MyD88 KO dendritic cells (DC)

TLR signalling activates DCs to secrete proinflammatory cytokines and up-regulate co-stimulatory molecule expression, thereby linking innate and adaptive immunity (5, 9, 19). It has also been suggested that during infections, DC could negatively regulate Th2 response in a MyD88 dependant manner (43).

Sera from infected and non-infected mice, were analysed for IL-4 (Th2 response), IL-12 and IFN- γ levels (Th1 response). IL-4 was not detectable. The results for IL-12 and IFN- γ dosages are shown in figure 7A. Non-infected MyD88 KO animals displayed significantly less IL-12

and IFN- γ and, in contrast to MyD88 +/- mice, *B.burgdorferi* infection did not increase the production of these cytokines. These results suggest that MyD88 KO mice are defective in Th1 cell differentiation, and that this defect is amplified during *B.burgdorferi* infection.

345 The Th2 differentiation of T cells leads to IL-4 production. In our model, we have tested the response of MyD88 +/- and MyD88 KO non-B cells to *B.burgdorferi*. LN from infected MyD88 KO animals were B-cell-depleted. The non-B cells, including T cells and antigen-presenting cells representing B cell environnement, were secondary stimulated *in vitro* with *B.burgdorferi*. As shown in figure 7B, and confirming previous data (24), supernatants
350 contained IL-4 but no IFN- γ and no IL-12, which was not the case in supernatants originating from cultures of MyD88 +/- non-B cells in the same conditions (figure 7C). Finally, we verified that IL-4 was indeed able to stimulate MyD88 KO B cells *in vitro* (figure 7D).

DC cell numbers in LN, based on CD11c expression, were similar in MyD88 +/- and MyD88 KO infected mice (not shown). *B.burgdorferi* infection is associated with an increase of the

355 CD86 activation marker expression on DC surface, in both MyD88 +/- and MyD88 KO mice (figure 7E). To further characterize MyD88 KO DCs response to *B.burgdorferi*, we generated mature DCs from bone marrow cultures with GM-CSF and analyzed the activation status of DCs in terms of the expression of the activation marker CD86 and IL12 production, with direct *B.Burgdorferi* stimulation. As a positive control, compared to MyD88 +/- DCs, MyD88

360 KO DCs cells upregulate CD86 in response to CD40 cross-linking but display an altered response to LPS as already described. The direct stimulation of MyD88 KO DCs with Bb does not upregulate CD86 (figure 7F), nor induces IL12 production (figure 7G), on the contrary to MyD88 +/- DCs. These results show that although DCs from infected MyD88 KO mice upregulate CD86 *in vivo*, they are not directly activated by *B.burgdorferi*. Thus, we can

365 speculate that a DCs defect in MyD88 KO mice might contribute to the Th1-Th2 imbalance.

Discussion

We have examined **the** polyclonal B cell activation induced by *B.burgdorferi* infection in MyD88 deficient mice and have made the ~~surprising and~~ important observation that it is dramatically increased, as compared to what is observed in heterozygous control animals. We have checked the level of the defect and found that it is not intrinsic to B cells, since 1) MyD88 deficiency rendered B cells unresponsive *in vitro* to *B.burgdorferi* and since 2) MyD88 KO and MyD88 +/- B cells behave similarly in a reconstituted WT environment. We also show that while CD4 cell function is required to drive polyclonal IgM overproduction, the MyD88 status of the T cells is not relevant. Finally, our experiments suggest that MyD88 KO DCs ~~are the most likely~~ **could be the source** of the expansion of B cells in **MyD88 KO infected mice** ~~and that MyD88 could act within DCs to restrict potentially pathological polyclonal expansion of B cells.~~

It has already been suggested that Th1 effector responses require TLR-mediated recognition and signalling, whereas Th2 responses were independent of the TLR pathway (34, 40). Our results agree with this view, and with data showing impaired IL-12 and INF gamma production in MyD88 KO mice during various experimental infections (11, 32, 39). At least two factors could be implicated in the activation of the CD4 Th2 cells in *B. burgdorferi* infected MyD88 KO mice : the lack of IL12 secretion and the production of IL-4 (30, 31). Indeed, it was proposed that, in absence of IL-12, the Th2 differentiation becomes a default pathway by derepression of GATA3, a key transcription factor for Th2 differentiation. In fact, DCs from MyD88 KO mice have lost the ability to produce proinflammatory cytokines, such as IL-12 in response to a large number of pathogen-associated molecular patterns (39, 40) and *in vitro* studies have suggested that MyD88 in DCs could be critical for Th cell differentiation (18, 40). On the other hand, IL-4, which is produced in minute amounts by naïve T cells and

390 activates T cells along an autocrine loop, also promotes GATA3 expression. Other possibilities must be considered, and among them the direct *B. burgdorferi* induced activation of the Notch pathway leading to a Th2 differentiation (3), a T-bet deregulation (25), and the direct activation by *B. burgdorferi* of an other MyD88 deficient non-lymphoid cells able to produce cytokines.

395 MyD88, originally described as a myeloid differentiation primary response protein, is an adaptor molecule in the IL-1R/TLR signalling by interacting with the Toll/IL-1R domain (15). Thereafter, IL-1R associated kinases (IRAK-1, IRAK-2, IRAK-M and IRAK-4) are recruited, and in turn recruit TNFR-associated factor 6 (TRAF-6), leading to the activation of NF- κ B and AP-mediated genes. In addition to its role in TLR activation pathway, MyD88 is
 400 an adaptor molecule for the IL-1 and IL-18 receptor signalling pathways (1). Responses of IL-1R deficient mice to several antigenic stimuli have already been described : IL1-R KO mice develop Th2-like response following infection with the Th1-inducing pathogen *L.Major* as well as after immunization with KLH, although the mechanisms of this Th2 shift are not clearly understood (38). IL-18 also stimulates Th1 differentiation, promoting IFN- γ , IL-12
 405 production and NK cell activation, this Th1 cell development being altered in IL18-R deficient mice (29). However the lack of *B. burgdorferi* induced hyper-IgM in ICE (IL-1 β converting enzyme, A. Woods, unpublished results) suggests that this phenomenon, hinges on a TLR pathway deficiency. Several MyD88 dependent TLRs are involved in the B cell response to *B.burgdorferi* (2, 4, 13, 49) : TLR2 via the recognition of lipoproteins, TLR5 and
 410 TLR9 via the bacterial flagellin and DNA, explaining that MyD88 deficiency abolishes direct activation of B cells and dendritic cells in response to *B.burgdorferi*. Indeed, the lack of hyper IgM in *B.burgdorferi* infected TLR2 KO mice could be the consequence of the binding of *B.burgdorferi* antigens to multiple TLRs. Thus, our results could reflect either 1) the blockade

of the MyD88 dependent TLR cascade or 2) the alteration of another unknown MyD88

415 dependant pathway (44). This issue will require further work.

Regarding the consequences of such high levels of polyclonal hypergammaglobulinemia in MyD88 KO infected mice, one could be worried about the possible occurrence of autoimmunity. These mice, compared to control WT mice, have increased levels of different autoantibodies including anti-DNA and RFs of the IgM class. The lack of autoantibody class
420 switching, combined with a possible lack of affinity maturation, could explain the absence of overt autoimmunity in these animals, at least within the time frame of our observation. In fact, compared to the IgM levels, the slightly increased level of total IgG in MyD88 KO *B.burgdorferi* infected mice, as previously described (7), is similar to the IgG levels of MyD88 +/- infected mice. What could be the interpretation of extremely high levels of IgM
425 with only slightly elevated IgG levels ? IgM overproduction in MyD88 KO infected mice is dependent on CD4 T cells and likely on IL4 production. Thus, the partial class switch defect is probably linked to CD4 T cell IL4 production without cognate T cell-B cell cooperation (which should have induced a more pronounced Ig switch) circumventing CD40L role. Also, these results may reflect a defect in the Ig class switch recombination (CSR) process in
430 MyD88 KO mice. Recently, in a mouse model of vaccination against human papilloma virus type 16 (HPV16), MyD88 KO mice developed only IgM. In this model, HPV16 caused naïve purified B cells to undergo CSR and express $\gamma 1$, $\gamma 2$, and $\gamma 3$ H chain transcripts in WT animals, but only the μ transcript was detected in MyD88 KO mice (46). However, MyD88 KO B cells still produced IgG in response to CD40 ligation and IL-4, demonstrating that they
435 are able to undergo CSR. Thus, in *B.burgdorferi* infected mice, CSR is partially allowed, and, owing to the Th2 profile, accounts for the slightly increased IgG1 production in MyD88 KO mice.

Our data contrast with those published recently by Pasare and Medzhitov (34) and with their conclusion that in addition to CD4+ T cell help, generation of T-dependent antigen specific antibody responses requires activation of TLRs in B cells. Neither T- dependent polyclonal nor specific antibody production is prevented by MyD88 deficiency in *B.burgdorferi* infection. However, Pasare et al. mostly considered anti-HSA response in MyD88 deficient B cells. Some differences in the results could be explained by the Ag and the condition of antigenic stimulation used (in our model, *in vivo* chronic bacterial infection).

In the end, we are left with a contrasting picture of MyD88's roles in this infectious model : on one hand, it controls the potentially harmful hypergammaglobulinemia and autoantibody production, on the other hand it authorizes the production of hazardous class switched autoantibodies. These results also alert to the relevance of therapeutic selective inhibition targeting TLRs signaling. Although TLRs have clearly been implicated in the genesis of autoimmune processes, the effect of the disruption of MyD88 interactions should be considered with caution (12).

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600 **Figure legends**

Figure 1

LN B cells in MyD88 KO infected mice compared with MyD88 +/- infected mice. B.burgdorferi infection is associated with drastic B cell activation and IgM production in
605 *MyD88 KO animals.*

A. Flow cytometry analysis of infected and uninfected mice lymph node cells. Viable lymphocytes were gated on forward scatter (FSC) and side scatter (SSC) parameters. B220/IgM+ staining reflects most B cells. Numbers indicate mean percentage for 5 (MyD88 +/-) to 8 mice (MyD88 KO).

610 **B.** Lymph node B220/IgM+ cells absolute numbers ($\times 10^6$) in infected (black bars) and uninfected (open bars) MyD88 +/- and MyD88 KO mice. Each value +/- SD represents the mean ratio from 5-8 mice. Statistical difference is designated by the symbol *, $P=0.0015$, Wilcoxon test.

C. Surface expression of CD86 on gated B220/IgM+ cells in MyD88 +/- and MyD88 KO
615 (thick line) compared to uninfected mice (thin line). Mean values for CD86 MIF ratios are : MyD88 +/- (n=5) $\times 1.3 \pm 0.29$, MyD88 KO (n=8) $\times 2.4 \pm 0.45$, $P<0.01$, Wilcoxon test.

D. Levels of serum IgM in infected MyD88 +/-, MyD88 KO and TLR2 KO mice compared to non-infected controls. IgM levels were measured by Elisa. The values represent the mean ratios +/- SD of the IgM concentrations between day of sacrifice (Ds : 3 to 4 weeks after
620 infection) and day 0 in infected mice (black bars) and uninfected mice (white bars). Numbers of MyD88 +/- and MyD88 KO tested animals are those represented in figure 1A. 3 TLR2 KO infected mice were compared with 2 non-infected controls. $P = 0.01$, Wilcoxon test.

E. Levels of total seric IgG in infected MyD88 +/-, MyD88 KO mice and TLR2 KO mice compared to non-infected controls. IgG levels were measured by Elisa. The values represent the mean ratios +/- SD of the IgG concentrations between day of sacrifice (Ds : 3 to 4 weeks after infection) and day 0 in infected mice (black bars) and uninfected mice (white bars).

Figure 2

IgG and IgE responses in B.burgdorferi infected MyD88 KO and MyD88 +/- mice, compared to non- infected controls

A. Levels +/- SD of total seric IgG1, IgG2b, IgG2c and IgG3 in infected (black bars) and non-infected mice (white bars). Numbers of mice are indicated in figure 2C. Sera were collected from mice 4 wks after infection (day of sacrifice : Ds). IgG isotypes were evaluated by Elisa.

B. Levels of IgE in infected and non-infected mice. 4 mice were tested in each group.

C. Effect of MyD88 deficiency on *B.burgdorferi*-specific IgG isotype distribution (anti-Bb Ig). Values represent the mean OD +/- SD observed for 1/200 serum dilution. Numbers of mice are indicated in each column. Statistical differences between the concentrations at the day of sacrifice (ds) of infected MyD88 KO and of MyD88 +/- mice are designated by the symbol ** P=0.001, and * P=0.03, Wilcoxon test. NS : no statistical difference.

Figure 3

IgM levels are not directly correlated to B.burgdorferi burden

MyD88 KO, TLR2 KO and C57BL/6 were inoculated with increased doses of *B.Burgdorferi* (10^3 to 10^7) to get a panel of bacterial burdens. Ankle tissues were assessed by quantitative PCR for *B.burgdorferi* DNA levels in mice sacrificed 20 days post-infection. Values reflect the

number of *B.burgdorferi fla* gene copies normalized per 10000 copies of a murine gene
(*gadph*)

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650 **Figure 4**

Induction of IgM autoantibody production after B.burgdorferi infection in Myd88 KO mice

Rheumatoid factors (RF), anti-dsDNA and anti-thyroglobulin IgM antibodies levels were measured by Elisa. Values represent the mean OD +/- standard deviation observed for the 1/100 (RF), 1/500 (anti-dsDNA) and 1/100 (anti-thyroglobulin) serum dilution respectively.

655 The same dilution for MRL/lpr pooled serum as for MyD88 +/- and MyD88KO mice was used as positive control. Five animals were tested in each group except for MyD88 KO infected mice (six animals).

Figure 5

660 *The absence of MyD88 in B cells is not sufficient to drive B cell hyperactivation in infected MyD88 KO mice. One or more MyD88 KO cellular partner appears necessary.*

(A-C). Purified B cells (anti-CD43 negative sorting) from MyD88 KO or MyD88 +/- pooled LN were cultured for 60h in the presence of sonicated *B.burgdorferi* (10 µg/ml) or anti-IgM (10 µg/ml). Unstimulated cells were used as controls (ns). It should be noted that unstimulated
665 purified B cells die in our culture conditions (60 h-culture). **A.** Side scatter versus forward scatter plots were used to monitor cell size. **B.** Histograms show proliferation of CFSE-labeled B cells with percentages indicating proliferating B cells. **C.** Surface expression of CD86 activation on B220/IgM+ cells.

D. Purified B cells (anti-CD43 negative sorting, $5 \cdot 10^7$ cells per mouse) from MyD88 +/- and
670 MyD88 KO mice were transferred to µMT mice (2 per group). After 24h, the recipient mice were infected with *B.burgdorferi* and analysed 3 weeks after infection for LN B220/IgM+ B cell percentages, CD86 expression on LN B cells and seric IgM and IgG levels.

Figure 6

675 Although being dependant on CD4⁺ T cell function, IgM hypergammaglobulinemia observed in infected MyD88 KO mice is not linked to the absence of MyD88 in T cells.

A. Numbers of CD4⁺ and CD8⁺ cells in LN of infected MyD88 KO (black) and MyD88 +/- (white) animals, determined by flow cytometry, relative to uninfected controls. Each point represents one infected animal compared to non-infected controls mean value.

680 B. MyD88 KO mice were injected intraperitoneally with anti-CD4 mAb (YTS177) to block CD4⁺ cells for at least 4 weeks (see methods). Each point represents a mouse. MyD88 KO infected and treated mice are compared with MyD88 KO infected and non-treated mice. Uninfected mice are shown as controls.

C. Serum IgM levels in anti-CD4 treated and untreated MyD88 KO mice, 4 weeks after infection with *B.burgdorferi*. Bars represent the mean ratios +/- SD of the IgM concentrations between day of sacrifice (Ds : 3 to 4 weeks after infection) and day 0 in anti-CD4 treated and untreated, uninfected (white) and infected (black) MyD88 KO animals. Differences between anti-CD4 treated and untreated MyD88 KO infected mice are significant (*, P =0.009, Wilcoxon test)

690 D. Purified T cells (anti-CD11b, anti-CD45R, anti-DX5 and anti-Ter119 negative sorting, 20.10⁶ cells) from MyD88 +/- and MyD88 KO mice were transferred to CD3εKO mice (3 and 4 per group). After 24h, the recipient mice were infected with *B.burgdorferi* and analysed 3 weeks after infection. All mice displayed significant numbers of transferred CD4 T cells (6%) in LN. The basal level of IgM in non infected CD3 KO animals was 342 +/- 10 µg/ml.

695 E. Regulatory T cells in uninfected MyD88 KO and MyD88 +/- mice. LN cells were gated on FSC and SSC parameters. Numbers indicate percentage of CD25^{high}CD45RB^{low} cells among CD4⁺ cells.

Figure 7

700 *Th1-Th2 cytokine imbalance and dendritic cells could drive B cell activation and IgM production in vivo in MyD88 KO infected mice*

A. Seric IL-12 and IFN- γ were measured by ELISA in infected (black symbols) and uninfected (white symbols) MyD88 KO and MyD88 +/- mice and compared to detectability threshold of the Elisa method used (black line, 15.6 pg/ml and 16 pg/ml respectively).

705 Numbers of mice were : MyD88 +/- uninfected n=5, MyD88 +/- infected n=10, MyD88 KO uninfected n=8, MyD88 KO infected n=8.

B, C. B cell depleted MyD88 KO (B) or MyD88 +/- (C) *in vivo* activated LN cells (anti-CD19 negative sorting) were cultured in the presence of sonicated *B.burgdorferi* (10 μ g/ml). Supernatants were tested for IL-12, IFN- γ , and IL-4 levels by Elisa. nd : non detectable.

710 D. Purified LN MyD88 KO B cells were cultured during 60h with sonicated *B.burgdorferi* (10 μ g/ml), IL-4 (0,1 ng/ml) or both; diagrams show expression of CD86 in stimulated (thick line) and control unstimulated cells (thin line).

E. Expression of CD86 on dendritic cells in MyD88 KO and MyD88 +/- infected mice. histograms show CD86 staining on CD11c positive cells from infected (thick line) and 715 uninfected (thin line) mice.

F. Bone marrow derived dendritic cells from MyD88 +/- and MyD88 KO mice were cultured for 60h with anti-CD40 Ab (10 μ g/ml), LPS (1 μ g/ml) or *B.burgdorferi* (1 or 2,5 μ g/ml). Diagrams show expression of CD86 on stimulated (thick line) and unstimulated (thin line) CD11c+ cells.

720 G. After stimulation depicted in (F), IL-12 concentration was evaluated in the supernatants by ELISA. n.d : non detectable.

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Table 1.

***B. burgdorferi* infection in MyD88 KO mice and controls**

725

Mouse strains	Arthritis Prevalence	Positive culture for <i>B. burgdorferi</i>	Anti- <i>B. burgdorferi</i> IgG
MyD88 KO	4/8	8/8	8/8
MyD88 +/-	2/5	5/5	7/7

Mice were infected with 10⁶ *B. burgdorferi* and necropsied 3 to 4 weeks post infection. Arthritis was considered as an early marker of infection. Anti-*B. burgdorferi* IgG response was considered positive when OD values reached background + 0,2.

730

Table 2.**LN lymphocytes in MyD88 KO and MyD88 +/- uninfected and infected mice**

	LN cell number (10⁶)	B220+/IgM+ (10⁶)	IgM+/IgD+ (10⁶)	CD4+ (10⁶)	CD8+ (10⁶)
MyD88 +/- uninfected	4+/-1.3	1.1+/-0.43	0.9+/-0.60	1.5+/-0.50	1.0+/-0.43
MyD88 +/- infected	13+/-8.2	7.5+/-3.27	6.6+/-3.14	4.9+/-1.75	3.0+/-1.07
MyD88 KO uninfected	5+/-2.6	1.5+/-0.80	0.9+/-0.58	1.7+/-0.92	1.2+/-0.73
MyD88 KO infected	46+/-14.6	28.3+/-7.55	23.1+/-3.82	11.4+/-4.35	7.7+/-1.22

Results are expressed as the mean +/- SD for groups of 5 to 8 mice. Cellular phenotypes were assessed as described for Figure 1 and Figure 6.

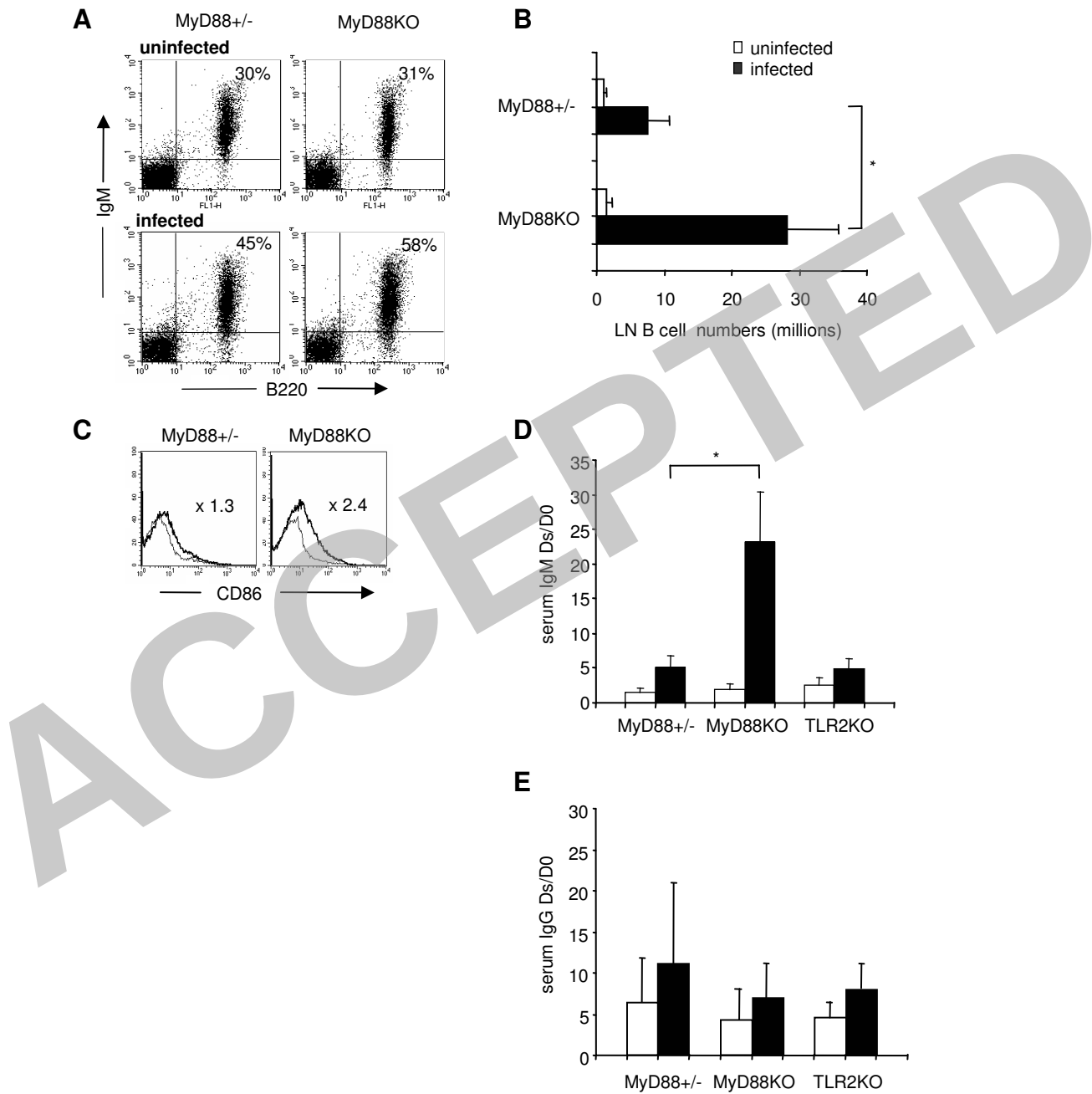


Figure 1

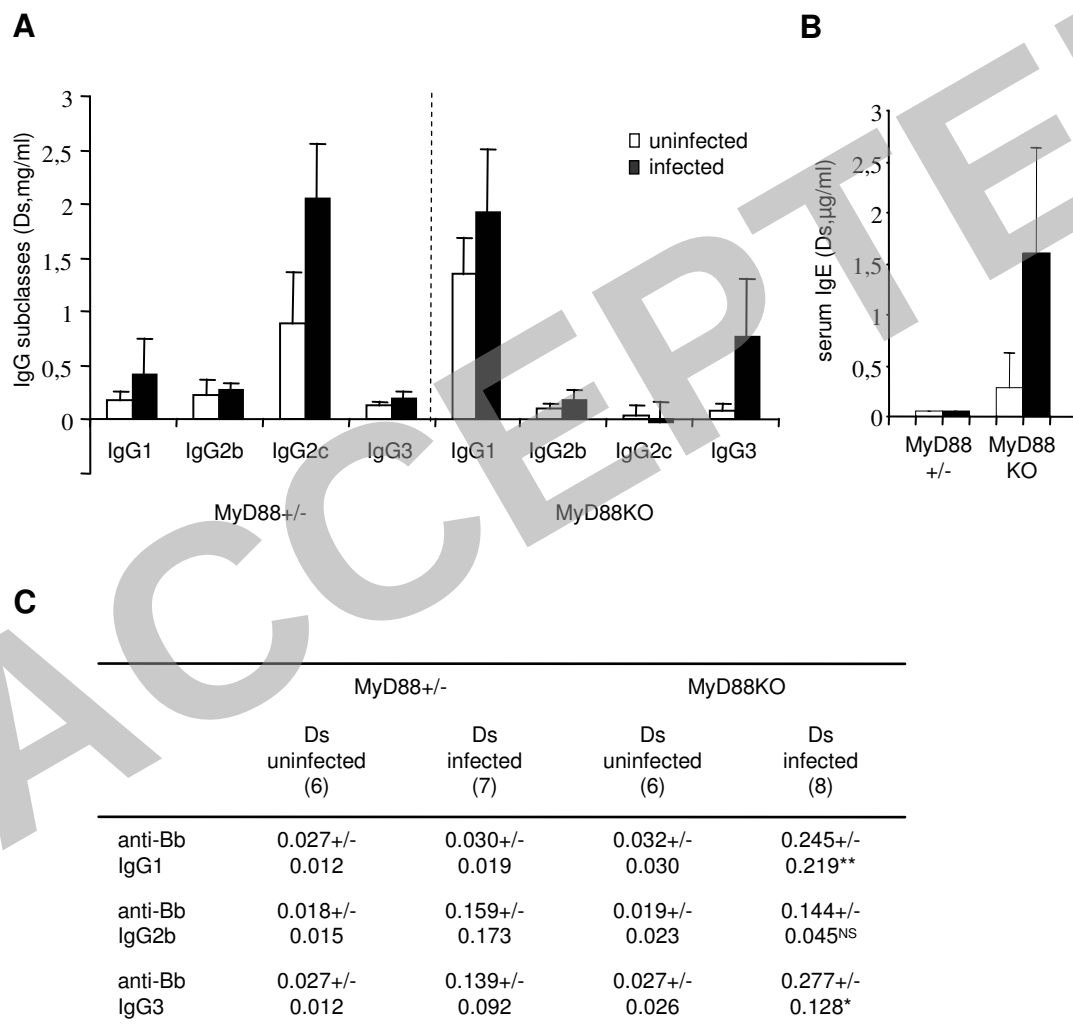


Figure 2

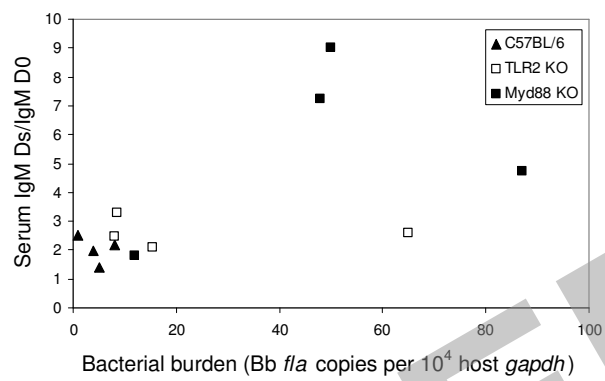


Figure 3

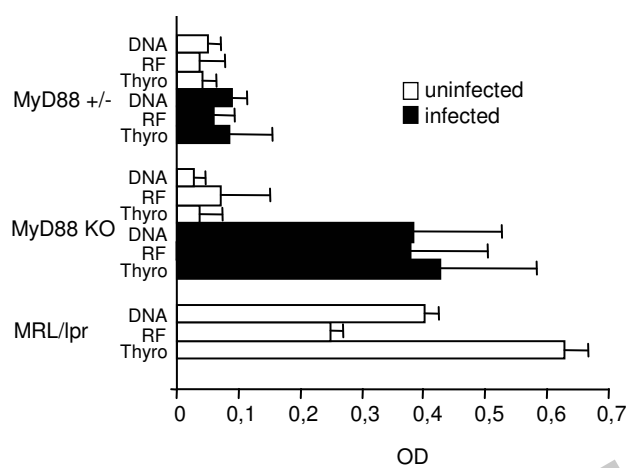


Figure 4

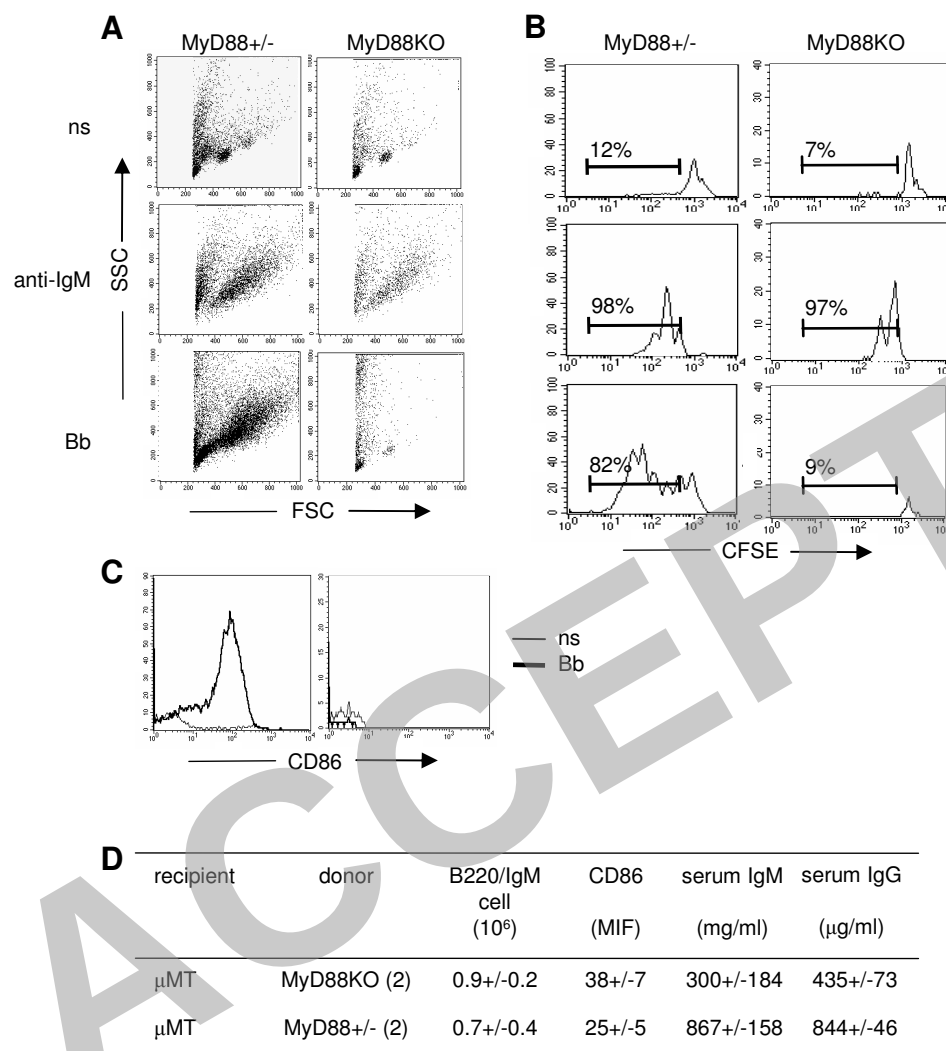


Figure 5

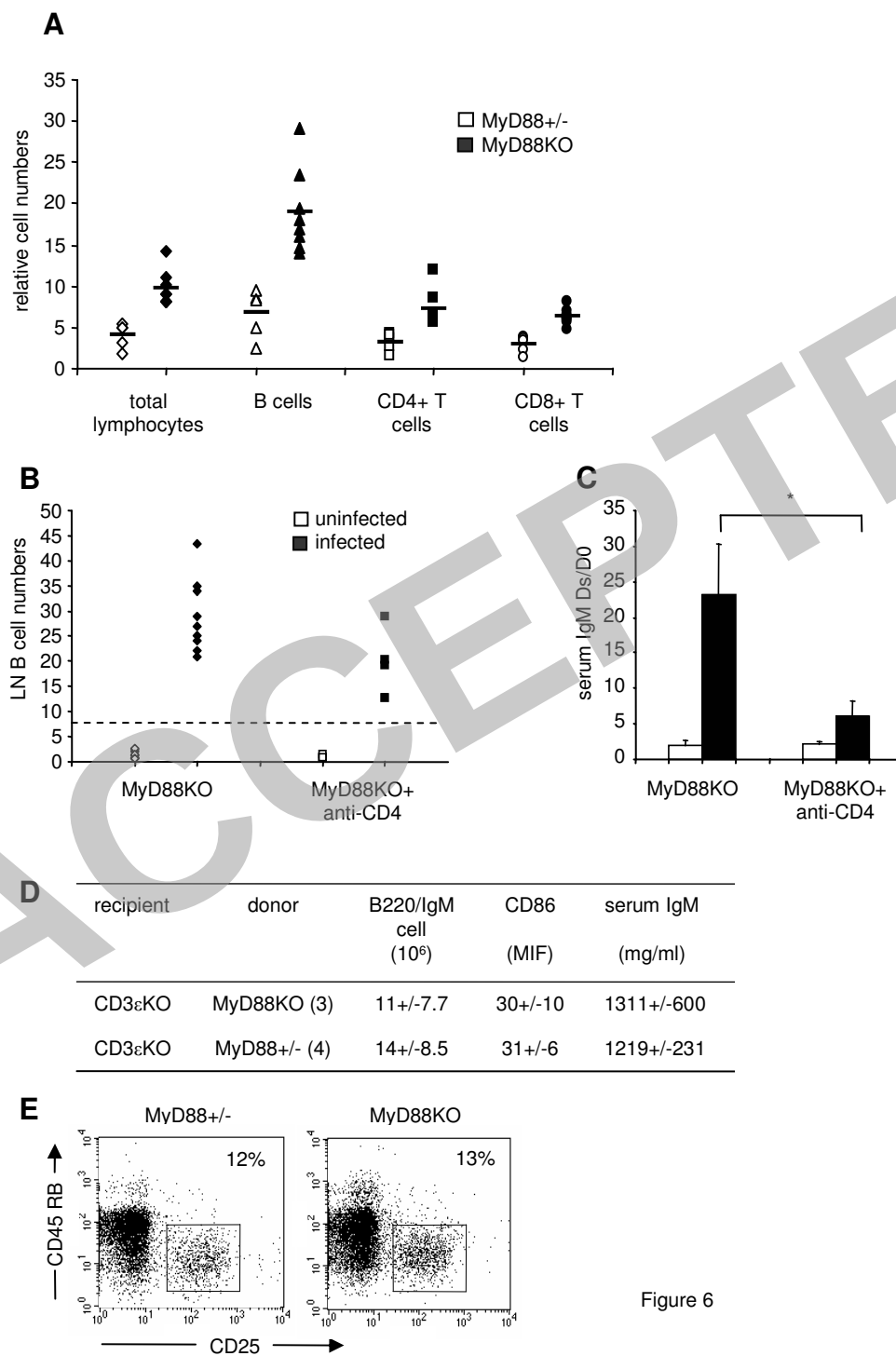


Figure 6

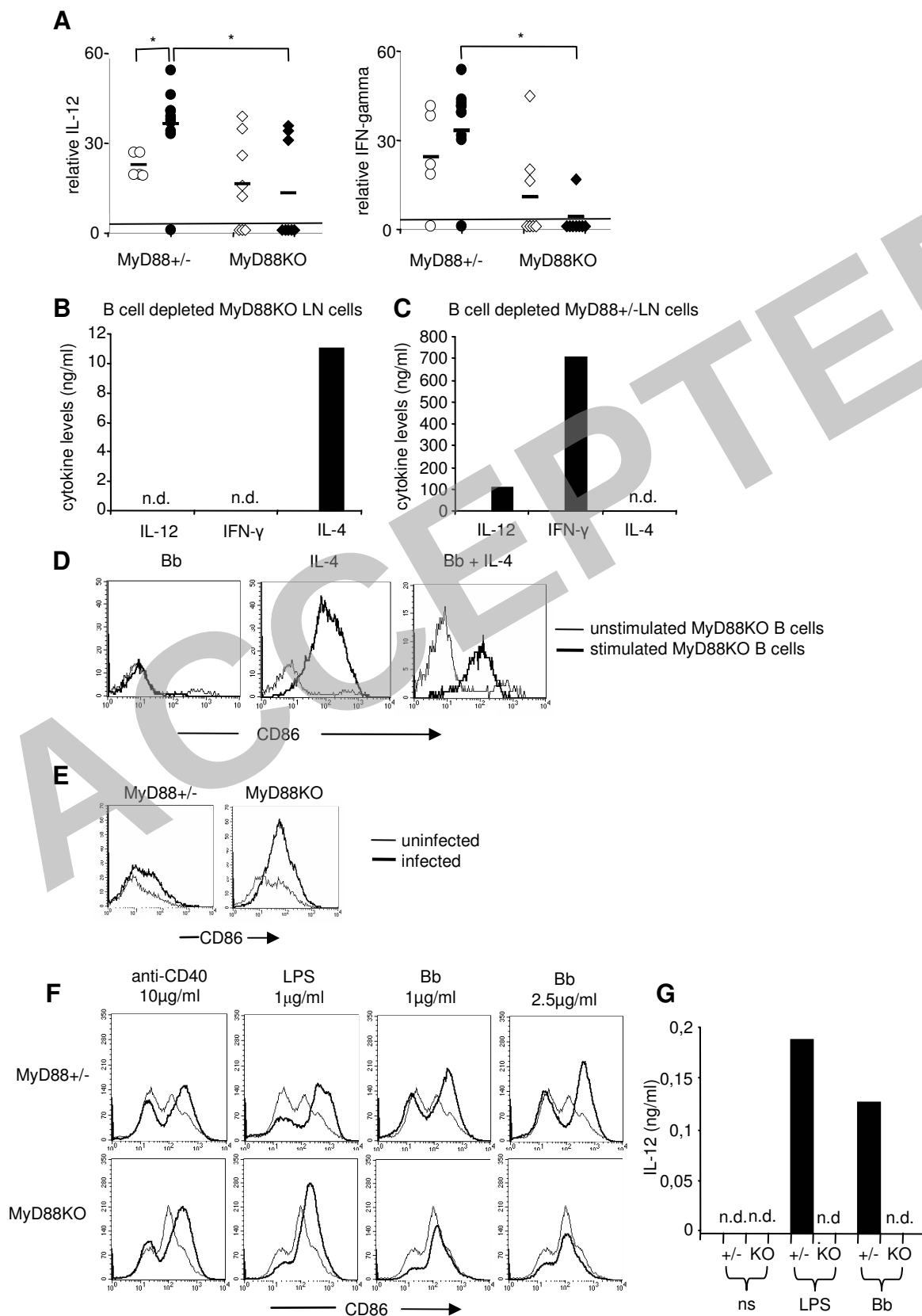


Figure 7