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Lipopolysaccharide is a fundamental structural component of the cell wall of Gram-negative bacteria (1). LPS harbors a conserved molecular pattern (lipid-A) that is recognized by innate immune system receptors that trigger the initial stages of antimicrobial host defense (2, 3). The course of infection mediated by Gram-negative bacteria is largely determined by the immune responses to LPS (4, 5). Although activation of the immune system during microbial invasion is generally protective, when excessive the reaction may harm the host through a mal-adaptive release of endogenously generated inflammatory mediators resulting in a life-threatening condition known as septic shock (6, 7).

The identification of genetic factors involved in the LPS response was initiated when allelic mutations of a single locus (LPS locus) in mice were shown to entirely abrogate the response to LPS (8–10). Later identification of the LPS gene through positional cloning (11) and production of TLR4 knockout mice confirmed that TLR4 (formerly known as the LPS locus) is needed to trigger the immune response to LPS (12–14). Those studies established that the signal-transducing receptor for LPS is TLR4. TLR4 belongs to the TLR family, a group of germline-encoded receptors that play a major role in innate immune recognition (15–17).

LPS recognition via TLR4 is complex and requires the help of different accessory molecules (15–17). After microbial infection, LPS associates with LPS-binding circulating proteins (18, 19). Although the exact mechanism of LPS recognition at the cell surface has not yet been elucidated, different studies propose that LPS associates with a TLR4/MD-2/CD14 complex (15–17, 20–22). After TLR4 activation by LPS, the MyD88 molecule is recruited to the plasma membrane, initiating a signaling cascade that leads to nuclear translocation of the transcription factors NF-κB and AP-1 (23) that in turn drive the production of TNF-α and cytokines such as IL-6. Engagement of TLR4 can also induce a MyD88-independent signaling transduction pathway, resulting in a delayed activation of NF-κB (16, 24, 25).

Recent studies have revealed that another TLR family receptor, RP105 (CD180), plays a role in B cell response to LPS (26, 27). In fact, the null mutation in the RP105 gene revealed impaired proliferative and humoral immune responses of RP105-deficient B cells to LPS (26). In parallel with TLR4, the surface expression and signaling of which seems to depend on the coexpression of the secreted extracellular protein MD-2, surface expression of RP105 is dependent on the coexpression of the MD-2 homolog MD-1 (21, 28). The RP105/MD-1 complex is preferentially expressed on the surface of mature B cells, but the mechanism through which RP105/MD-1 and TLR4 receptors cooperate in LPS response in B cells remains to be elucidated (26).

The dual ability of B cells to generate an Ag-specific immune response and to be activated through Toll-like receptors, establishes B cells as an important bridge between the innate and adaptive immune systems. In fact, LPS stimulation of murine B cells enhances the Ag presentation ability and is accompanied by B cell proliferation and secretion of large quantities of LPS-neutralizing Abs (29, 30). Although LPS has been used as a potent B cell stimulator, the molecular mechanisms involved in triggering B cell activation by LPS are poorly described.

Susceptibility to LPS in both humans and mice has been associated with allelic variations at the TLR4 locus (31). However, susceptibility to infections is usually under complex genetic control, and it is possible that naturally occurring allelic variation at
different genes controls the response to LPS. With the intent to identify natural polymorphisms in genes that control the response to LPS, we performed a genome-wide search using the F2 intercross generation of the LPS high responder mouse strain C57BL/6 and the low responder mouse strain BALB/c. Our results revealed that the MHC class II locus is a major genetic factor in controlling B cell responsiveness to LPS.

Materials and Methods

Mice

The C57BL/6 and BALB/c mice were bred and maintained in conventional housing at the Instituto Gulbenkian de Ciência. All mice analyzed in this study were sex-matched adults. F1 mice were generated in reciprocal crosses between C57BL/6 and BALB/c and F1 (C57BL/6 × BALB/c) mice were used to generate an F2 progeny. B6.C-H2-<d>/b By is a congenic strain carrying the MHCd haplotype in the C57BL/6 genetic background and is here referred to as B6.C. The C.B10-H2-<b>/bIiMed is a congenic strain carrying the MHCd haplotype in the BALB/c genetic background and is here referred to as C.B10. Both strains where purchased from The Jackson Laboratory. MHC class II−/− mice and MHC class II−/+ transgenic (Tg) Ead (referred to as I-A transgenic (Tg) Ead mice express the class II E molecule exclusively in the thymus.

All procedures were in accordance with national regulations on animal experimentation and welfare.

LPS and Abs

Commercial LPS from Salmonella typhimurium was purchased from Sigma-Aldrich. Purified LPS was extracted from commercial LPS using a phenol re-extraction method as described by Hirschfeld et al. (32). Purification of LPS eliminates the ability of signaling through murine TLR2 that is commonly observed in nonpurified LPS preparations (32). LPS purification was validated by the inability to induce cell response in splenocytes from TLR4−/− mice (data not shown). Anti-mouse CD19-FITC (1D3), anti-mouse CD45/B220-FITC (RA3-6B2), anti-mouse CD69-PE (H1.2F3), and streptavidin-PE were purchased from BD Pharmingen. Goat anti-mouse IgM UNLB Ab (1020-01) and goat anti-mouse IgM AP-coupled Ab (1020-40) were purchased from Southern Biotechnologies Associates. Anti-mouse CD180 (RP105) (RP/14) was purchased from eBioscience.

Cell purification

Single-cell suspensions from spleen and draining lymph nodes were prepared by straining of the tissues through a nylon mesh. Purified B cell populations were obtained by high speed sorting (MoFlo; Cytomation) after labeling using anti-mouse CD19 or B220 Abs. The purity of sorted cells was always >98%.

Cell activation

Single-cell suspensions from spleen, draining lymph nodes, and purified B cell populations were cultured at 37°C with 5% of CO2 for 24 h. Cells were plated at a density of 5 × 10^5 per well in 96-well flat-bottom plates with RPMI 1640 supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, 10 mM HEPES (pH 7.4), 50 μM 2-ME, 100 U penicillin, and 100 μg/ml streptomycin (all from Invitrogen Life Technologies) in the presence or absence of purified or commercial LPS. Surface staining after activation used anti-mouse CD69-PE and anti-mouse CD19-FITC or CD45/B220-FTC Abs. Dead cells were excluded from analysis with propidium iodide. All data were acquired with a FACSCalibur cytometer (BD Biosciences) and analyzed with CellQuest (BD Biosciences) and FlowJo (Tree Star) software.

B cell proliferation assay

Purified B220+ cells (5 × 10^5) from spleen and draining lymph nodes were cultured in flat-bottom 96-well plates with or without 10 μg/ml purified LPS, as described above. After 42 h, cultures were pulsed with [3H]thyminid (ICN; sp. act., 5 Ci/mol), cells were harvested 6 h later, and thymidine incorporation was measured following conventional techniques. Data represent the mean and SD of six cultures expressed as cpm per culture.

ELISPOT

Numbers of IgM secreting cells were enumerated by a modification (32) of the Sedgwick and Holt technique (34). Briefly, 5 × 10^4 purified B220+ cells from spleen or draining lymph nodes were cultured in flat-bottom 96-well plates with or without 10 μg/ml purified LPS for 4 days. At the fifth day, one-half of the culture from each well was plated in serial dilutions in 96-well ELISA plates precoated with goat anti-mouse IgM unlabeled Ab (5 μg/ml). Plates were then incubated overnight at 37°C in 5% CO2. After three washes with PBS and PBS-Tween 20, goat anti-mouse IgM AP-coupled Ab was added, and plates were incubated overnight at 4°C. After three more washes with PBS-Tween 20, the aminomethyl phosphonol-5-bromo-4-chloro-3-indolyl phosphate substrate solution was added, and plates were again incubated overnight in the dark at 4°C. Plates were washed with distilled water, and blue spots were counted under a microscope and expressed as the number of IgM-secreting cells.

Genotyping

Genomic DNA was extracted from mouse tails according to standard procedures. A 142 progeny from the F2 (BALB/c × C57BL/6) generation was genotyped using conventional PCR protocols for 82 microsatellite markers, polymorphic for the parental strains. Markers were chosen according to their chromosomal position as given by the Whitehead MIT Centre for Genome Research (Cambridge, MA), to cover uniformly the whole of the mouse genome. The genotypes for each locus were determined by DNA marker amplification through conventional PCR techniques. Amplification products were analyzed in agarose gels (Cambrex BioScience Baltimore).

FIGURE 1. The LPS-induced activation phenotype. Flow cytometry profiles of CD69 expression in CD19+ splenocytes representative of C57BL/6, BALB/c, and F1 (C57BL/6 × BALB/c) mice after stimulation in vitro with 10 μg/ml commercial LPS for 24 h. The values in the upper right quadrant indicate the percentage of the CD19+ population that was considered to be activated as compared with nonstimulated controls.
with concentrations varying between 0.8 and 4%, according to the length of the polymorphism of the amplified fragment and stained with ethidium bromide (Sigma-Aldrich). For each marker, each individual of the F2 generation was typed as homozygous for one or the other of the parental strains or as heterozygous when presenting both the parental strain alleles.

Statistical analyses

Comparison of the means for the LPS-induced B cell activation phenotype among C57BL/6, BALB/c, and F1 mouse strains was performed using one-way ANOVA. Evidence of significant differences between each group (2 df) was considered for \( p \leq 0.01 \). Quantitative trait locus analysis was performed using the R/QTL software (35). This program calculates logarithm of odds (LOD) \(^3\) scores over intervals between linked markers, representing the likelihood of genetic association for markers along the chromosome with the phenotype. The program also compares recessive, dominant, and additive models of allele actions. Levels of statistical significance were determined from permutation tests, and significant linkage (36) was considered for LOD \( \geq 3.4 \) (\( p \leq 0.001 \)), whereas suggestive linkage was considered for LOD \( \geq 1.9 \) (\( p < 0.05 \)). Where applicable, the Student t test was used to evaluate statistically significant results. The following symbols were used to represent significance levels: *, \( p < 0.05 \); **, \( p < 0.01 \); ***, \( p < 0.001 \).

Results

Genetic mapping of LPS-induced B cell activation

B cell response to LPS stimulation in vitro includes up-regulation of activation surface markers, cell proliferation, and IgM secretion. We observed that the proportion of CD69 expression among CD19\(^+\) splenic B cells after 24 h of LPS stimulation in vitro (Fig. 1) was consistently higher in the C57BL/6 mice (95.1 ± 1.7%) as compared with the BALB/c mice (68.9 ± 8.2%), whereas the F1 (C57BL/6 x BALB/c) generation presented an intermediate phenotype (87 ± 4.2%). To study the genetic control of LPS-induced B cell activation, we analyzed an F2 (C57BL/6 \( \times \) BALB/c) \((\nabla)\) and 139 F2 (BALB/c \( \times \) C57BL/6; ■) mice.

\(^3\) Abbreviations used in this paper: LOD, logarithm of odds; Tg, transgenic; QTL, quantitative trait loci.

D4MIT301 (42.5 cM) with a LOD score of 2.77. This marker shows close linkage to the TLR4 locus (33.0 cM). It is likely that the TLR4 polymorphisms previously described between C57BL/6 and BALB/c mouse strains are associated with functional differences that could contribute to the observed LPS response phenotype. Unexpectedly, we found that the second controlling locus mapped to the MHC region on mouse chromosome 17, with the highest LOD score of 3.4 at marker D17Mit24 (20.4 cM; Fig. 3A).

No evidence for linkage to any other chromosomal region was found. The C57BL/6-derived allele on the chromosome 4 locus

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No evidence for linkage to any other chromosomal region was found. The C57BL/6-derived allele on the chromosome 4 locus
shows an additive effect on the phenotype, whereas the C57BL/6-derived allele mapping to the MHC region on chromosome 17 exhibited recessive behavior (Fig. 3B). The C57BL/6 alleles at both loci increase the phenotype (Fig. 3B), strongly suggesting that they have a joint effect in LPS responsiveness phenotype of the C57BL/6 strain, but the nature of this interaction remains to be determined. It is known that commercial preparations of LPS can be contaminated with other bacterial products able to induce cell activation. Therefore, we extracted purified LPS to obtain a preparation that exclusively activated B cells through the LPS-binding receptors. We noted that the observed difference in response of C57BL/6 and BALB/c mouse strains was more pronounced when using purified LPS, implying that the phenotype under analysis was linked to B cell activation through LPS-binding receptors (Fig. 4).

**Congenic mapping confirms a LPS-responsive region in close linkage with the MHC locus**

To confirm that the locus identified in close linkage to the MHC genetic region had a role in LPS-induced B cell response, we analyzed different responsiveness traits in purified B220+ splenic cells derived from two MHC-congenic mouse strains, B6.C and C.B10 (Fig. 5). The results showed that introgression of a fragment of 11.1 cM of chromosome 17, comprising the MHC genetic region from the BALB/c strain onto a C57BL/6 genetic background (B6.C-congenic strain; Fig. 5) confers decreased expression of CD69 (Fig. 6A), cell proliferation (Fig. 6C), and IgM secretion (Fig. 6E) in LPS-stimulated B cells as compared with progenitor C57BL/6 cells. Albeit at lower responsiveness level, the introgression of an analogous fragment of 7.4 cM from the C57BL/10 strain onto a BALB/c genetic background (C.B10-congenic strain; Fig. 5) confers higher frequency of CD69-expressing cells (Fig. 6B), increased cell proliferation (Fig. 6D), and higher numbers of IgM-secreting cells (Fig. 6F) after LPS stimulation, as compared with parental BALB/c mice. The low LPS responsiveness to purified LPS observed in both BALB/c and C.B10 strains (Fig. 6, B, D, and F) is probably due to the sharing of the TLR4 allele from BALB/c which is not represented in the C57BL/6 and B6.C strains (Fig. 6, A, C, and E). Thus, these data suggest that even in the BALB/c low LPS-responsive genetic background, the MHC haplotype has a detectable effect on the responsiveness to LPS (Fig. 6, B, D, and F). The observed differences in congenic strains are not attributable to differences in the surface expression level of MHC class II molecules (data not shown), suggesting that MHC molecules expression level is not involved in the LPS responsiveness phenotype. These results confirm that allelic variants of the region on chromosome 17 spanning the MHC locus contain genetic factor(s) that partially restore(s) the difference in CD69 expression observed in LPS-stimulated B cells of the parental mouse strains. Furthermore, the results show that the action of such a genetic factor, other than controlling LPS-induced B cell activation, exhibits a measurable effect at the level of B cell proliferation and Ab production, suggesting that genetic variation in this region may influence the course of the LPS immune response at the systemic level.

**Expression of MHC class II molecules limits B cell response to LPS**

MHC class II genes are included in the congenic region of the C.B10- and B6.C-congenic strains (Fig. 5) and have been claimed to participate in the response to LPS (37). To test whether MHC class II genes are implicated in LPS-induced activation of B cells, we compared the response of B220+ purified cells from spleen and lymph nodes, in MHC class II−/− and C57BL/6 mice. Purified B cells from MHC class II−/− mice showed a higher response to LPS than C57BL/6 mice, translated in higher percentage of CD69+ B cells, higher cell proliferation and higher numbers of IgM-secreting cells (Fig. 7). These results indicate that the lack of expression of MHC class II genes in B cells leads to increased B cell response to LPS receptor triggering. To show that higher response to LPS in MHC class II−/− mice was not due to absence of CD4 T cells, we tested I-A−/− Tg Eaα mice which express MHC class II molecules exclusively in the thymus and show restoration of the CD4 T cell compartment. In fact, purified B220+ cells from such mice do not restore wild-type response to LPS, strongly suggesting that the higher LPS response of MHC class II−/− mice is due to the lack of MHC class II molecules on B cells (Fig. 8).

**The MHC haplotype controls expression of RP105 (CD180)**

In search for molecular components that could explain the observed MHC control of B cell responsiveness to LPS stimulation, we studied the surface expression of RP105 on freshly isolated B cells. RP105 is a LPS-responsive TLR molecule that is mainly expressed in mature B cells. Our results revealed that expression of RP105 measured both as percent of positive cells and mean fluorescence intensity correlates with MHC haplotype (Fig. 9). In fact, B6.C mice show lower levels of RP105 expression than C57BL/6 mice, whereas C.B10 mice show higher levels than BALB/c mice. Such results suggest that along with
unidentified genetic background factors, the MHC haplotype has on its own a measurable effect on the surface expression of RP105. In accordance with the observed LPS responsiveness phenotype, the MHC class II^{+/−} mouse B cells also showed an up-regulation of RP105 expression (data not shown), reinforcing the notion of a functional link between MHC class II gene products and B cell responsiveness to LPS. We also measured TLR4 RNA expression in nonstimulated purified B cells, but the observed differences between MHC congenic strains and their parental strains did not reach statistical significance (data not shown). However, it remains to be determined whether TLR4 surface expression in these mouse strains follows the observed RP105 RNA expression patterns.

The results here reported show that MHC class II molecules are involved in B cell activation via LPS-binding receptors and indicate that polymorphisms at the MHC class II genes constrain B cell responsiveness to LPS stimulation.

Discussion
The molecular mechanisms leading to innate stimuli recognition, signaling transduction and subsequent cellular events in B cells, have been extensively investigated. However, there is little information on genetic components mediating the cross-talk of cellular innate immune functions and adaptive immune functions in B cells. This genetic study shows that the difference in LPS responsiveness observed between the C57BL/6 and BALB/c mouse strains was linked to two genetic regions, the TLR4 region and the MHC locus. Although it is expected that the TLR4 region would be linked to this phenotype, it was surprising to verify that the MHC locus showed the strongest linkage (LOD score, 3.4) and had
The LPS response of purified B220⁺ splenocytes was measured as the percent of CD69-expressing cells after 24 h of incubation in the presence or absence of purified LPS (5 μg/ml). Data refer to the average values of pooled splenocytes from five C57BL/6, five MHC class II−/−, and five MHC class II−/− Tg Ea mice, which express the MHC class II E molecule exclusively in the thymus and therefore carry a restored CD4 T cell compartment. Values are representative of two independent experiments, and statistically significant results are marked by stars as described in Materials and Methods.

Ascertaining whether polymorphisms in the MHC II genes constrain B cell responsiveness to LPS via TLR4 surface expression. Several studies have shown that some MHC II molecules interfere with the LPS response at the level of surface recognition or intracellular signaling. Different lines of evidence suggest that recognition of LPS at cell surface may be affected by interactions with MHC class II molecules. Some early studies have shown that monoclonal anti I-A/E Abs directly inhibit B cell responses to LPS with a gradual loss of inhibitory activity over time (30, 38, 39). It is possible that these Abs may mediate their effect either by blocking the LPS receptor complex at the cell membrane or because binding to Ia Ags would result in the redistribution of other surface molecules, including LPS receptors (30). Our results would also support the hypothesis that MHC II pathway could have inhibitory effects on the LPS pathway at the level of intracellular signaling. On the other hand, MHC class II molecules could directly interact with LPS, leading to decreased cell activation. Several studies have shown that some results do not exclude the hypothesis that the MHC II pathway could also have inhibitory effects on LPS responsiveness at the level of surface recognition or intracellular signaling. Different lines of evidence suggest that recognition of LPS at cell surface may be affected by interactions with MHC class II molecules.

The notion that MHC class II somehow interferes with B cell response to LPS has been reported earlier (30), and a recent study proposes that the LPS responsiveness in other cell types such as human PBMC and mouse macrophages is also affected by the absence of MHC class II expression (37). Interestingly, expression of MHC class II molecules was reported to enable LPS response of those cell types, whereas we observed a decreased B cell response to LPS. This discrepancy suggests that the mechanism through which MHC class II molecules interfere with the LPS response may be different in different cell types.

Our results have demonstrated that MHC class II genetic configurations displaying lower responsiveness to LPS also show lower surface expression of the LPS recognition complex component RP105 (CD180). This finding favors the hypothesis that action of MHC class II molecules in constraining B cell ability to respond to LPS is modulated through surface expression of RP105, in such a way that better surface expression of RP105 leads to better responsiveness to LPS. The mechanism by which such modulation occurs remains to be clarified. One possibility is that RP105 expression at the cell surface is down-regulated in B cells that have experienced Ag presentation through the MHC class II pathway. Such a possibility would support a model where Ag presentation and T cell interaction would render B cells short or long term committed to adaptive functions and poor responders to LPS stimulation. The finding that MHC class II genetic configuration plays a role in RP105 controlling cell surface suggests that they may be as well involved in the controlling expression of other components of the innate immune system. However, quantification of TLR4 RNA in unstimulated B cells was not informative to ascertain whether polymorphisms in the MHC II genes constrain B cell responsiveness to LPS via TLR4 surface expression.

Although the data presented here suggest that MHC controlled LPS responsiveness is at least in part explained by modulation of innate immunity molecules expression, it is not excluded that other mechanisms could also exist that explain additional roles of MHC class II molecules in B cell response to LPS. In particular, our
bacterial polysaccharides are capable of binding MHC class II molecules inside APCs and are presented to T cells through the MHC class II endocytic pathway (40). The capacity of polysaccharides to induce T cell responses opens new perspectives on the MHC class II presentation paradigm. Although there is no evidence that LPS or LPS-derived molecules are presented to T cells through the MHC class II complex, Forestier et al. (41) demonstrated that different types of internalized LPS accumulate in an MHC class II-positive lysosomal compartment in both murine and human B cells. The same study shows that Brucella abortus LPS coprecipitates with MHC class II molecules in an haplotype-independent manner, constituting evidence for a direct interaction between the MHC class II molecules and LPS.

The action of MHC class II molecules in B cell activation by LPS is still not clear. Different studies have shown an existing relationship between LPS and the MHC class II gene products at different levels but have not come together to elucidate the nature of such interaction. We provided genetic evidence confirming that MHC class II molecules constrain B cell response to LPS and that this occurs in an all-or-none manner. These findings may have implications to the study of human susceptibility to innate stimuli by pathogens.

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Disclosures

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