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Coexpression of TLR2 or TLR4 with HLA-DR Potentiates the Superantigenic Activities of *Mycoplasma arthritidis*–Derived Mitogen

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*Mycoplasma arthritidis*–derived mitogen (MAM) is a member of the superantigen family that structurally differs from other members while still capable of initiating cognate APC/T cell interaction. In addition to the critical role of MHC class II molecules, it has been suggested that TLR2 and TLR4 may cooperate with MHC class II during MAM-induced responses. In this study, we investigated the direct involvement of TLR2 and TLR4 in MAM binding and presentation to T cells. Our results showed that MAM fails to bind to TLR2- and TLR4-transfected cells. However, coexpression of TLR2 or TLR4 with HLA-DR significantly increases MAM binding and the subsequent T cell activation compared with cells expressing HLA-DR alone. The upregulated MAM binding and activity in HLA-DR/TLR–transfected cells is abrogated by an anti–HLA-DR Ab. Interestingly, we also found that MAM complexed with soluble HLA-DR is capable of binding to both TLR2 and TLR4. The enhancing effect of TLR2 or TLR4 on MAM-induced T cell proliferation was not due to TLR ligand contamination in the MAM preparation. Taken together, these results strongly suggest that binding of MAM to HLA-DR leads to a conformational change in MAM structure allowing its interaction with TLR2 and TLR4 and a better recognition by T cells. *The Journal of Immunology*, 2014, 192: 000–000.
Our results indicate that ligation of MAM with HLA-DR triggers a conformational change that promotes MAM binding to TLR2 and TLR4 leading to a significant enhancement of T cell activation. Given the present findings, MAM could be considered as a pertinent tool to study the cross-talk between innate and adaptive immunity.

Materials and Methods
Abs and reagents
Anti–hTLR4 (HTA125 an IgG2a mAb) (24) was provided by Dr. K. Miyake (Saga Medical School, Saga, Japan); anti–hTLR2 (TL2.1, an IgG2a mAb) was purchased from eBioscience (San Diego, CA); and anti–HLA-DR mAb (L243, IgG2a), which recognizes a conformational epitope on the DRα-chain, was obtained from the American Type Culture Collection (Manassas, VA). Anti-staphylococcal enterotoxin B mAb (8C12, an IgG2a mAb) was generated in our laboratory and was used as isotype control. Reconstituant MAM was generated and purified as we described previously (5). MAM also was biotinylated by the Pierce EZ-linked NHS-biotin kit (Pierce), according to the manufacturer’s protocol. FBS and hygromycin were purchased from Wisent. Zeocin purchased from InvivoGen, and penicillin (P), streptomycin (S), and β-lactamase (G) were procured from Life Technologies (Burlington, ON, Canada). Recombinant MAM was generated and purified as described previously (18). Purified MAM was able to bind to HEK-293 cells expressing HLA-DR, TLR2, or TLR4 expression by cytometry (Fig. 1A). The transfected HEK-293 cells were sorted and first analyzed for HLA-DR molecules that can affect MAM-induced T cell activation. Stably transfected HEK-293 cells were used along this study, because they do not initially express these MHC class II, TLR molecules or any accessory molecules that can affect MAM-induced T cell activation. Stably transfected HEK-293 cells were sorted and first analyzed for HLA-DR, TLR2, or TLR4 expression by cytometry (Fig. 1A). The transfected cells were then used to study the binding capacity of biotinylated MAM. As shown in Fig. 1B, although there is a significant binding of MAM to HEK-293 cells expressing HLA-DR, it was unable to bind to TLR2- or TLR4-transfected cells. Because there is no detectable MAM binding to TLR2- and TLR4-transfected cells, we then investigated whether coexpression of HLA-DR molecules could influence MAM binding. For this purpose, HEK-293 cells stably expressing HLA-DR were cotransfected with TLR2, TLR4, or empty vector. Stably cotransfected HEK-293 cells were then used to study the binding capacity of biotinylated MAM. Results presented in Fig. 2B and 2C show that MAM binding was significantly upregulated in cells cotransfected with both HLA-DR and TLR2 or TLR4 as compared with cells expressing only HLA-DR.

Because we have previously shown that anti–HLA-DR Ab (L243), but not anti-MHC class I or anti-CD40, completely inhibited the MAM binding (28), the L243 Ab was used to evaluate the cooperation between HLA-DR and TLR2 or TLR4 in MAM binding. As shown in Figs. 3A and 3B, treatment with L243 inhibited MAM binding to HLA-DR– as well as to HLA-DR/TLR2– and HLA-DR/TLR4–transfected cells in a dose-dependent manner (Fig. 3B). The major difference between the observed inhibitions is that lower doses of L243 (1 μg/ml) were required to completely inhibit MAM binding to HLA-DR–transfected cells as compared with 5 μg/ml for HLA-DR/TLR2– and HLA-DR/TLR4–cotransfected cells.
These results indicate that HLA-DR is the main MAM receptor and strongly support the cooperation between HLA-DR and TLR2 or TLR4 in MAM binding.

**MAM ligation with soluble HLA-DR influences its binding to TLR2 and TLR4 molecules**

It was proposed earlier that Sag binding to MHC class II molecules leads to a conformational change that allow Sag/MHC class II complexes to interact with TCR on T cells and induces subsequent T cell activation (29). This hypothesis was supported by our co-crystal analysis (MAM/HLA-DR) (15), and the ternary complex, TCR/MAM/HLA-DR (16). To verify whether the conformational change induced in MAM upon its binding to MHC class II could influence the MAM binding to TLRs, we generated soluble HLA-DR and used it for the binding experiments. Fig. 4A shows that there was no detectable binding of MAM to empty vector–, TLR2- or TLR4-transfected cells. However, the addition of soluble HLA-DR allows significant binding of MAM to both TLR2- and TLR4-transfected cells (Fig. 4B), confirming the cooperation of HLA-DR in MAM binding to TLR molecules.

**Coexpression of TLR2 or TLR4 with HLA-DR enhances MAM-induced T cell activation**

The cooperation demonstrated between the MHC class II and the TLRs could have biological implications. Indeed, it has previously been shown that TLR2 and TLR4 could affect cytokine production by macrophages and monocyte possibly via MHC class II signaling. In our study, to characterize the role of TLR in MAM-induced cell activation, transfected and cotransfected HEK-293 cells were cocultured with MAM-responsive murine 3DT cell clones that express Vβ8.2 (6). Cocultured cells were left unstimulated or stimulated with different concentrations of MAM. After 48 h, levels of IL-2 were assessed in cell supernatants as

**FIGURE 1.** No detectable binding of MAM to TLR2- or TLR4-expressing cells. (A) Expression levels of HLA-DR, TLR2, and TLR4 in stably transfected HEK-293 cells. Cells were first incubated with L243, TL2.1, or HTA125 Abs against HLA-DR, TLR2, and TLR4, respectively, followed by Alexa 488–conjugated goat anti-mouse IgG. IgG2a Ab was used as an isotype match control. Data are representative of three independent experiments. (B) Transfected cells (0.3 × 10⁷/100 μl) were incubated without MAM-biot. (negative control, empty plot) or with MAM-biot. (20 nM, gray plot), followed by streptavidin–PE. Cells were analyzed by flow cytometry for MAM binding. Data are representative of three independent experiments.

**FIGURE 2.** Expression of TLR2 and TLR4 enhances MAM binding to HLA-DR/HEK-293–transfected cells. (A) Expression levels of HLA-DR, TLR2, and TLR4 in stably transfected HEK-293 cells. Cells were stained using L243, TL2., or HTA125 Abs against HLA-DR, TLR2, and TLR4, respectively. IgG2a Ab was used as an isotype match control. Data are representative of three independent experiments. (B) Transfected cells (0.3 × 10⁷/100 μl) were incubated without MAM-biot. (negative control, empty plot) or with MAM-biot. (20 nM, gray plot), followed by streptavidin–PE. Cells were analyzed by flow cytometry. Data are representative of three independent experiments. (C) Histograms represent the mean ± SEM of three independent MAM-binding experiments. *p < 0.05.
a parameter of T cell activation. Similarly to our previously reported data (6), MAM induced T cell activation of 3DT cells when presented by HLA-DR. Interestingly, the coexpression of TLR2 (Fig. 5A) or TLR4 (Fig. 5B) together with HLA-DR molecules on HEK-293 cells significantly increased the activation of cocultured T cells upon stimulation with MAM in a dose-dependent manner. MAM presented by HEK-293 cells transfected with empty vector or cells transfected with only TLR2 or TLR4 failed to induce any detectable IL-2 production. It is worth noting that, in contrast to other cell types such as human monocytes, monocytic cell line, FIGURE 3. HLA-DR mAb completely blocks MAM binding. (A) Transfected cells (0.3 × 10⁶/100 μl) expressing HLA-DR or HLA-DR in combination with TLR-2 or TLR-4 were incubated without MAM-biot. (negative control, empty plot) or with MAM-biot. (20 nM) in the absence (black plot) or presence (gray plot) of L243 anti–HLA-DR Abs (2 μg/ml) for 2 h on ice, followed by streptavidin–PE. Cells were analyzed by flow cytometry for MAM binding. (B) Histograms represent mean ± SEM of three independent experiments in which different concentrations of anti–HLA-DR were used. *p < 0.05.

FIGURE 4. Soluble HLA-DR allows MAM binding to TLR2 and TLR4: MAM-biot. was added to HEK-293 cells transfected with empty vector or cells transfected with only TLR2 or TLR4, alone (A) or in combination with soluble HLA-DR (B) and incubated for 1 h at 37°C. Washed cells were then incubated with streptavidin–PE. After 1 h, cells were washed and analyzed by flow cytometry.
and fibroblast-like synoviocytes (30, 31), we could not perceive any signaling event in our transfected and cotransfected HEK-293 cells following addition of MAM. Therefore, the IL-2 response observed in the above experiments was not due to IL-1 or IL-6 production by HEK-293 because no such cytokines were detected in the cell supernatants (data not shown). Along the same line of evidence, the enhancement of MAM activity in HLA-DR/TLR2– or HLA-DR/TLR4–cotransfected cells was not due to a MAM-induced increase in HLA-DR expression in these cells, because a stimulation with MAM failed to affect HLA-DR expression (data not shown). Taken together, our results demonstrate a greater IL-2 production in T cells upon their stimulation with MAM presented by HEK-293 cells expressing both HLA-DR and TLRs (TLR2 and TLR4) when compared with cells expressing HLA-DR alone. Moreover, MAM was able to bind cells expressing TLR2 or TLR4 only in the presence of soluble HLA-DR molecules. The enhancement of MAM binding was completely abrogated by pretreatment with anti–HLA-DR mAb. On the biological level, cells expressing both HLA-DR and TLR molecules and presenting MAM to T cells in cocultures induced a more pronounced activation of T cells as compared with cells expressing only HLA-DR on their surface. This enhanced response was proven specific to MAM because TLR ligands failed to mount any response when used as stimulants.

It has been previously demonstrated that MAM was capable of activating THP1, a human monocytic cell line (HLA-DR+/TLR2+/TLR4+), and that such activation was inhibited by Abs directed against both TLRs. In addition, macrophages derived from C3H/HeJ mice that exhibit a hyporeactive form of TLR4 (nonresponsive to LPS) and from C3H/HeN TLR2 knockout mice (TLR2−/−/TLR4+/-) were shown to produce inflammatory cytokines upon their stimulation with MAM, whereas C3H/HeJ TLR2KO cells lost such ability (18). Even though these interesting findings outline a cross-talk between MAM and TLRs, they did not dem-

**FIGURE 5.** Coexpression of TLR2 or TLR4 with HLA-DR enhanced MAM-induced T cell activation: HEK-293 cells stably transfected with empty vector, HLA-DR, TLR2, HLA-DR/TLR2 (A), TLR4, or HLA-DR/TLR4 (B) (2 × 10⁴ cells/200 μl) were cocultured with 8 × 10⁵ cells/200 μl of a murine VB8.2 (3DT). Cells were stimulated in the presence or absence of MAM at the indicated concentration. After 48 h of incubation, the supernatants were collected, and the IL-2 release was assessed with bioassay using CTLL-2 cells, followed by XTT metabolization assay. IL-2 levels are represented by arbitrary units per milliliter. Results represent the mean ± SEM of four independent experiment. *p < 0.05 comparing TLR positive with negative cells.

Enhanced MAM-induced T cell activation is dependent on HLA-DR but totally independent of TLR2 or TLR4 ligands

To further evaluate the cooperation between TLR2 or TLR4 and HLA-DR molecules for T cell activation induced by MAM, the cocultures were treated with different doses of L243. As shown in the Fig. 6, L243 completely inhibited T cell activation when MAM was presented by HLA-DR alone. In addition, when MAM was presented by HEK-293 expressing HLA-DR in combination with TLR2 (Fig. 6A) or TLR4 (Fig. 6B), L243 Ab was able to abrogate, but to a lesser extent, the MAM-induced T cell activation.

To rule out the possibility that the observed T cell activation was due to contaminants in the MAM preparation that could ligate TLRs, namely the LPSs, T cell clones cocultured with transfected HEK-293 cells were treated with MAM and/or LPS from the Porphyromonas gingivali, ligand of TLR2 (32) or from the E. coli, ligand of TLR4 (33). As shown in Fig. 6, both LPSs were not able to induce the proliferation of T cells in coculture with HEK-293–transfected with TLR2 (Fig. 6C) or TLR4 (Fig. 6D). Moreover, both LPSs could not synergize with MAM in activating T cells cocultured with HEK-293 expressing HLA-DR and TLR2 (Fig. 6C) or TLR4 (Fig. 6D). Indeed, IL-2 levels detected upon MAM stimulation were not altered when the stimulation was undertaken with a combination of MAM and both types of LPSs. These results also were confirmed using polymyxin B (10 μg/ml) to inhibit LPS-induced effects (5267 ± 383 U/ml by MAM versus 4349 ± 383 U/ml IL-2 by MAM + polymyxin B). Furthermore, on the basis of the heat sensitivity of MAM and LPS, MAM being heat-sensitive (34) and LPS being heat-resistant (35), heating MAM at 95°C for 15 min (36) abrogated its capacity to induce T cell activation in the coculture with HLA-DR/TLR2– or HLA-DR/TLR4–transfected HEK-293 cells (data not shown). These results indicate that the enhanced MAM-mediated T cell activation in the presence of both HLA-DR and TLRs (TLR2 and TLR4) was not due to a contamination of MAM preparation with TLR ligands, outlining as such the biological interaction between MAM and TLRs themselves in the presence of HLA-DR molecules. Given our data, we suggest a model outlining the cooperation between MHC class II and TLR2 or TLR4 molecules for MAM binding and activity (Fig. 7).

**Discussion**

The present study highlights the role of TLRs 2 and 4 as important coreceptors for MAM. Along with HLA-DR, TLR2 and TLR4 are able to increase MAM binding and its presentation to T cells. Indeed, data presented in the current investigation indicate that MAM exhibited an increased binding to cells coexpressing both HLA-DR and TLR2 or TLR4 when compared with cells expressing HLA-DR alone. Moreover, MAM was able to bind cells expressing TLR2 or TLR4 only in the presence of soluble HLA-DR molecules. The enhancement of MAM binding was completely abrogated by pretreatment with anti–HLA-DR mAb. On the biological level, cells expressing both HLA-DR and TLR molecules and presenting MAM to T cells in cocultures induced a more pronounced activation of T cells as compared with cells expressing only HLA-DR on their surface. This enhanced response was proven specific to MAM because TLR ligands failed to mount any response when used as stimulants.
onstrate the direct binding between both types of molecules. Data from our current study show that MAM is not capable of directly binding TLR2 or TLR4 using HEK-293 cells expressing either of these TLRs and immunostaining techniques. However, our study demonstrate that MAM exhibit an increased binding to HEK-293 cells expressing both HLA-DR and TLR2 or TLR4 molecules as compared with cell expressing only HLA-DR, a finding strongly suggesting the cooperation between TLRs and HLA-DR molecules for MAM binding. To outline the significance of the increased MAM binding to cells cotransfected with HLA-DR and TLR2 or TLR4 compared with cells expressing HLA-DR alone, anti–HLA-DR mAb was added and shown to completely inhibit

FIGURE 6. Enhanced MAM-induced T cell activation is HLA-DR-dependent but totally independent of TLR2 or TLR4 ligands. (A and B) Cells stably transfected with HLA-DR, HLA-DR/TLR2 (A), or HLA-DR/TLR4 (B) (2 × 10^5 cells/200 µl) were cocultured with 8 × 10^4 cells/200 µl cells of a murine VB8.2 (3DT)–positive T cell line. Cells were stimulated with MAM in the presence or absence of different doses of anti–HLA-DR (L243). After 48 h of incubation, the supernatants were collected, and the IL-2 releases were assessed with bioassay using CTLL-2 cells, followed by XTT metabolization assay. IL-2 levels are represented by arbitrary units per milliliter. Results represent the mean of triplicate of one experiment. (C and D) Cocultured cells were stimulated with MAM (250 ng/ml) in the presence or absence of 100 ng/ml LPS from P. gingivalis (ligand of TLR2) (C) or from E. coli (ligand of TLR4) (D). After 48 h of incubation, the supernatants were collected and the IL-2 release was assessed with bioassay using CTLL-2 cells, followed by XTT metabolism assay. IL-2 levels are represented by arbitrary units per milliliter. Results represent the mean of triplicate of a representative experiment of two independent experiments.

FIGURE 7. Schematic model of MAM interactions with MHC class II, TLR2 or TLR4, and TCR.
the binding of MAM to HLA-DR cells and HLA-DR/TLR2 or HLA-DR/TLR4 cells.

Therefore, our data suggest that MAM upon its binding to HLA-DR undertake a conformational change allowing its interaction with TLR2 and TLR4. These findings are in accordance with results described above, where MAM was capable of interacting with mononuclear cells expressing TLR2 and TLR4 (18). Indeed, we consider that the cross-talk that was previously reported between MAM and TLRs in monocytes and macrophages and leading to cell activation (18) is really due to the well-known presence of MHC class II molecules in these cells, in particular the HLA-DR (37). To further confirm our interpretation of these data, macrophages derived from SLJ mice that lack MAM-reactive MHC class II alleles as well as Chinese hamster ovary cells, which do not express any MHC molecules and that were transfected with CD14/TLR2 or CD14/TLR4, were shown to be irrespective to the stimulation by MAM. However, these cells could be activated by LPS or macrophage-activating lipopeptide-2, the ligands of TLR4 and TLR2, respectively (18). Therefore, the previously described observations outlining the interaction of MAM with TLRs in MHC class II-positive cells support our findings that MHC class II molecules themselves positively influence MAM binding to TLR2 and TLR4.

Interestingly, the cooperation between TLRs and HLA-DR molecules was not restricted to the binding to MAM but included also the biological response upon stimulation with MAM. It is well established that MAM, like other Sags, bind to MHC class II molecules on APCs and is presented as such to T cells by interacting with their TCR, and more specifically with the region Vβ 3.1, 13.1, 14, and 17 in humans and Vβ 6, 8.1, and 5.1 in mice (38). MAM induces different Vβ repertoire depending on the HLA-DR haplotype, which indicates that the Vβ specificity of MAM is determined by the MAM/HLA-DR complex (39, 40). In human T cells, MAM was shown to induce an increased intracellular Ca2+ concentration (41). In addition, a wide array of cytokines was triggered by MAM in human cells derived from peripheral blood or spleen (42, 43). MAM was shown to induce a type 2 cytokine profile in mice that express both TLR2 and TLR4 and a type 1 cytokine profile in mice that express only TLR2 (23, 44). In our study, we wanted to investigate the biological outcome of MAM binding to TLR2 and TLR4 cooperating with HLA-DR molecules on surface of APCs. Indeed, MAM presented by cells expressing only TLR2 or TLR4 failed to induce any detectable T cell response, whereas it triggered slight T cell activation reflected by IL-2 production when presented by cells transfected with HLA-DR. Most importantly, a significant T cell activation was observed when MAM was presented by cells coexpressing HLA-DR/TLR2 or HLA-DR/TLR4. This enhancement is not due to an increased HLA-DR expression as similar levels of HLA-DR were observed in the HEK-293 cells after MAM stimulation (data not shown). To confirm the specificity for MAM in this T cell response, we stimulated the system above using LPS. Our data showed that adding LPS to the coculture system has no effect on T cell activation. These results strongly suggest a biological synergism between HLA-DR and TLR molecules with respect to stimulation with MAM. The cooperation between TLRs and MHC class II molecules was first outlined in 2000, where macrophages exhibiting low or no MHC class II expression failed to respond to LPS stimulation (45). More recently, Frei et al. (46) have shown that MHC class II molecules colocalize with TLR2 and enhance TLR2- as well as TLR4-induced responses upon stimulation with bacterial lipoprotein or LPS, respectively. An interesting study in 2011 reported a peculiar cross-talk between MHC class II molecules and TLRs, particularly TLR3, 4, and 9. Indeed, Liu et al. (47) have demonstrated that mice knocked out for their MHC class II molecules exhibited a reduced TLR-mediated immune response, namely TNF, IL-6, and IFN-γ, when challenged with TLR4, TLR3, or TLR9 ligands. The authors further outlined the importance of MHC class II in full activation of the TLR system in vitro using peritoneal macrophages and dendritic cells derived from MHC−/− mice. These cells produced a decreased immune response when stimulated with TLR ligands as compared with WT cells, highlighting as such the specific role of MHC class II in TLR signaling (47). In addition to the role of MHC class II molecules in TLR-mediated immune responses, TLRs themselves could be also implicated in MHC class II signaling. Activation of TLR4 was shown to upregulate the autophagy-dependent presentation of Ag by MHC class II molecules (48). Our data in this study outline another role of TLR in MHC class II signaling by cooperating with the latter molecules for MAM binding and upregulating as such the MAM-induced activation of T cells.

In view of data presented in this study, we can suggest a model of interaction between MHC class II molecules, TLRs and MAM. Binding of MHC class II to MAM induces a conformational change in this latter allowing its interaction with TLR2 and TLR4 and subsequently a better presentation to T cells (Fig. 7). Conformational changes occurring upon ligand/receptor interactions have long been reported in literature. For instance, binding of Ag/MHC complex to TCR induces structural changes in this latter allowing the initiation of the activation cascade (49). We previously reported that MAM uniquely dimerizes HLA-DR molecule and itself adopts novel fold with homodimer formation (15). MAM’s unique binding could be causing some unexplored conformational changes to the complex of MAM/HLA-DR. The additional binding of MAM/HLA-DR to TLR2/4 provides more complexity to the conformation of the tricomplex MAM/HLA-DR/TLR2/4 that could be providing more binding sites to MAM, allowing as such a better interaction with TCR on T cells. However, the exact nature of this association needs to be explored by crystallographic study of the trimer complex. In addition, many questions remain to be resolved concerning the intracellular signaling implicated in the TLR/MHC class II cross-talk, its effect on APCs themselves, and the possible association with other adaptor molecules.

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Disclosures
The authors have no financial conflicts of interest.

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