The RP105/MD-1 complex is indispensable for TLR4/MD-2-dependent proliferation and IgM-secreting plasma cell differentiation of marginal zone B cells

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Abstract
Marginal zone (MZ) B cells mount rapid T-cell-independent (T-I) immune responses against microbial components such as LPS. While Toll-like receptor 4 (TLR4) is essential for LPS responses, MZ B cells uniquely express high levels of another LPS sensor Radioprotective 105 (RP105). However, little is known about how RP105 is used by MZ B cells. In this study, we investigated TLR4- or RP105-dependent MZ B cell responses by utilizing agonistic monoclonal antibodies (mAbs) to each receptor. Cross-linking TLR4 and RP105 at the same time with the mAbs induced robust IgM-secreting plasma cell generation as lipid A moiety of LPS. In contrast, stimulation with either mAb alone did not elicit such responses. RP105-deficient MZ B cells failed to produce IgM-secreting plasma cells in response to lipid A. TLR4 or lipid A stimulation of MZ B cells up-regulated their B lymphocyte-induced maturation protein 1 (Blimp-1) and X-box-binding protein 1 (Xbp-1) mRNA expression. RP105 stimulation alone did not give these responses and in fact decreased TLR4-mediated their expression. Compared with wild-type (WT) MZ B cells, RP105-deficient MZ B cells exhibited increased levels of Blimp-1 and Xbp-1 mRNA expression in response to lipid A. Lipid A or TLR4 plus RP105 stimulation induced massive proliferation and expression of Bcl-xL and c-Myc in WT but not RP105-deficient MZ B cells. These responses contributed to TLR4-mediated anti-apoptotic responses in MZ B cells. Thus, RP105 contributes in a unique way to the TLR4-dependent survival, proliferation and plasma cell generation of MZ B cells.

Keywords: innate immunity, LPS, T cell-independent immune response

Introduction
The innate immune system quickly recognizes and responds to microbial products to provide a first line of defense against pathogens. Mediators of these innate responses include Toll-like receptor (TLR) family proteins that recognize many pathogen products (1). They are characterized by extracellular leucine-rich repeat motifs (LRMs) and intracellular Toll/IL-1 receptor (TIR) domains. TLR4 was the first TLR to be identified and its extracellular domain forms a heterodimeric complex with the secreted MD-2 protein (2, 3). Neither TLR4−/− or MD-2−/− mice responds to LPS, so both members of the TLR4/MD-2 complex are essential for LPS responses (4, 5). Recent crystal structure analyses revealed that MD-2 has a large hydrophobic cavity that could accommodate LPS (6). LPS binding to MD-2 induces homodimerization of the 1:1 TLR4/MD-2 complex, in a tail-to-tail orientation with two TLR4 molecules (7).

B cells can contribute to innate immunity by rapidly producing antibodies and uniquely express another pair of TLR family proteins important for LPS responses. Radioprotective 105 (RP105), a homolog of TLR4, is an LRM containing
Roles for RP105/MD-1 in marginal zone B cells

Spleen B cells are divisible into two major populations, follicular (FO) and marginal (MZ) B cells (B220CD21low CD23high and B220CD21high CD23low-neg) (19, 20). Uniquely located near the splenic marginal sinus, MZ B cells play an important role in host defense and initiate rapid T-cell-independent (T-I) immune responses to pathogen products such as LPS (21). In fact, encounter with TLR ligands results in robust proliferation and Ab production. In contrast, FO B cells reside in the splenic follicle near the T-cell zone and participate in T-cell-dependent (T-D) immune responses. MZ B cells express higher densities of TLR4/MZ-2 and RP105/MZ-1 than FO B cells (16, 22, 23). In keeping with this, MZ B cells can respond to low concentrations of LPS, rapidly proliferate and produce large amounts of Ab. However, much less is known about precise roles for TLR4/MZ-2 and RP105/MZ-1 in these B cell subsets.

We have now utilized unique agonistic mAbs to TLR4 or RP105 and gene-targeted mice to explore the importance of these LPS sensors in T-I immune responses. TLR4 and RP105 independently transmit their signals in spleen B cells. Although TLR4 is essential for LPS recognition and responses, TLR4 stimulation of MZ B cells is not sufficient for their survival, proliferation and generation of IgM-secreting plasma cells. On the contrary, the RP105/MZ-1 complex is known to be critical for RP105 signaling in spleen B cells (11–13). RP105 forms a complex with MD-1, a homolog of MD-2 (14). RP105−/− or MD-1−/− spleen B cells show reduced proliferative responses to LPS (10, 15). RP105- and MD-1-deficient mice are severely impaired with respect to hapten-specific Ab production against LPS (16). Additionally, recent crystal structure analyses indicate that MD-1 may recognize LPS with low affinity (17, 18). These results suggest that RP105/MD-1 and TLR4/MD-2 are functionally linked. However, how these LPS sensors cooperate together is poorly understood.

B-cell preparation and proliferation in vitro

Spleen cells were prepared from 8- to 10-week-old mice, and their B cells were isolated by magnetic depletion of cells other type with CD43-specific magnetic microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). The purified other B cells were isolated by magnetic depletion of cells. Spleen cells were prepared from 8- to 10-week-old mice, and their B cells were isolated by magnetic depletion of cells other type with CD43-specific magnetic microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). The purified other B cells were isolated by magnetic depletion of cells.

Methods

Mice

C57BL/6 mice were purchased from Japan SLC (Hamamatsu, Japan) and were used at 8–10 weeks of age. C57BL/6, RP105−/− (15) and TLR4−/− (4) mice were maintained in microisolator cages under specific pathogen-free conditions and maintained in the animal facility of University of Toyama. All experiments were performed according to the guidelines for the care and treatment of experimental animals at University of Toyama (Approved No. S-2010MED-26).

Reagents

Lipid A from Salmonella minnesota was purchased from Sigma–Aldrich (St Louis, MO, USA). CpG-B was purchased from InvivoGen (San Diego, CA, USA). Annexin V-FITC was purchased from BD Bioscience (San Diego, CA, USA).

Antibodies and flow cytometry

Purified anti-mouse RP105 mAb (clone RP/14), anti-mouse MD-1 mAb (clone MD113) and anti-mouse TLR4 mAb (clone UT12) were prepared as previously described (10, 24, 25).

The following antibodies for flow cytometry were purchased from BD Bioscience: phycoerythrin (PE)-conjugated anti-CD138 (clone 281-2) and FITC-conjugated anti-CD21/CD35 (clone 7G6).

The following antibodies for flow cytometry were purchased from eBioscience (San Diego, CA, USA): FITC- or APC-conjugated anti-CD45R/B220 (clone RA3-6B2), PE-conjugated anti-RP105 (clone RP/14), PE-conjugated anti-MD-1 (clone MD14), PE-conjugated anti-TLR4/MD-2 (clone MTS510) and PE-Cy7-conjugated anti-CD23 (clone B3B4).

The cells (1 × 10⁶) were incubated with purified anti-mouse FcγR (clone 2.4G2) to block binding of the labeled antibodies to FcγR. After 15 min, the cells were stained with predetermined optimal concentrations of the respective antibodies; 7-aminomycin D (BD Bioscience) was used to exclude dead cells. Flow cytometry analyses were conducted on a FACSCalibur and FACSCanto (Becton Dickinson & Co., Mountain View, CA, USA), and the data were analyzed with Flowjo software (Treestar, San Carlos, CA, USA).

Cell sorting

For sorting for FO and MZ B cells, spleen cells were stained with FITC-conjugated anti-CD21/CD35, APC-conjugated anti-CD45R/B220 and PE-Cy7-conjugated anti-CD23. Cells were sorted on a FACSAria (Becton Dickinson & Co.). Sorting gates and post sort analyses are presented in Fig. 1(A).

Enzyme-linked immunosorbent assay

Purified B cells (1 × 10⁵ per 200 µl per well) were cultured in a 96-well plate in RPMI 1640 medium (Life Technologies,
Grand Island, NY, USA) supplemented with 8% heat-inactivated fetal calf serum, 50 μM 2-ME, penicillin (50 U ml\(^{-1}\) / ml) and streptomycin (50 μg ml\(^{-1}\) / ml). The purified anti-RP105, anti-TLR4, anti-MD-1, CpG-B or lipid A was added at the same time the cells were plated. Each culture was set up in triplicate. The IgM concentration in the culture supernatants was titrated by ELISA. Briefly, ELISA plates were coated with unlabeled isotype-specific anti-mouse Ig. IgM in the B cells were detected with biotin-conjugated isotype-specific anti-mouse IgM (Southern Biotech, Birmingham, AL, USA) and HRP-conjugated streptavidin (Southern Biotech). HRP-conjugated streptavidin was detected with o-phenylenediamine (Sigma–Aldrich) and ODs of 490 nm were determined with a Micro Plate Reader (Bio-Rad, Hercules, CA, USA).

Preparation of RNA and cDNA
Total RNA was isolated with RNeasy mini kit (Qiagen, Hilden, Germany) or TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer’s instructions. RNA was reverse transcribed with a TaqMan Reverse Transcription Reagents (Applied Biosystems, Carlsbad, CA, USA) following the manufacturer’s instructions.

Real-time quantitative PCR
Real-time quantitative PCR (RT-qPCR) was performed with a TaqMan Gene Expression Master Mix (Applied Biosystems) and analyzed with an Mx3000P (Agilent Technologies, Santa Clara, CA, USA) following the manufacturer’s instructions. Relative transcript abundance was normalized for that of Hprt mRNA. The information for primers used for real-time PCR is listed in Supplementary Table 1, available at International Immunology Online.

Western blotting
The cells were lysed for 60 min on ice in lysis buffer (50 mM Tris–HCl buffer (pH 7.5), 150 mM NaCl, 1% Triton X-100, 5 mM EDTA and protease inhibitor). Cell lysates were clarified by centrifuged at 12,000 \( \times \) g, 4°C for 15 min. Supernatants were separated by SDS–PAGE and transferred onto PVDF membrane. After blocking with 5% skim milk, the membranes were incubated for 1 h with anti-Bcl-xL antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or anti-β-actin (Santa Cruz Biotechnology) in solution 1 of Can Get Signal (Southern Biotech, Birmingham, AL, USA) and HRP-conjugated streptavidin was detected with o-phenylenediamine (Sigma–Aldrich) and ODs of 490 nm were determined with a Micro Plate Reader (Bio-Rad, Hercules, CA, USA).

Statistical analysis
The data are given as means values ± SDs. Statistical significance was evaluated by one-way analysis of variance followed by post-hoc Tukey’s test. \( P < 0.05 \) was considered statistically significant.

Results
RP105/MD-1 is highly expressed on MZ B cells
Before investigating the relative importance of RP105/MD-1 and TLR4/MD-2 in specialized B cell subsets, FO and MZ B cells were isolated from spleens (Fig. 1A) and evaluated their mRNA expression by RT-qPCR (Fig. 1B). MD-1 and TLR4 mRNA levels were similar in FO and MZ B cells, but expression of RP105 and MD-2 in MZ B cells were higher than in FO B cells. We also examined cell surface expression of these complexes by flow cytometry (Fig. 1C). The MTS510 mAb
specifically recognizes the TLR4/MD-2 complex. As others and we reported previously (16, 22, 23), FO B cells have trace amounts of TLR4/MD-2 by staining with MTS510 mAb. TLR4/MD-2 expression on MZ B cells was slightly higher than that on FO B cells. The UT12 mAb recognizes TLR4 alone (25) and it gave similar results to the MTS510 mAb. RP105 and MD-1 were uniformly expressed on these B cell subsets, while MZ B cells had high densities of RP105 and MD-1.

**TLR4 plus RP105 ligation of MZ B cells induces robust plasma cell generation and IgM production**

Our experiments then focused on potential functions for RP105/MD-1 and TLR4/MD-2 on the B-cell subsets. To explore cooperation between RP105/MD-1 and TLR4/MD-2, we utilized agonistic mAbs specific for the two receptors. Both mAbs similarly induced massive proliferation of spleen B cells from wild-type (WT) mice (Supplementary Figure 1A is available at *International Immunology* Online). Lipid A induced expansion of spleen B cells from WT mice, but TLR4+/− spleen B cells did not proliferate upon lipid A stimulation. As we previously reported, proliferative responses of RP105−/− spleen B cells to lipid A were reduced by at least an order of magnitude (Supplementary Figure 1B is available at *International Immunology* Online) (15, 16). We now show that RP105−/− or TLR4−/− spleen B cells respond normally to anti-TLR4 or anti-RP105 mAb, respectively, suggesting that RP105/MD-1 and TLR4/MD-2 use distinct signaling pathways to promote spleen B-cell proliferation independently.

Then, we stimulated FO and MZ B cells from WT spleen with either lipid A, anti-RP105, anti-TLR4 or anti-RP105 plus anti-TLR4 and analyzed CD138 (Syndecan-1) expression on the stimulated cells (Fig. 2A). CD138 is normally expressed on bone marrow pre-B cells and IgM-secreting plasma cells but not on mature B cells. Lipid A stimulation increased the percentages of B220+ CD138+ cells among both FO and MZ B-cell subsets (Fig. 2A). Upon lipid A stimulation, absolute numbers of CD138+ cells generated from MZ B cells were ~7-fold higher than those from FO B cells (Fig. 2B) since the percentage of CD138+ cells and the number of cultured cells in MZ B cells were higher than those in FO B cells (Fig. 2A and C). The percentages of CD138+ cells in FO and MZ B cells increased with anti-TLR4 as lipid A (Fig. 2A). However, absolute numbers of CD138+ cells induced by anti-TLR4 were fewer than those by lipid A (Fig. 2B). This was because anti-TLR4 alone recovered less numbers of cultured cells than lipid A (Fig. 2C). Also, anti-RP105 stimulation induced few CD138+ cells in FO and MZ B cells (Fig. 2A and B). Previous studies showed that both TLR4/MD-2 and RP105/MD-1 are required for spleen B cell responses to lipid A, so we stimulated FO and MZ B cells with anti-TLR4 plus anti-RP105. Interestingly, while percentages of CD138+ cells resulting from this combination were lower than with anti-TLR4 alone-stimulated cells in both FO and MZ B cells (Fig. 2A), the number of CD138+ cells was dramatically increased by co-stimulation of anti-RP105 in the anti-TLR4-stimulated MZ B cells (Fig. 2B). This was because anti-TLR4 plus anti-RP105 stimulation recovered a large number of cultured cells as compared with TLR4 ligation alone in MZ B cells (Fig. 2C).

MZ B cells rapidly produce IgM in response to T-I antigens (21). As previously reported, lipid A stimulation induced more IgM production in MZ B cells compared with FO B cells in 72 h cultures (20) (Fig. 2D). Anti-TLR4 induced a small amount of IgM in MZ B, whereas FO B cells did not produce significant amount of IgM by this stimulation. Anti-RP105-stimulated FO and MZ B cells did not produce IgM. Consistent with induction of CD138+ cells, anti-TLR4 plus anti-RP105 stimulation of MZ B cells produced as much IgM within 72 h as lipid A (Fig. 2D, right panel). FO B cells produced only small amounts of IgM with stimulation this protocol (Fig. 2D, left panel). These results demonstrate that RP105 can cooperate with TLR4 to induce robust plasma cell generation and IgM production from MZ B cells. Anti-TLR4 plus anti-RP105 stimulation may mimic lipid A stimulation in rapid T-I immune responses.

**RP105/MD-1 is indispensable for lipid A-induced plasma cell generation and IgM production by MZ B cells**

TLR-like complex requirements for terminal differentiation of MZ B cells were then explored with RP105−/− mice. We stimulated WT or RP105−/− MZ B cells with anti-TLR4 plus anti-RP105, lipid A or the CpG-B TLR9 ligand. CpG-B was included to permit comparison with an unrelated type of TLR ligand stimulation. The percentage of CD138+ cells in RP105−/− MZ B cells was similar to that in WT MZ B cells in response to anti-TLR4 plus anti-RP105 stimulation (Fig. 3A). Induction of CD138+ cells in response to lipid A was impaired in RP105−/− MZ B cells compared with WT MZ B cells. Furthermore, cell number of CD138+ cells in RP105−/− MZ B cells was severely reduced compared with WT MZ B cells in response to anti-TLR4 plus RP105 or lipid A (Fig. 3B) since RP105−/− MZ B cells recovered few cultured cells compared with WT MZ B cells (Fig. 3C). Induction of CD138+ cells in response to CpG-B was not impaired in RP105−/− MZ B cells (Fig. 3A and B). Consistent with this index of CD138+ cell generation, RP105−/− MZ B cells had defective IgM production induced by anti-TLR4 plus anti-RP105 or lipid A but not CpG-B (Fig. 3D).

We previously reported that anti-MD-1 mAb treatment suppressed anti-RP105 or lipid A-induced spleen cell responses (10). Induction of CD138+ cells in response to lipid A was slightly impaired by anti-MD-1 in MZ B cells (Fig. 4A). Anti-MD-1 treatment did not reduce the percentages of CD138+ cells induced by either anti-TLR4 plus anti-RP105 or CpG-B. However, anti-MD-1 stimulation decreased cell number of CD138+ cells or cultured cells and IgM production induced by anti-TLR4 plus anti-RP105 or lipid A but not CpG-B in MZ B cells (Fig. 4B–D). Thus, RP105/MD-1 on MZ B cells is very important for plasma cell generation and IgM production induced by TLR4- but not TLR9-mediated signals.

**RP105 stimulation does not up-regulate B lymphocyte-induced maturation protein 1 and X-box binding protein 1 mRNA expression and in fact negatively regulates these responses to TLR4 ligation**

B lymphocyte-induced maturation protein 1 (Blimp-1) has been proposed to be a master regulator of plasma cell differentiation (26–28). X-box binding protein 1 (Xbp-1) is also
required and seems to be a proximal regulator of the secretory phenotype in plasma cells (27). We tested the possibility that RP105 controls Blimp-1 and Xbp-1 expression in the B cell subsets (Fig. 5). MZ B cells had higher level of Blimp-1 expression than FO B cells in non-stimulation (Fig. 5A, left panel). Lipid A or anti-TLR4 but not anti-RP105 stimulation increased transcripts for Blimp-1 in both FO and MZ B cells. Interestingly, co-stimulation of anti-RP105 significantly decreased TLR4-mediated Blimp-1 mRNA expression in both the B cell subsets (Fig. 5A, left panel). Similar levels of Xbp-1 transcripts were seen in unstimulated MZ and FO B cells (Fig. 5A, right panel). Levels of Xbp-1 mRNA expression were significantly increased by lipid A or anti-TLR4 in MZ B but not FO B cells. Anti-RP105 stimulation alone did not increase the level of Xbp-1 mRNA but decreased Xbp-1 expression in anti-TLR4-stimulated both FO and MZ B cells. Furthermore, with lipid A or anti-TLR4 plus anti-RP105 stimulation, RP105−/− MZ B cells had enhanced levels of Blimp-1 and Xbp-1 mRNA expression relative to their WT counterparts (Fig. 5B).

Pax5 and Bcl-6 proteins are important repressors of Blimp-1 expression (27, 28). However, we did not observe elevated levels of Pax5 or Bcl-6 mRNA with anti-RP105
stimulation in MZ B cells, indicating that these transcriptional factors are not involved in the RP105-mediated suppression of Blimp-1 (Supplementary Figure 2 is available at International Immunology Online). Lipid A, anti-TLR4 or anti-TLR4 plus anti-RP105 stimulation similarly decreased Pax5 and Bcl-6 mRNA expression in MZ B cells (Supplementary Figure 2 is available at International Immunology Online). Since Blimp-1 can repress Pax5 and Bcl-6 transcription (28), this was presumably due to the increased levels of Blimp-1. These results indicate that TLR4 is important for up-regulation of Blimp-1 and Xbp-1 mRNA expression in MZ B cells. In contrast, RP105 stimulation alone did not up-regulate Blimp-1 and Xbp-1 expression. RP105 uniquely appears to negatively regulate TLR4-mediated their expression.

**RP105 stimulation by itself does not induce proliferation of MZ B cells but dramatically augments TLR4-dependent their proliferation**

MZ B cells exhibit a robust proliferative response following stimulation with LPS (21). Our findings suggested that RP105/MD-1 on MZ B cells might be required for TLR4/MD-2-dependent proliferation. Therefore, we investigated the ability of FO and MZ B cells to proliferate in response to lipid A, anti-TLR4, anti-RP105 or anti-TLR4 plus anti-RP105 (Fig. 6). As previously reported, lipid A-induced proliferative responses of MZ B cells were larger than those of FO B cells (20) (Fig. 6A). While either anti-TLR4 or anti-RP105 stimulation induced weak proliferative responses of MZ and FO B cells, respectively, we observed robust proliferation in response to anti-TLR4 plus anti-RP105 in both the B cell subsets. Proliferative responses to anti-TLR4 plus anti-RP105 were much larger than with lipid A. Since FO and MZ B cells appeared to differentially respond to anti-TLR4 and anti-RP105 (Fig. 6A), we further stimulated them with different doses of the mAbs (Fig. 6B). Interestingly, anti-RP105 induced proliferation of FO B but not MZ B cells. Contrastively, anti-TLR4-induced proliferation was observed in MZ B but not FO B cells. Then, we explored the roles of RP105 in MZ B-cell proliferation. It is of note that proliferative responses to anti-TLR4 plus anti-RP105 or lipid A were severely impaired in RP105−/− MZ B cells (Fig. 6C). Thus, although anti-RP105 by itself does not induce proliferation of MZ B cells, RP105 signals robustly augment TLR4-dependent proliferation of MZ B cells. This response may compensate RP105-mediated suppression of CD138 and Blimp-1 expression. In addition, the B cell subsets appear to differently respond to TLR4 and RP105 ligation.

**RP105 is required for lipid A-induced expression of Bcl-xL and c-Myc in MZ B cells**

Bcl-xL is an NF-κB target gene and plays a crucial role in TLR-triggered cell survival and proliferation (29–31). Furthermore, Vav proteins up-regulate Bcl-xL expression in a common signaling pathway downstream of RP105 and TLR4 in spleen B cells (32). Therefore, we investigated the
expression of Bcl-xL in stimulated FO and MZ B cells (Fig. 7A). The expression of Bcl-xL was not detected in the unstimulated FO and MZ B cells. Lipid A stimulation induced Bcl-xL protein expression in FO and MZ B cells within 60 min, but the expression level of Bcl-xL in FO B cells was smaller than that in MZ B cells. In FO B cells, anti-RP105 induced larger Bcl-xL expression than anti-TLR4. In contrast, anti-TLR4 or anti-RP105 stimulation similarly induced low levels of Bcl-xL in MZ B cells. Interestingly, TLR4 plus RP105 stimulation augmented either mAb alone-mediated Bcl-xL expression in MZ B cells. We also assessed the expression of Bcl-xL in cultured WT and RP105−/− MZ B cells (Fig. 7B). Lipid A-simulated RP105−/− MZ B cells were severely impaired in respect to Bcl-xL expression. Furthermore, decreased expression of Bcl-xL was observed in RP105−/− MZ B cells stimulated with anti-TLR4 plus anti-RP105.

c-Myc is also important for TLR-mediated MZ B cell survival (33). In FO B cells, levels of c-Myc mRNA expression were increased by anti-RP105 but not anti-TLR4 or lipid A stimulation (Fig. 7C). As observed in Bcl-xL expression (Fig. 7A), TLR4 plus RP105 stimulation did not augment RP105-mediated c-Myc expression in FO B cells. In MZ B cells, lipid A but not anti-TLR4 or anti-RP105 stimulation significantly increased c-Myc mRNA expression. Furthermore, anti-TLR4 plus anti-RP105 stimulation slightly increased levels of c-Myc mRNA compared with either mAb alone in MZ B cells. We also assessed the expression of c-Myc mRNA in WT and RP105−/− MZ B cells (Fig. 7D). RP105−/− MZ B cells were impaired in TLR4 plus RP105- or lipid A-mediated c-Myc expression.

These results strongly suggest that RP105 is required for TLR4-dependent induction of Bcl-xL and c-Myc expression in MZ B cells. In addition, RP105 stimulation alone uniquely increases their expression in FO B cells.

RP105 is required for TLR4-mediated anti-apoptotic responses in MZ B cells

To demonstrate the significance of RP105-mediated Bcl-xL and c-Myc induction, we finally examined whether RP105 stimulation contributes to increase anti-apoptotic responses in MZ B cells (Fig. 8). At 24 h of culture, over 80% of cultured WT or RP105−/− MZ B cells were Annexin V− apoptotic cells in medium alone (Fig. 8A). Lipid A stimulation decreased the percentage of Annexin V− apoptotic cells, and this was impaired in RP105−/− MZ B cells. Anti-TLR4 or anti-RP105 stimulation slightly decreased percentages of Annexin V− cells as compared with medium alone. Consistent with induction of Bcl-xL and c-Myc expression, anti-TLR4 plus anti-RP105 stimulation reduced the percentage of Annexin V− cells as lipid A, and this was dependent on RP105. These anti-apoptotic responses were more obvious at 48 h of culture (Fig. 8B). The percentages of Annexin V− cells induced by longer stimulation of either mAb alone were similar to those by 24 h
stimulation in WT MZ B cells, whereas lipid A or anti-TLR4 plus anti-RP105 stimulation dramatically augmented the anti-apoptotic responses. These anti-apoptotic responses were severely impaired in RP105−/− MZ B cells. These results clearly suggest that RP105 is required for TLR4-mediated anti-apoptotic responses in MZ B cells. These RP105-mediated events likely permit MZ B cell survival, proliferation and generation of Ab-secreting plasma cells in cooperation with TLR4 signals (Fig. 9).

Discussion
MZ B cells initiate rapid and robust T-I IgM responses to blood-borne antigens (21). This unique characteristic has partly been interpreted to reflect higher levels of the LPS sensor RP105/MD-1 (16, 22, 23). However, precise roles for this receptor complex have been poorly defined. Here, we show that RP105 ligation on MZ B cells dramatically augmented TLR4-dependent survival, proliferation, plasma cell generation and IgM secretion. RP105-deficient MZ B cells were severely impaired with respect to rapid lipid A-induced IgM secretion. TLR4 but not RP105 stimulation alone upregulated cell surface CD138 and Blimp-1/Xbp-1 mRNA expression. Interestingly, RP105 stimulation negatively regulated TLR4-mediated their expression in MZ B cells. RP105 was required for augmentation of TLR4-induced Bcl-xL and c-Myc expression, which may facilitate anti-apoptosis, cell survival and proliferation of MZ B cells. These results suggest that RP105 has a major role for cell survival and proliferation of MZ B cells in cooperation with TLR4 signals to produce efficient IgM-secreting plasma cells (Fig. 9). The new information now makes it possible to propose RP105/MD-1 mechanisms in T-I type immune responses. Additionally, we observed that FO and MZ B cells were differentially regulated by TLR4 and RP105 signaling. This may give an important information to understand the functional differences between the B cell subsets.

Upon LPS stimulation, TLR4 recruits several adaptor molecules to the TIR domain of its intracellular portion (1). Unlike other TLR family proteins, RP105 lacks the TIR domain (8), suggesting that RP105 and TLR4 might use distinct signaling components. Our experiments indeed revealed that proliferative responses initiated by RP105 ligation are not dependent on TLR4 signaling (Supplementary Figure 1B is available at International Immunology Online). Furthermore, TLR4-deficiency did not influence RP105-dependent spleen B cell proliferation (Supplementary Figure 1B is available at International Immunology Online). While LPS may be recognized by both receptors, they likely transmit different signals. RP105 activates a similar signaling pathway to B-cell receptor signaling, involving Lyn/CD19/Vav, Bruton tyrosine kinase and phosphatidylinositol 3-kinase (11, 13, 32). It is now
before differentiation into plasma cells, activated B cells undergo massive proliferation (27). We demonstrated that RP105 is required for TLR4-mediated Bcl-xL and c-Myc expression in MZ B cells (Fig. 7). RP105 signaling must be required to augment TLR4-dependent cell survival and proliferation. RP105 has been shown to promote NF-κB activation (13), c-Myc and Bcl-xL are NF-κB target genes, which are important for TLR4-mediated cell survival and proliferation (12, 32, 33). Vav protein is phosphorylated by RP105 ligation in the A20 B cell line (32). Interestingly, Vav1/2−/− B cells are severely impaired in anti-RP105- or LPS-induced Bcl-xL expression (32). Therefore, Vav/NF-κB/Bcl-xL and Vav/NF-κB/c-Myc axes may have a crucial role for RP105-mediated survival and proliferation of MZ B cells. Further studies are required to investigate the details of RP105 signaling pathways. It would also be interesting to explore if NF-κB and Vav activation are critical for TLR4 plus RP105-mediated responses in MZ B cells.

TLR4 but not RP105 signals are involved in induction of CD138 expression and up-regulation of Blimp-1 and Xbp-1 mRNA in MZ B cells (Figs 2A and 5A). Moreover, RP105 suppresses anti-TLR4-induced expression of CD138 and up-regulation of Blimp-1/Xbp-1 mRNA (Figs 2A and 5A). While Blimp-1 is a master regulator for plasma cell differentiation (28), at the early phase of LPS responses, MZ B cells may require RP105-mediated survival and proliferative responses at the expense of TLR4-mediated Blimp-1 up-regulation (Fig. 9). These responses may compensate RP105-mediated suppression of cell surface CD138 and Blimp-1/Xbp-1 mRNA expression. In myeloid cells, RP105/MD-1 can be a negative regulator of LPS responses by directly associating with TLR4/MD-2 (34). This interaction results in inhibition of LPS binding to MD-2 on HEK293-transfected cells. However, this inhibitory mechanism is unlikely to be used by MZ B cells since RP105 does not negatively regulate all the lipid A responses in MZ B cells. RP105 is required for TLR4-mediated proliferation, induction of Bcl-xL and c-Myc expression and anti-apoptotic responses (Figs 6–8). It is possible that a transcription factor other than Pax5 or Bcl-6 is responsible for RP105-mediated Blimp-1/Xbp-1 repression (Supplementary Figure 2 is available at International Immunology Online). Another possibility is that a signaling molecule downstream of RP105 directly inhibits TLR4 signaling. It is now important to study transcriptional factors and genes regulated by TLR4 and RP105 signaling in MZ B cells.

RP105-induced B cell responses were partly shared between FO and MZ B cells. Both MZ and FO B cells robustly proliferated in response to TLR4 plus RP105 ligation (Fig. 7). RP105 ligation suppressed TLR4-mediated CD138 and Blimp-1/Xbp-1 mRNA expression in both the B cell subsets (Figs 2A and 5A). In contrast, RP105 linked with TLR4 to augment Bcl-xL and c-Myc expression in MZ B but not FO B cells (Figs 7A and 7C). We also found some differences between FO and MZ B cells in TLR4 or RP105-mediated responses. While TLR4 ligation induced proliferation of MZ B cells, this response did not occur by RP105 ligation (Fig. 6B). This was not reflected by high expression of RP105/MD-1 on MZ B cells (Fig. 1C). RP105 but not TLR4 stimulation induced proliferation of FO B cells (Fig. 6B). These results clearly indicate that these B cell subsets may be differentially affected by TLR4 and RP105 signaling. At this moment, precise roles of RP105 in FO B cells are unclear, but...
further investigation will reveal the whole pictures of roles for TLR4 and RP105 in FO and MZ B cells.

TLR4 plus RP105-mediated robust proliferation seems to be important for generation of plasma cells in MZ B cells. This combination of mAbs also enabled FO B cells to proliferate, but they did not efficiently produce IgM-secreting plasma cells by this combination (Fig. 2B and D). The expression levels of Blimp-1 in FO B cells were much lower than those in MZ B cells (Fig. 5A). Furthermore, RP105 does not link with TLR4 to up-regulate Bcl-xL and c-Myc expression in FO B cells (Fig. 7A and C). Thus, efficient generation of IgM-secreting plasma cells may require not only proliferation but also sufficient levels of Blimp-1 expression and RP105-mediated Bcl-xL and c-Myc induction in cooperation with TLR4.

A recent crystal structure analysis of bovine RP105/MD-1 revealed that RP105/MD-1 forms an unusual tetrameric complex of two RP105 and two MD-1 molecules (35). This 2:2 RP105/MD-1 homodimer is assembled in a head-to-head orientation, resulting in a large distance between their C termini. This structure is totally different from those of other TLRs. TLR4/MD-2 homodimers are assembled in a tail-to-tail orientation, resulting in close proximity between their C termini and induction of signal transduction (7). Given its proposed structure, RP105 is unlikely to independently transmit signals. However, our results show that MZ B cells require RP105 activation for rapid T-I immune responses. Clearly, more structure–function information related to RP105/MD-1 is needed.

As suggested previously, RP105/MD-1 could be physically associated with TLR4/MD-2 (34). Therefore, it would be interesting to investigate this possibility on either FO B or MZ B cells. The interaction on MZ B cells might be stronger than that on FO B cells. Due to the limited number of MZ B cells per spleen, such biochemical experiments would be technically challenging.
In conclusion, while the TLR4/MD-2 complex is essential for recognition of LPS, the RP105/MD-1 complex is also indispensable for rapid and vigorous T1-type immune responses. These observations are informative about cooperative and distinct roles for pathogen receptors used by a highly specialized B-cell subset that constitutes a first line of defense.

Supplementary data

Supplementary data are available at International Immunology Online.

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