Research article

Mouse B1 cell line responds to lipopolysaccharide via membrane-bound CD14

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The role of membrane-bound CD14 in the response of mouse B1 cell lines to lipopolysaccharide (LPS) was studied. The surface profile of mouse TH2.52 B cells was positive for CD5, IgM, B220, CD11b and F4/80, suggesting that TH2.52 cells carried the typical phenotype of B1 cells. Furthermore, TH2.52 B1 cells were found to express membrane-bound CD14, which plays a critical role in LPS recognition. TH2.52 B1 cells responded to a very low concentration of LPS and exhibited: (i) augmentation of DNA synthesis; (ii) activation of nuclear factor (NF)-κB; and (iii) phosphorylation of extracellular signal regulated kinase 1/2 (Erk1/2). They were markedly inhibited by anti-CD14 antibody. Therefore, the expression of membrane-bound CD14 was suggested to provide high sensitivity to LPS for TH2.52 B1 cells.

Introduction

The immune system contains a subset of B-cells that are phenotypically and functionally distinct from the conventional B-cell population.[1–4] This subset expresses a low level of CD5 antigen and is called $CD5^+$ B-cells. The B-cell population including $CD5^+$ B-cells and $CD5^-$ B-cells that are very similar to $CD5^+$ B-cells is now referred to as B1-cells. B1 cells are the predominant B-cells during fetal life. In adults, B1 cells predominate in peritoneal and pleural cavities[5,6] and in the lamina propria of the gut,[7] but account for only a few percent of splenic B-cells and are rare in the lymph nodes, thymus and peripheral blood.[8] In contrast to conventional B2-cells, mature B1-cells characteristically possess a self-replenishing capacity.[3,9] Although B1-cells constitute only a few percent of the total peripheral B-cells, about one-half of serum IgM and IgA, and a significant amount of IgG, have been shown to be derived from B1-cells.[10] B1 antibodies are characterized by broad specificity and low affinity,[11] and react against multivalent antigens of bacterial cell wall components,[12,13] such as LPS,[14] phosphatidylcholine,[15] α1-3 dextran.[16] The antibodies are also reactive to *Enterobacter* and *Serratia* spp. in the *Enterobacteriaceae* family.[17] The antigen specificity of B1 antibodies suggests that B1-cells constitute a primitive immune system and participate in the protective immunity against enteric bacteria by forming a first line of defense.[11,18] The mouse TH2.52 B-cell line is a hybridoma cell line between mouse splenic B-cells and mouse M12.4.1 B lymphoma. The stimulation of TH2.52 cells with LPS results in differentiation of IgM-secreting cells and secretion of cytokines.[19,20] In the present study, we found that the TH2.52 B-cell line with the surface profile of B1 cell type expressed membrane-bound CD14. The role of CD14 in the response of TH2.52 B1 cells to LPS is discussed.

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Materials and Methods

Antibodies

Anti-mouse CD14 monoclonal antibody (mAb; 4C1) which blocks the binding of LPS to CD14 was provided

by Y. Adachi. Other antibodies used were as follows: phycoerythrin (PE)-conjugated rat anti-CD5 mAb; rat anti-mouse CD45R/B220 mAb; anti-CD14 mAb (mC5-3; PharMingen, San Diego, CA, USA); biotin-conjugated goat anti-mouse IgM antibody (Southern Biotechnology Associates, Birmingham, AL, USA); PerCP-conjugated streptavidin (Becton Dickinson, San Jose, CA, USA); FITC-conjugated rat anti-CD11b mAb (Immunotech, Marseilles, France); FITC-conjugated rat anti-F4/80 mAb (Cosmo Bio, Tokyo, Japan); rat anti-mouse RP105 mAb (Wako, Osaka, Japan); and FITC-conjugated goat anti-rat IgG antibody (Cappel, Aurora, OH, USA).

Cells and cell culture

Mouse M12.4.1 B lymphoma and its hybridoma with mouse splenic B-cells, TH2.52 B-cells[19] were cultured in RPMI 1640 medium containing 5% heat-inactivated fetal calf serum (Gibco-BRL, Grand Island, NY, USA) at 37˚C under 5% CO_2.

Immunofluorescent staining and laser flow cytometry

TH2.52 B-cells were stained with an appropriate dilution of fluorescent antibodies against various cell surface markers. The cell surface profile was analyzed with a laser flow cytometer (FACSCalibur, Becton Dickinson). The fluorescence intensity was expressed on a log scale.

Detection of mRNA levels for CD14 by reverse transcriptase-polymerase chain reaction (RT-PCR)

Oligonucleotide primers used for RT-PCR were (5′-GCAGGGCTCCGAATAGAATCC-3′) for CD14-1 and (5′-GGAAGCCAGAGACACCATCG-3′) for CD14-2.[21] Total RNA was extracted from TH2.52 cells and its parent M12.4.1 B lymphoma cells. RT-PCR was performed as described previously.[22]

DNA synthesis

DNA synthesis in TH2.52 cells was assayed by [^{3}H]-thymidine incorporation into the nucleus. The cells (3 x 10^3/100 µl) were plated in 96-well plates and incubated with various concentrations of LPS in the presence or absence of anti-CD14 mAb (4C1) at 1 µg/ml for 24 h. [^{3}H]-Thymidine (1 µCi/well, Amersham, UK) was added to the cultures; 18 h later, the cells were harvested on a glass fiber filter. Radioactivity was counted as cpm with a Beckman β-counter.

Luciferase reporter gene assay for NF-κB activation

TH2.52 cells (2 x 10^5/ml) were seeded in 35-mm plastic dishes, and on the following day the cells were transfected for 8 h with 0.5 µg of luciferase reporter genes driven by 5 times tandem repeats of NF-κB (pNF-κB-Luc plasmid, PathDetect system, Stratagene, La Jolla, CA, USA) and 0.5 µg of pCMV-β-gal plasmid[23] by the Effectene transfection reagent, based on a proprietary non-liposomal lipid (Qiagen, Hilden, Germany). The transfected cells were washed to remove the plasmids and further incubated for 72 h. They were stimulated with LPS (10 ng/ml) in the presence or absence of anti-CD14 mAb for 8 h, and lysed with lysis reagent from Promega (Madison, WI, USA) for measurement of luciferase activity. The cell lysates (10 µl) were mixed with the luciferase substrate (100 µl), and the luciferase activity was determined by a luminometer. The activity of β-galactosidase was measured by using *o*-nitrophenyl-galactopyranoside as substrate and was used for normalizing transfection efficiencies. Values are expressed as the mean of triplicates ± standard deviation (SD).

Immunoblotting

TH2.52 cells were seeded in 35-mm plastic dishes (4 x 10^5 cells/dish) and incubated with LPS (1 ng) for 30 min. Briefly, cells were lysed in lysis buffer containing 0.5 M Tris-HCl, 4% SDS, 2 mM mercaptoethanol and boiled for 5 min at 100˚C. Aliquots were electrophoresed under reducing conditions in a 4–20% gradient polyacrylamide gel and transferred to a polyvinylidene difluoride membrane filter. The membranes were incubated with a panel of rabbit polyclonal antibody against extracellular signal regulated kinase 1/2 (Erk1/2) and phospho Erk1/2 (New England Biolabs, Beverly, MA, USA) for 1 h. The membranes were further treated with horseradish peroxidase-conjugated protein G for 1 h. Immune complexes on the blots were detected with an enhanced chemiluminescence substrate (New England Nuclear, Boston, MA, USA).

Statistical analysis

Experimental results are expressed as the mean ± SD in triplicate. Statistical significance was determined by Student's *t*-test.

RESULTS

Cell surface profile of TH2.52 cells

The cell surface profile of TH2.52 B-cells was characterized by laser flow cytometry using mAbs against various

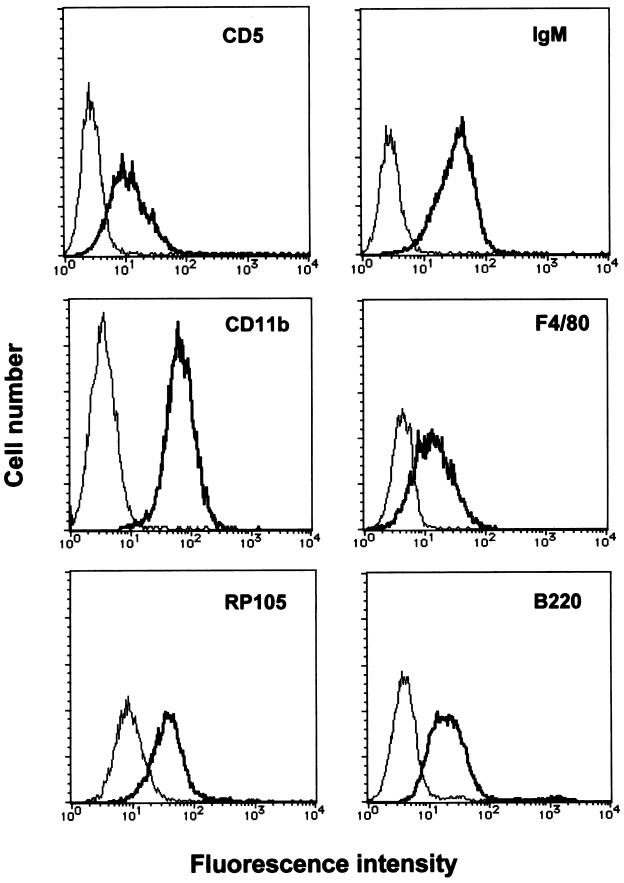

Fig. 1. Cell surface profile of TH2.52 cells. Laser flow cytometric histograms of CD5, IgM, CD11b, F4/80, B220 and RP105 expression on TH2.52 cells are shown. TH2.52 cells were stained with specific antibodies (thick line) and irrelevant control antibodies (thin line).

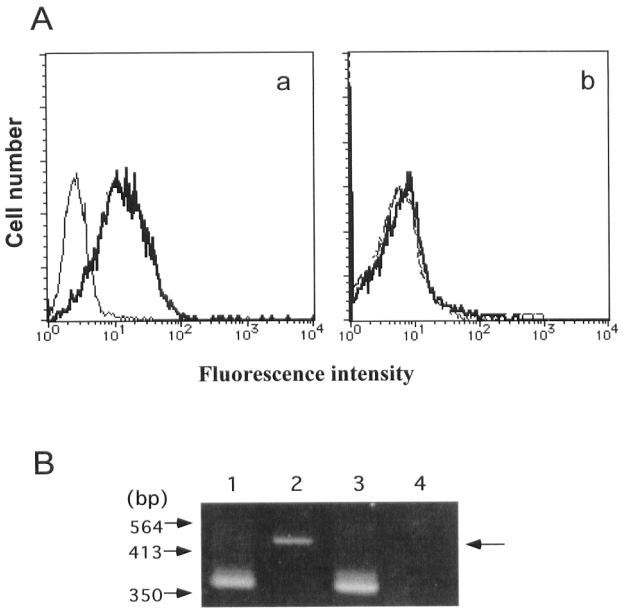

Fig. 2. Detection of CD14 on TH2.52 B1 cells and its partner M12.4.1 B lymphoma cells. (A) Laser flow cytometric histograms of CD14 expression on TH2.52 cells (a) and M12.4.1 B lymphoma cells (b). (B) Detection of CD14 mRNA in TH2.52 cells and M12.4.1 B lymphoma cells. The mRNA products of the house-keeping gene GAPDH (lanes 1 and 3) and CD14 gene (lanes 2 and 4) in TH2.52 cells (lanes 1 and 2) and M12.4.1 B lymphoma cells (lanes 3 and 4) are shown.

cell surface markers (Fig. 1). The surface profile of TH2.52 cells was positive for CD5, surface IgM, B220, CD11b, F4/80 and RP105, suggesting that TH2.52 cells possessed the characteristics of B1-cell phenotypes. Surprisingly, we found that CD14 was expressed on the surface of TH2.52 B1 cells (Fig. 2A). The intensity of CD14 expression was almost the same as that of the mouse RAW 264.7 macrophage cell line. In addition, the profile of the partner M12.4.1 B-cells was $CD5^+$, IgM^-, $B220^+$, $CD11b^-$, $RP105^+$ and $F4/80^-$. Reverse transcriptase/PCR analysis using TH2.52 cells resulted in the amplification of a single product with the predicted size (about 413 bp long; Fig. 2B). On the other hand, neither membrane-bound CD14 nor its mRNA was detectable in the partner M12.4.1 B-cells.

The inhibition of LPS-induced activation by anti-CD14 antibody

TH2.52 B1-cells were found to express membrane-bound CD14 which plays a critical role on LPS recognition.[24] In order to elucidate the participation of CD14 in the response of TH2.52 B1-cells to LPS, we studied the effect of anti-CD14 mAb (4C1) on LPS-induced activation of TH2.52 B1 cells (Fig. 3A). LPS at 1 and 10 ng/ml induced the marked enhancement of DNA synthesis in TH2.52 cells (P <0.01), although higher concentrations of LPS did not affect DNA synthesis significantly. The enhancement of DNA synthesis in TH2.52 B1 cells by LPS at 1 and 10 ng/ml was inhibited markedly in the presence of 4C1 mAb which blocks the binding of LPS to CD14 (P <0.01). An irrelevant mAb did not affect it. The effect of anti-CD14 mAb (4C1) on LPS-induced NF-κB activation of TH2.52 B1 cells was studied (Fig. 3B). NF-κB activation was measured by the NF-κB-dependent luciferase reporter gene activity. LPS markedly enhanced NF-κB-dependent reporter gene activity in TH2.52 cells. 4C1 mAb completely blocked the enhancement by LPS. 4C1 mAb alone did not affect reporter gene activity. Furthermore, the effect of anti-CD14 mAb on LPS-induced phosphorylation of Erk1/2 was studied (Fig. 3C). Although LPS definitely induced the phosphorylation of Erk1/2 in TH2.52 B1 cells, 4C1 mAb completely inhibited the phosphorylation of Erk 1/2. In addition, anti-CD14 antibody (mC5-3) which does not block the binding of LPS to CD14 did not inhibited its phosphorylation.

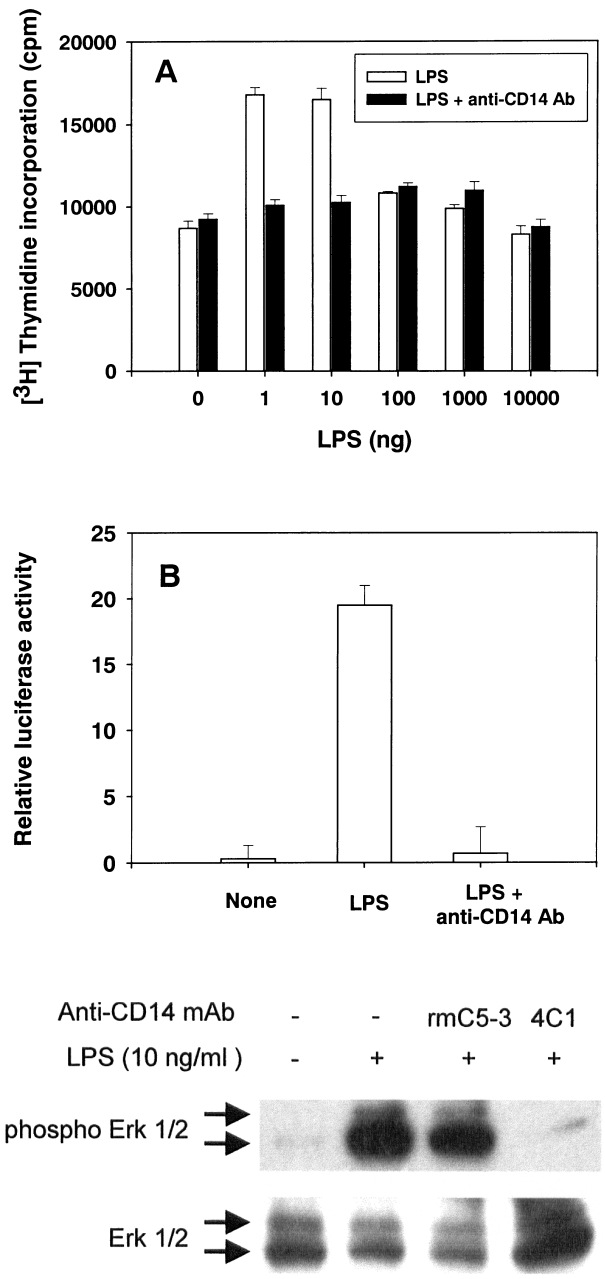

Fig. 3. The inhibition of LPS-induced activation by anti-CD14 mAb. (A) [^{3}H]-Thymidine incorporation by TH2.52 cells in cultures with various concentrations of LPS in the presence or absence of anti-CD14 mAb (4C1) which blocks the binding of LPS to CD14. (B) NF-κB activation of TH2.52 cells in cultures of LPS in the presence or absence of anti-CD14 mAb (4C1). (C) The phosphorylation of Erk1/2 in the presence or absence of anti-CD14 mAb (4C1). Anti-CD14 mAb (mC5-3) which does not block the binding of LPS to CD14 was also used. Note that mC5-3 mAb does not inhibit the phosphorylation of Erk1/2.

DISCUSSION

In the present study, we demonstrated that TH2.52 B-cells express membrane-bound CD14 and respond to LPS through it. The TH2.52 cell line is the hybridoma between mouse M12.4.1 B lymphoma and mouse splenic B-cells,[19] and expresses characteristic surface markers, such as CD5, CD11b, B220 and F4/80. This phenotype corresponds to B/macrophage cells which Borrello and Phipps[25] reported as B1 lineage cells. The expression of CD14 on TH2.52 B1-cells would not be strange because TH2.52 cells express F4/80 and CD11b which are macrophage-related markers. The expression of CD14 on TH2.52 B1 cells is also supported by the findings that mouse CD5$^+$ B-cells are more sensitive than conventional B-cells in response to low concentrations of LPS[26] and that autoimmune-prone human B1-cells express CD14.[27] The expression of CD14 on TH2.52 cells might be responsible for the B1 character. Recently, it has been reported that CH12.LX, a murine B-cell line, expresses CD14 and responds to LPS.[28] However, it is not clear whether CH12.LX cells belong to B1-cells or not.

Considering the pivotal role of CD14 in LPS recognition,[24] the expression of CD14 on TH2.52 B1 cells might provide the high sensitivity to LPS. In fact, TH2.52 B1 cells responded to a very low concentration of LPS, and LPS-induced activation of TH2.52 B1 cells was almost completely blocked by anti-CD14 mAb. CD14 molecules expressed on TH2.52 B1 cells might collect LPS at a low concentration to their cell surface and present it efficiently to LPS receptors. On the other hand, conventional B2-cells expressing no CD14 might require a higher concentration of LPS for their activation. Thus, the expression of CD14 may confer the high sensitivity to LPS on CD14-expressing TH2.52 B1-cells. This idea is strongly supported by the fact that transfection of CD14 to pre-B-cells[29] and vascular endothelial cells[30] dramatically enhanced their sensitivity to LPS.

Unlike conventional B2-cells, B1-cells have various novel characteristics, such as localization of B1-cells in the peritoneal and pleural cavity,[5,6] self-replenishing capacity,[3,9] and production of natural antibody with broad specificity and low affinity.[11] These peculiar characteristics of B1-cells might be explained by their high sensitivity to LPS. They would proliferate after exposure to LPS as well as specific Ag. Furthermore, non-specific activation of B1-cells by LPS might induce polyclonal activation of B1-cells, independent of T-cells. It is reasonable that B1-cells produce natural antibody with low affinity and broad specificity. It is of interest that B1-cells produce anti-LPS antibody.

TH2.52 B1 cells were found to express CD11b, RP105, and TLR4 (data not shown), which were the possible candidates of LPS receptor. The present study clearly demonstrates that CD14 accelerates LPS-induced cell activation. However, the LPS receptor on TH2.52 B1 cells, to which CD14 transfers LPS, is still unclear. The TH2.52 B1 cells used in the present study might be useful for characterization of mouse B1-cells and further understanding of their response to LPS.

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