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Anti-CD14 antibody reduces LPS responsiveness *via* TLR4 internalization in human monocytes

Donghee Kim[a], Jae Young Kim[b,*]

[a] Lee Gil Ya Cancer and Diabetes Institute, Gachon University, Incheon, Republic of Korea
[b] Department of Biological Science, Gachon University, Incheon, Republic of Korea

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ABSTRACT

CD14 is an LPS sensing receptor that is primarily expressed in monocytes. CD14 binds and transfers bacterial LPS to the surface TLR4:MD-2 complex to enable its recognition. After recognizing LPS, this complex produces the first intracellular signals *via* TIRAP and MyD88, after which surface TLR4/LPS complex is rapidly internalized and produces additional signals *via* TRAM and TRIF. It was recently suggested that CD14 is a key regulator of LPS-induced TLR4 endocytosis and second signaling. In the present study, we showed that surface TLR4 expressions of human primary monocytes and cell line THP-1 were significantly reduced after treatment with anti-CD14 Ab. Among three anti-CD14 Abs with different epitope specificities used in this study, My4, which has an epitope specificity for LPS binding domain of the CD14 molecule, was found to be the most potent at reduction of surface expression of TLR4 as well as CD14. To test the reason for this reduction, we performed an *in vitro* internalization assay using anti-TLR4 Ab conjugated with toxin. The results of this analysis indicated surface CD14 ligation-mediated TLR4 internalization, and the mechanism of the internalization was found to be partially clathrin-dependent. We next examined NF-κB/AP-1 activation and TNF-α production of THP-1XBlue-CD14 cells in response to LPS challenge with or without My4 pre-treatment. The results revealed that NF-κB/AP-1 activation and TNF-α production of cells treated with My4 were significantly impaired when compared to the control. Our results suggest that membrane CD14 ligation-mediated TLR4 internalization is a novel mechanism for effective down-regulation of surface expression of TLR4 and subsequent reduction of LPS response of human monocytes.

1. Introduction

CD14 is a glycoprotein that exists in the membrane of monocytic cells as a glycosylphosphatidylinositol (GPI)-anchored receptor or in serum as a soluble form of receptor (Ulevitch and Tobias, 1995). CD14 is an LPS sensing receptor that binds to LPS and moves to the surface TLR4:MD-2 complex, where it helps TLR4 recognize even small amounts of LPS (Gioannini et al., 2004). After recognizing LPS, this complex produces intracellular signals *via* toll-interleukin 1-receptor domain containing adaptor protein (TIRAP) and myeloid differentiation factor 88 (MyD88), and these adaptor proteins induce proinflammatory cytokine expression through activation of transcription factors NF-κB and AP-1 (Akira and Takeda, 2004; Kagan and Medzhitov, 2006). Subsequently, surface TLR4/LPS complex is rapidly internalized and co-localized in the same place within the cells (Husebye et al., 2006). It was thought that such internalization of TLR4/LPS is for LPS detoxication and TLR4 recycling (Akashi et al., 2003), and that the primary role of CD14 is only to bring LPS to TLR4, which causes activation of the intracellular signaling pathway. However, LPS-induced TLR4 endocytosis was found to be essential for the second signaling pathway, in which internalized TLR4 produces intracellular signals *via* TRIF-related adaptor molecule (TRAM) and TIR domain-containing adaptor protein-inducing interferon-β (TRIF) (Kagan et al., 2008; Tanimura et al., 2008), and these adaptor molecules induce type I interferon expression through activation of the transcription factor, interferon regulatory factor-3 (IRF3) (Akira and Takeda, 2004). In addition, Zanoni et al. (2011) recently revealed that, in contrast to wild type cells, CD14-deficient macrophages and dendritic cells do not engage LPS-induced TLR4 endocytosis and CD14 is needed for LPS-induced TLR4 endocytosis and second signaling through TRAM and TRIF as a key regulator.

We previously found that surface TLR4 expression was significantly reduced in $CD14^+$ monocytes that had been purified from human peripheral blood mononuclear cells using magnetic beads conjugated with anti-CD14 Ab. Therefore, in this study, we attempted to elucidate the mechanisms of this reduction.

* Corresponding author at: Department of Biological Science, Gachon University, Incheon 406-799, Republic of Korea. Tel.: +82 32 820 4551; fax: +82 32 820 4549.
E-mail address: jykim85@gachon.ac.kr (J.Y. Kim).

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2. Materials and methods

2.1. Reagents and chemicals

Lipopolysaccharides (LPS) from 055:B5 *Escherichia coli*, chlorpromazine, filipin III and 1,25-dihydroxyvitamin D3 (vitamin D3) were obtained from Sigma–Aldrich (St. Louis, MO, USA). PE-conjugated TLR4 (HTA125), CD4 and CD14, and PE-cy5-conjugated CD11b/Mac-1 antibodies (Abs) were purchased from BD Biosciences Pharmingen (San Diego, CA, USA). Saporin-conjugated goat anti-mouse IgG (mAb-ZAP) was purchased from Advanced Targeting Systems (San Diego, CA, USA). RPMI-1640 medium for cell culture was obtained from Welgene Inc. (Daegu, Korea). Fetal bovine serum (FBS) was obtained from Lonza Ltd. (Basel, Switzerland).

2.2. Cell lines and cell culture

The human myelomonocytic cell line THP-1 was obtained from ATCC (Manassas, VA, USA), and THP-1XBlue-CD14 cells were obtained from InvivoGen (San Diego, CA, USA). THP-1 cells were grown in RPMI-1640 supplemented with 10% heat-inactivated FBS, 1% antibiotic-antimycotic (Invitrogen Corp., Gibco BRL, MD, USA), 10 mM HEPES buffer (Invitrogen Corp.), β-mercaptoethanol (Invitrogen Corp.) at 37 °C in a 5% CO_2 humidified incubator. THP-1XBlue-CD14 cells were cultured in RPMI 1640 supplemented with 10% heat-inactivated FBS, 200 μg/ml Zeocin and 250 μg/ml G418.

2.3. Flow cytometric analysis

THP-1 cells (100 μl of 1×10^7 cells/ml) were treated with anti-CD14 Ab for 30 min. Anti-CD14 Ab-treated or -untreated THP-1 cells were stained with the following monoclonal Abs: PE-conjugated TLR4, CD4 and CD14, and streptavidin-PE/Cy5-conjugated CD11b at 4 °C in the dark for 20 min. The cells were analyzed on a Cytomics FC500 MLP (Beckman Coulter Inc., Fullerton, CA, USA). In some experiments, cells were treated with 100 nM vitamin D3 to induce cell surface CD14 expression for 3 days.

2.4. Immunofluorescence microscopy

Cells (100 μl of 1×10^6 cells/ml) were treated with FITC-conjugated My4 or PE-conjugated TLR4 Ab for 10 min. After washing, stained cells were treated with PE-conjugated TLR4 or FITC-conjugated My4, respectively. For nuclei staining, cells were incubated with Hoechest (Invitrogen Corp.) for 5 min. Cells were resuspended with a fluorescent mounting medium (DAKO Cytomation, Glostrup, Denmark) and smeared onto a glass slide. The cells were covered with cover slip and examined under Nikon Eclipse TE2000U fluorescent microscope.

2.5. In vitro internalization assay

Anti-TLR4 Ab (HTA125), My4, MEM18, 61D3 and CD11b Ab are mouse monoclonal IgG type Abs. Each primary antibody or isotype Ab at 10 μg/ml was pre-incubated with the Mab-ZAP or Goat IgG-SAP at 1 μg/ml in PBS for 1 h. Goat IgG-SAP was used as a non-targeted saporin control for the Mab-ZAP secondary Abs. THP-1 cells were pre-incubated with 100 nM vitamin D3 or same amount of ethanol for 48 h, and then, cells were harvested and plated in 96-well microplate at 2×10^4 cells/80 μl macrophage serum free medium (SFM, Gibco BRL). After 8 h incubation, either primary–secondary Ab mixture or isotype control Ab mixture was added to total volume of 100 μl and cells were incubated for 72 h. Cell viability and proliferation were determined with Ez-Cytox cell viability assay kit (Daeil Lab Service Co., Seoul, Korea) based on the cleavage of the tetrazolium salt to water-soluble formazan by succinate-tetrazolium reductase. Briefly, cells were treated with anti-CD14 Ab for 66 h. After treatment, cells were added 100 μl macrophage SFM including with 10 μl of Ez-Cytox solution for 6 h in the 37 °C incubator. Then absorbance was measured using the ELISA Reader (μ-Quant, Bio-Tek Instruments, Winooski, VT, USA) at 450 nm. Percent cell viability was calculated by assigning the average of the readings from isotype Ab controls with Mab-ZAP mixture as 100% cell viability. Anti-CD11b Ab was used as a positive control for internalization (Montero et al., 2009).

2.6. Endocytosis inhibition assay

Cells were treated with endocytic inhibitors to distinguish clathrin-dependent from -independent endocytosis. For clathrin-dependent endocytosis, cells were pre-incubated with 0–200 μg/ml chlorpromazine (CPZ) for 1 h at 37 °C. For caveolar endocytosis, cells were pre-incubated with 0–50 μg/ml filipin III for 1 h. Subsequently, My4 was added and the incubation was continued for another 10 min, after which the cells were analyzed by FACS.

2.7. NF-κB/AP-1 activation reporter assay

To measure NF-κB/AP-1 activation we used THP1Xblue-CD14 cells, which are reporter cells expressing cell surface CD14 and embryonic alkaline phosphatase gene under the control of a promoter inducible by the transcription factors NF-κB and AP-1. Cells were maintained around 1×10^6 cells/ml in RPMI 1640 medium supplemented with 10% FBS, 200 μg/ml Zeocin and 250 μg/ml G418. Cells were seeded onto 96-well culture plates at 2×10^5 cells/well and stimulated with LPS for 24 h. After stimulation a 20-μl aliquot of supernatant was collected and then added to 180 μl of a Quanti-Blue (InvivoGen) solution for color development at 37 °C. After 2 h-color development absorbance measurements were performed using an ELISA Reader (μ-Quant, Bio-Tek Instruments) at 655 nm.

2.8. TNF-α and IFN-β measurements

The quantities of TNF-α, and IFN-β in the culture supernatants were determined by ELISA, using human IFN-β (PBL, Piscataway, NJ, USA) and TNF-α ELISA kits (eBioscience, Vienna, Austria) according to the manufacturer's instructions. Briefly to test TNF-α production, 100 μl cytokine standard or 50 μl sample diluents plus 50 μl test samples were added to well. Biotin conjugate (50 μl) was added to all wells and incubated at RT for 2 h. Then the wells were washed 6 times with washing buffer. Streptavidin-HRP (100 μl/well) was then added and plates were further incubated for 1 h at RT. After washing plates 6 times, 100 μl of TMB substrate solution was added to the wells and plates were incubated in the dark for 15 min. Following the incubation, 100 μl stop solution was added to each well and plates were read at 450 nm. To test IFN-β production, 50 μl sample diluents were added to each well and 50 μl cytokine standard or 50 μl test samples were added to each well and incubated at RT for 1 h. Then the wells were washed 3 times with washing buffer. Antibody solution (100 μl/well) was then added and plates were further incubated for 1 h at RT. After washing 3 times, HRP solution was added and plates were further incubated for 1 h at RT. After washing 3 times 100 μl of TMB substrate solution was added and plates were incubated in the dark for 15 min. Following the incubation, stop solution (100 μl/well) was added to each well and plates were read at 450 nm.