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Suppression of immunoglobulin production in human peripheral blood mononuclear cells by monocytes *via* secretion of heavy-chain ferritin

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ABSTRACT

In vitro antigen stimulation of peripheral blood mononuclear cells (PBMCs) does not induce immunoglobulin (Ig) production. However, pretreatment of PBMCs with L-leucyl-L-leucine methyl ester (LLME) prior to *in vitro* stimulation removes the suppression of Ig production. In the present study, we attempted to identify the target cells of LLME and determine the mechanisms by which Ig production in PBMCs is suppressed. We found that $CD14^+$ monocytes are involved in the suppression of Ig production in PBMCs. Furthermore, we confirmed that heavy-chain ferritin derived from $CD14^+$ monocytes suppresses Ig production in PBMCs, possibly through iron sequestration.

Introduction

We have established an *in vitro* immunization method for the induction and propagation of B cells that produce antigen-specific antibodies. Many researchers have successfully established B cells that produce antigen-specific antibodies using peripheral blood mononuclear cells (PBMCs) from patients with various diseases; however, this was achieved by expansing memory B cells in PBMC samples collected from these patients (Shoji et al. 1996). We have successfully elicited antigen-specific antibody production in naïve B cells using PBMCs from healthy donors and the cholera toxin B subunit as the antigen (Ichikawa et al. 1999).

In our *in vitro* immunization protocol, to induce immunoglobulin (Ig) production in PBMCs, these cells were first pretreated with L-leucyl-L-leucine methyl ester (LLME) to remove the PBMCs suppressing Ig production. Following this, LLME-treated PBMCs were stimulated with a specific antigen in the presence of IL-2, IL-4, and an adjuvant, resulting in the induction of antigen-specific antibodies (Ichikawa et al. 1999).

LLME is a lysosomotropic agent that is captured through receptor-mediated endocytosis and eliminates lysosome-rich cells. Thus, LLME induces apoptosis in various cells such as NK cells, suppressor T cells, macrophages, and monocytes (Thiele and Lipsky 1986). The elimination of these cells by LLME would make it possible to induce Ig production in PBMCs. The mechanisms of LLME-induced apoptosis in these cells have been studied extensively (Brieva et al. 1984; Kuwano et al. 1986).

In the present study, we attempted to clarify the mode of action of LLME and the regulatory mechanisms for Ig production in PBMCs. We confirmed that monocytes were one of the target cells of LLME under our experimental conditions (Thiele and Lipsky 1986), and found that monocytes suppressed Ig production in antigen-stimulated PBMCs possibly *via* the secretion of heavy-chain ferritin. Although monocytes have previously been shown to regulate spontaneous Ig production by human PBMCs through secretion of IL-6

Abbreviations: DFO, deferoxamine mesylate; Ig, immunoglobulin; LLME, L-leucyl-L-leucine methyl ester; LSM, lymphocyte separation medium; PBMCs, peripheral blood mononuclear cells.

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and IL-10 (Kanda and Tamaki 1999), we found a novel role of monocytes and their secretary factor, heavy-chain ferritin, in the regulation of Ig production in PBMCs.

Materials and methods

Antigens and reagents

Mite extract was purchased from LSL (Tokyo, Japan). Recombinant human IL-2 was purchased from R&D Systems (Cambridge, MA). Recombinant human IL-4, GM-CSF, and TNF-α were purchased from Pepro Tech (London, UK). LLME was obtained from Bachem (Torrance, CA). Heavy-chain ferritin derived from the human liver was purchased from Calbiochem (San Diego, CA). Deferoxamine mesylate (DFO) was obtained from Sigma (St. Louis, MO).

Isolation of PBMCs

Peripheral blood samples were obtained from 10 donors (1 female and 9 males). PBMCs were separated by density-gradient centrifugation using a lymphocyte separation medium (LSM; Organon Teknika, Durham, NC). In brief, 25 mL of peripheral blood from healthy donors was layered onto 15 mL of LSM and centrifuged at $400 \times g$ for 30 min at room temperature. PBMCs were then collected, washed 3 times with enhanced RDF (eRDF) medium (RPMI1640:DMEM:F12 in 2:1:1, Kyokuto Pharmaceutical, Tokyo, Japan), and treated with 0.25 mM LLME for 20 min at room temperature. After washing with the culture medium, these cells were subjected to *in vitro* stimulation. All the experiments were done using PBMCs from 10 donors, and the representatives were shown.

In vitro stimulation

In vitro stimulation of human PBMCs was performed in 24-well culture plates (Becton Dickinson Labware, Flanklin Lake, NJ). LLME-treated PBMCs ($3–5 \times 10^6$ cells) were stimulated with mite extract (10 μg/mL) in the presence of IL-2 (10 units/mL) and IL-4 (10 ng/mL) and cultured in eRDF medium supplemented with 10% heat-inactivated FBS (SAFC Biosciences, Lenexa, KS) and 2-mercaptoethanol (2ME, 50 μM) for 8 days. For proteomic analysis, LLME-treated PBMCs were antigen stimulated and cultured in serum-free medium consisting of a 1:1 mixture of eRDF and RITC80-7 media (Research Institute for the Functional Peptides, Yamagata, Japan).

Measurement of IgM concentration by ELISA

Microtiter plates (Nunc, Naperville, IL) were coated with 100 μL of 1000-fold diluted anti-human IgM antibody (Tago, Burlingame, CA) and incubated for 2 h at 37 °C. The plates were then washed 3 times with 2.24×10^{-2} M phosphate buffer containing 1.37×10^{-1} M NaCl and 0.05% Tween 20 (TPBS). Aliquots of serially diluted supernatants of *in vitro* stimulated PBMCs were added to the plates and incubated overnight at 4 °C. Following this, the plates were washed 3 times with TPBS, and 100 μL of 2000-fold diluted horseradish peroxidase-conjugated anti-human IgM (TAGO) goat antibody was added to the plates, and they were incubated for 2 h at 37 °C. The plates were then washed 3 times with TPBS. A substrate solution [0.1 M citrate buffer (pH 4.0) containing 0.003% H_2O_2 and 0.3 mg/mL ABTS (Wako, Osaka, Japan)] was added to the plates, and they were incubated for 20 min. Finally, absorbance at 405 nm was measured with an ELISA reader.

Measurement of ferritin concentration by ELISA

Ferritin concentration was quantitatively determined by using the AssayMax Human Ferritin ELISA Kit (Assaypro, St. Charles, MO). In brief, the samples were added to the wells and incubated for 2 h. After the wells were washed 5 times with wash buffer, 50 μL of biotinylated anti-ferritin antibody was added, followed by incubation for 1 h. After the wells were washed 5 times with wash buffer, 50 μL of streptavidin–peroxidase conjugate was added, followed by incubation for 30 min. After the wells were washed 5 times with wash buffer, 50 μL of chromogen substrate was added, followed by incubation for 10 min. After the incubation, 50 μL of stop solution was added. Finally, absorbance was measured with an ELISA reader. We subtracted the reading at 570 nm from that at 450 nm to correct optical imperfections.

Generation and maturation of dendritic cells

Immature DC (iDC) and mature DC (mDC) were obtained by the treatment of monocytes (5×10^6 cells) with 20 ng/mL GM-CSF and 20 ng/mL IL-4, and 20 ng/mL GM-CSF, 20 ng/mL IL-4 and 30 ng/mL TNF-α, respectively, for 7 days.

Flow cytometric analysis

Cell-surface antigen expression was analyzed using an EPICS XL flow cytometer (Beckman Coulter, Miami, FL). All the antibodies (PE-conjugated anti-CD14, anti-CD56, anti-CD80, anti-CD83, and anti-CD86) were purchased from Beckman Coulter.

Cell isolation using the MACS system

Cells were isolated with the MACS system (Miltenyi Biotec K.K., Tokyo, Japan). $CD4^+$, $CD8^+$, $CD56^+$, and $CD14^+$ cells were removed or enriched either by a negative- or a positive-selection protocol. Efficiency of respective cell depletion and enrichment was >95%.

Proteomic analysis

Culture supernatants (20 μg) of nontreated and LLME-treated PBMCs were subjected to 20% SDS-PAGE and silver staining. Protein bands exhibiting differential expression were excised and subjected to in-gel tryptic digestion (Thermo Fisher Scientific, Waltham, MA). Aliquots of these products were subjected to MALDI-TOF mass spectroscopy (PerSeptive Biosystem MALDI-TOF Voyager-DE RP Biospectrometry workstation, Applied Biosystems, Framingham, MA). Peptide masses were searched against Swiss-Prot and NCBI databases using the Aldente search tool, as described previously (Zhang et al. 2006).

Western blot analysis

Proteins were separated by 15% SDS-PAGE and transferred to a Hybond-P membrane (GE Healthcare, Amersham Place, UK). The membrane was blocked with 5% skim milk and incubated with 1 μg/mL of rabbit polyclonal anti-ferritin antibody (ab7332, Abcam, Cambridge, MA). The membrane was then washed with TPBS and incubated with 2000-fold diluted anti-rabbit IgG horseradish peroxidase-conjugated antibody (GE Healthcare) as the secondary antibody. Proteins were detected using the ECL-Plus Western blotting detection system (GE Healthcare) and visualized with the LAS-1000 luminoimage analyzer (Fuji Film, Tokyo, Japan).