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Human endotoxin tolerance is associated with enrichment of the CD14+ CD16+ monocyte subset

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ABSTRACT

Prior exposure to lipopolysaccharides (LPS) induces a state of cell resistance to subsequent LPS restimulation, known as endotoxin tolerance, mainly by repressing the expression of pro-inflammatory cytokines. We established an endotoxin tolerance model in human monocytes Endotoxin-tolerant cells showed a decrease in IκBα degradation and diminished expression of Tumor necrosis factor (TNF) (both messenger RNA [mRNA] and protein content). The myeloid differentiation factor 88 (MyD88)/MyD88 splice variant (MyD88s) ratio, an indirect way to test the Toll-like receptor 4 (TLR4) MyD88-dependent signaling cascade, did not change in endotoxin-tolerant cells when compared to LPS-stimulated or -unstimulated ones. Remarkably, cell population analysis indicated a significant increase of the CD14+ CD16+ subset only under the endotoxin-tolerant condition. Furthermore, endotoxin-tolerant cells produced higher amounts of C–X–C motif chemokine 10 (CXCL10), a typical MyD88-independent cytokine.

Introduction

Human blood monocytes are not a homogeneous population of cells; they can be separated based on the expression of cell surface markers. Differential expression of CD14 and CD16 could define at least two subsets of monocytes with distinct functional properties. In general, CD14+ monocytes produce more cytokines. Additionally, monocytes with different combinations of CD14 and CD16 antigens on their surfaces produce characteristic cytokine profiles after lipopolysaccharide (LPS) challenge (Belge et al. 2002).

Pre-exposure of isolated adherent human monocytes to LPS leads to hyposensitivity to secondary LPS stimulation, known as endotoxin tolerance (Cavaillon and Adib-Conquy 2006). To date, the underlying molecular mechanisms of endotoxin tolerance are not completely understood. LPS, a glycolipid component of the cell wall of Gram-negative bacteria, is a model molecule used to study inflammatory responses caused by exposure to bacteria (Vassallo et al. 2012). Toll-like receptor 4 (TLR4) is the main receptor for LPS (Takeda and Akira 2005); for signaling, TLR4 employs the Toll/interleukin-1 receptor (TIR)-domain containing adaptor-inducing Interferon-beta (IFN-β) (TRIF) or myeloid differentiation factor 88 (MyD88)-dependent signaling pathways. TRIF-dependent signaling activates IFN regulatory factor 3 (IRF3) and the expression of type II IFN, such as C–X–C motif chemokine 10 (CXCL10) and Chemokine (C–C motif) ligand 5 (CCL5) (Yamamoto et al. 2003). Contrariwise, MyD88-dependent signaling activates the nuclear factor-kappa beta (NF-κB) and c-Jun NH_2-terminal kinase (JNK) pathways (Janssens and Beyaert 2002) and the transcription of inflammatory genes such as TNF, interleukin-1 beta (IL-1β), IL-6, and IL-12 (Burns et al. 1998).

MyD88s is a product of an alternative splicing (exon 2 deletion) of MyD88 (Janssens et al. 2002). In mouse, MyD88s regulates the IL-1β and LPS signaling pathways due to its inability to activate downstream signaling molecules (Burns et al. 2003); in humans, upregulation of MyD88s mRNA was reported in monocytes from

Abbreviations: IκBα, I-kappa-B-alpha; IRF, IFN regulatory factor; JNK, c-Jun NH_2-terminal kinase; LPS, lipopolysaccharide; MyD88, myeloid differentiation factor 88; PBMC, peripheral blood mononuclear cells; TLR4, toll-like receptor 4; TRIF, TIR domain containing adaptor-inducing IFN-β.

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patients with sepsis (Adib-Conquy et al. 2006). Other studies in septic patients showed changes in blood monocyte phenotypes, characterized by the proliferation of a CD16+ subset (Fingerle et al. 1993; Skrzeczynska et al. 2002). Differential expression of surface molecules on monocytes such as CD16 is likely related with the LPS response.

In this report, we induced endotoxin tolerance in freshly isolated, adherent human monocytes and examined which monocyte subpopulation best fits to a characteristic hypo-responsiveness state.

Materials and methods

Isolation and cell culture

Four female and five male healthy donors (without any type of treatment) were recruited at the Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán (INCMNSZ) Blood Bank. Peripheral blood monocyte cells (PBMC) were collected using Ficoll (Amersham Biosciences, Uppsala, Sweden) density gradients from leukocyte concentrates from healthy donors. Ten million PBMC were resuspended in RPMI 1640 medium (GIBCO Invitrogen Corp., Grand Island, NY, USA) supplemented with 2 mM L-glutamine and 10% fetal bovine serum (FBS) and incubated in 5% CO_2 at 37 °C in tissue culture 50 mm Petri dishes (Corning) during 1 h. Non adherent cells were removed by gentle washing and adherent cells remained in culture for experiments and these are called hereafter monocytes. After 24 h of incubation period monocytes remained unstimulated or were presensitized to generate an endotoxin-tolerant condition with 10 ng/ml LPS from *Escherichia coli* serotype 0111:B4 (Sigma Chemical Co., St. Louis, MO, USA) for 24 h. After 48 h of incubation period, cells were washed twice with warm RPMI-1640, and the following conditions were included: Monocytes remained unstimulated (a) control, were treated with 10 ng/ml with sonicated LPS (b) single LPS stimulus, were treated with 10 ng/ml LPS + 10 μg/ml polymyxin B (Sigma) as a negative control because polymyxin B is an antibiotic binding to lipid A, which is the component of LPS responsible for receptor binding and cellular signaling, therefore polymyxin B block LPS activity (c) LPS + Polymyxin B, or were challenged a second time with 10 ng/ml LPS (d) LPS tolerant cells. IκBα content was analyzed 20 min after stimulation, expression of Tumor necrosis factor (TNF) messenger RNA (mRNA) was evaluated 60 min after stimulation, and TNF immunoreactivity was evaluated 3 h after stimulation.

RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from 10×10^6 cells using Trizol® (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's directions. RNA quantification was determined by absorbance measurement at 260 nm/280 nm in an ND-1000 NanoDrop®. For RT, no <1 μg of total RNA was mixed with Oligo-dT (12–18 mer) (Invitrogen Life Technologies, USA), 200 U of Superscript II (Invitrogen Life Technologies, Brazil), first strand buffer and mixed deoxyriboNucleotide triphosphate (dNTP) (Invitrogen Life Technologies, Brazil). Real time relative expression was performed in capillaries with 20 μl of reaction mix; quantitative Polymerase chain reaction (qPCR) for TNF was carried out by using a specific probe from the Universal ProbeLibrary (UPL) (Roche Applied Science, Salt Lake City, UT, USA) and unique primers. PCR master mix for TNF contained Light Cycler TaqMan Master, 0.5 μM PCR primer mix, and 0.2 μM of specific Human (UPL). All reactions were performed and analyzed in a LightCycler 2.0 instrument (Roche Applied Science). Results were normalized against Hypoxanthine-guanine phosphoribosyltransferase (HPRT) (UPL probe) expression.

Cytoplasmic extracts and Western blotting analysis

After incubation of monocytes in tissue culture dishes, under LPS and tolerance stimulation conditions previously described, cells were washed three times with ice-cold PBS and harvested. Supernatants were collected, and pellets were frozen following resuspension in 10 mM Hepes pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, and 1 mM DTT. Cytoplasms were separated from nuclei by centrifugation (14,000 × *g* × 10 min), collected, and resuspended in anti-protease buffer (Roche Diagnostics GmbH, Mannheim, Germany). After adjusting protein concentration from extracts, samples were utilized immediately or stored at −70 °C. For Western blotting analysis, equal protein amounts of cytoplasm extracts were boiled in Laemmli buffer, resolved in 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred on a 0.45-μm Immuno-Blot Polyvinylidene fluoride (PVDF) membrane (Merck Millipore, Darmstadt, Germany). After blocking with nonfat milk, membranes were washed in TBS-T and probed with the rabbit anti-MyD88 polyclonal antibody raised against a peptide corresponding to amino acids 233–248 of human MyD88 (Chemicon International, Temecula, CA, USA) or with rabbit anti-IκBα polyclonal antibody raised against the C-terminus of human IκB-α (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Following washing in Tris buffered saline with Tween 20 (TBS-T), membranes were incubated with secondary Horseradish peroxidase (HRP)-conjugated anti-rabbit IgG. Bands were detected using Supersignal West Pico chemiluminescent substrate reagents (Pierce PERBIO, Rockford, IL, USA) according to the manufacturer's instructions.

Milliplex assay

Supernatants were collected, frozen, and stored at −80 °C until cytokine measurement. Assays were performed using a kit (Millipore's MILLIPLEX® Human Cytokine/Chemokine kit) following the manufacturer's instructions. We assayed supernatants of nine donors for TNF, macrophage inflammatory protein-1 beta (MIP-1β), Granulocyte-macrophage colony-stimulating factor (GM-CSF), CXCL10, and cytotoxic lymphocyte maturation factor 2 p40 (IL-12p40).

Flow cytometry

Expression of TLR4, CD14, CD16, and intracellular TNF were evaluated in monocytes from five individuals by flow cytometry. Cells were treated as previously described to generate endotoxin-tolerant cells. For TNF detection, cells were pretreated with Brefeldin A (Sigma). After incubation, cells were harvested and washed with staining buffer 2% (phosphate buffered solution [PBS] 2% fetal calf serum [FCS]). Supernatant was discarded and cells were resuspended in staining buffer and incubated with saturant amounts of anti-TLR4 PECy7 (eBioscience, San Diego, CA, USA), Anti-CD14 (APC) (eBioscience), and anti-CD16 (FITC) (BD Pharmingen BD Biosciences, San Jose, CA, USA) antibodies for 15 min in the dark at room temperature. Cells were washed, centrifuged, and permeabilized with flow cytometry (FACS) permeabilizing solution BD Permz (BD Biosciences) for 15 min at room temperature. For intracellular TNF detection, we added anti-TNF (PE) (BD Pharmingen BD Biosciences) antibody for 30 min in the dark at room temperature. Cells were washed, centrifuged, and resuspended in 1% paraformaldehyde (Sigma) to be analyzed within 24 h. Event acquisition was performed using BD FACSDiva software (BD Bioscience) and a BD FACS Canto II flow cytometer (BD Bioscience). For each