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Early Activation Markers of Human Peripheral Dendritic Cells

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ABSTRACT: Two major populations of dendritic cells (DCs), myeloid and plasmacytoid, can be isolated from human peripheral blood, and are distinguished by differential expression of the cell surface markers CD11c and CD123. These two populations of DCs also are different in their expression of Toll-like receptor (TLRs), which are involved in their activation. To investigate the early events during activation of peripheral DCs, the cells were stimulated *in vitro* with ligands for TLR-4 (as in lipopolysaccharides [LPS]) or TLR-9 (CpG-containing oligonucleotide [CpG]). The earliest change in protein expression detected after stimulating peripheral DCs with lipopolysaccharide (LPS) or CpG was increased production of the chemokine interleukin (IL)–8. Enhanced production of IL-8 occurred already within 2 hours of stimulation in both myeloid dendritic cells (M-DCs) and plasmacytoid dendritic cells (P-DCs), and preceded expression of the well established activation marker CD40. Although both populations of DCs secreted IL-8 upon activation, the levels of IL-8 produced was several times higher within the M-DCs compared with the P-DCs population. Before activation, both subsets of DCs expressed the IL-8 receptor type B (CD128b); but after stimulation the IL-8 receptor was down-regulated in both populations of DCs. Increased expression of MHC class II molecules is generally regarded as an early activation marker of DCs. However, only the P-DCs showed a significant up-regulation of MHC class II after stimulation. The M-DC population up-regulated MHC class II without any prior activation; thus care should be taken using increased expression of MHC class II molecules as an early activation marker of peripheral M-DCs after activation *in vitro*.

In conclusion, we propose that during activation of human DCs the production of IL-8 and loss of CD128b are the earliest signs of activation preceding both MHC class II, CD40, CD80, and CD86 expression. *Human Immunology 68, 324–333 (2007).* © American Society for Histocompatibility and Immunogenetics, 2007. Published by Elsevier Inc.

KEYWORDS: CpG; LPS; MHC expression; IL-8; Myeloid dendritic cell; Plasmacytoid dendritic cell

ABBREVIATIONS

CpG	CpG-containing oligonucleotide
DC	dendritic cell
IL	interleukin
LPS	lipopolysaccharide
M-DC	myeloid dendritic cell
PAMP	pathogen-associated molecular pattern
P-DC	plasmacytoid dendritic cell
PRR	pattern recognition receptor
TLR	toll-like receptor

INTRODUCTION

Dendritic cells (DCs) play a central role in our immune system [1]. The DCs are the most important cell type responsible for antigen presentation, and as such they control both initiation and maintenance of adaptive immune responses, as well as induction of peripheral tolerance [2–5].

Immature DCs are normally found in peripheral blood. Upon activation these cells will up-regulate surface molecules such as MHC class II, adhesion and co-stimulatory molecules, thereby adopting a mature phenotype [6]. The DCs sample and sense their environment and pass this information on to the immune system. When pathogenic organisms or substances are encountered, the presence of non-self material will immediately be communicated to the immune system. Pathogens are primarily recognized by a large number of pathogen-associated molecular patterns (PAMPs) that are identified by conserved pattern recognition receptors (PRRs), such as the Toll-like receptors (TLRs) [7]. Several TLRs have been identified and

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proven to be crititically involved in provoking maturation of DCs [8, 9].

In addition, TLRs are also differentially expressed on diverse DC subsets [9, 10]. For example, in human peripheral blood, the two major subpopulations of DCs, the myeloid (M-DCs) and the plasmacytoid (P-DCs) dendritic cells, differently express TLR4 and TLR9 [9, 10]. The initial phenotypic changes that occur after DC activation are important and interesting to study to gain insight in the regulation of our immune system. However, the early events after myeloid and plasmacytoid DC activation remain relatively unknown. Here, we describe some early activation events at the protein level after *in vitro* stimulation of DCs using ligands to TLRs.

MATERIALS AND METHODS

Cells and Reagents

Peripheral blood buffy coat preparations were obtained from the Blood Bank of the University Hospital of Malmoe (Sweden). Blood dendritic isolation kit II (MACS), LD and MS columns were from Miltenyi Biotec, Bergisch Gladbach, Germany.

Lipopolysaccharide (LPS, from *Escherichia coli* O111:B4) was from Sigma (St. Louis, MO). The CpG oligodeoxynucleotide (ODN) sequences, 5′-tcgtcgttttgtcgttttgtcgtt-3′ and 5′-ggGGGACGATCGTCgggggg3′, phosphorothioate (PTO) linkages between the bases are shown in lower case and phosphodiester linkages are shown in capital letters, were obtained from MWG-BIOTECH AG (Ebersberg, Germany). The ODNs were suspended in sterile dH_20 and stored and handled under aseptic conditions. A Limulus Amebocyte Lysate (LAL) kit (Charles River Endosafe, Charles River Laboratories, Inc., Charleston, SC) was used to determine the amount of endotoxin in the CpG preparation and was shown to be, in 5 μg/ml solution of CpG, below detection limit of the assay (0.5 ng LPS/ml).

N-hydroxysuccinimide biotin (NHS-biotin) was purchased from Bio-Rad Laboratories (Richmond, CA). RPMI 1640 with L-glutamine, gentamicin, Dulbecco's PBS without Ca^{2+} and Mg^{2+} and human serum "off the clot" Type AB, were all purchased from PAA Laboratories (Linz, Austria). Ficoll-Paque was purchased from Amersham Pharmacia Biotech AB (Uppsala, Sweden). Fragmin was obtained from Pfizer (Täby, Sweden). Human IgG (h-IgG) (Gammanorm) was purchased from Biovitrum AB (Stockholm, Sweden).

The BD Cytofix/Cytoperm Plus (with GolgiStop) cytokine intracellular staining, Cytometric Bead Array (CBA) human inflammation kit, Annexin V-FITC Apoptosis detection Kit I and 7-Amino-Actinomycin D (7-AAD) were all purchased from Becton Dickinson (San Diego, CA).

Isolation of Human Peripheral Blood Dendritic Cells

Peripheral blood mononuclear cells (PBMCs) were separated using peripheral blood buffy coat preparations from healthy donors by density-gradient centrifugation on Ficoll-Paque [11] with added Fragmin (20 units/ml).

Isolation of human peripheral DCs was performed by a two-step procedure using the human blood dendritic cell isolation kit II (MACS) using LD and MS columns following the manufacturer's instructions. Briefly, PBMC were incubated with non–DC depletion cocktail (containing biotin labeled anti BDCA-1 and microbeads conjugated with monoclonal antibodies against human CD14 and CD19) in PBS containing 0.5% bovine serum albumin (BSA) and 2 mmol/L EDTA for 15 minutes at 4°C, washed and applied on a MACS column. The flow through fraction containing pre-enriched DCs, were incubated with DC enrichment cocktail (microbeads conjugated with antibodies against biotin, human BDCA-4 and BDCA-3) in PBS containing 0.5% BSA and 2 mmol/L EDTA for 15 minutes at 4°C and washed once. Labeled dendritic cells were adsorbed on a MACS column and eluted after removal of the column from the magnetic device. To achieve highest purity of the DC population, the positively selected cells were after elution, separated over a second MACS column as a standard procedure.

In Vitro Stimulation of Isolated Peripheral Blood Dendritic Cell Preparations

Cells were suspended in culture medium; RPMI 1640 supplemented with 10% human serum "off the clot," Type AB, and 100 μg/ml of gentamicin. Isolated dendritic cells were incubated at a final concentration of 1×10^6/ml in Eppendorf tubes rotated with short rotation intervals using a Triomix-rotator (Triolab AB, Sweden) in the presence or absence of LPS 1 μg/ml or CpG 5 μg/ml.

The CpG sequences used in this study were 5′-tcgtcgttttgtcgttttgtcgtt-3′, which corresponds to the B-class ODN-2006 [12] and 5′-ggGGGACGATCGTCgggggg3′, which corresponds to the A-class ODN 2216 [13], both used in previous studies. The CpG sequence of the B-class was used throughout in this paper except in experiments inducing production of α-interferon where the CpG sequence of the A-class was used. All cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2 for up to 8 hours. Stimulated or un-stimulated isolated dendritic cells were upon end of incubation tested for signs of necrosis or apoptosis using 7-AAD and Annexin-FITC. Briefly, according to the manufacturer's instruction, cells, final concentration 1×10^6/ml, were washed twice in cold PBS and resuspended in 1 × binding buffer, 5 μl

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