Free Radical Biology & Medicine 52 (2012) 635–645

Contents lists available at SciVerse ScienceDirect

Free Radical Biology & Medicine

journal homepage: www.elsevier.com/locate/freeradbiomed

Original Contribution

Modulatory effects of low-dose hydrogen peroxide on the function of human plasmacytoid dendritic cells

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ARTICLE INFO

Article history:
Received 22 July 2011
Revised 11 November 2011
Accepted 14 November 2011
Available online 8 December 2011

Keywords:
Plasmacytoid dendritic cells
Oxidative stress
Inflammation
Immune regulation
Free radicals

ABSTRACT

Under normal conditions, plasmacytoid dendritic cells (pDCs) are located in peripheral lymphoid organs or circulate in the blood, from where they can migrate to sites of infection or inflammation. In inflamed tissues, pDCs can be exposed to elevated levels of reactive oxygen species produced by inflammatory cells and we presume that oxidative stress could affect the cellular responses of pDCs to microenvironmental stimuli. To explore this possibility, human pDCs isolated from peripheral blood of healthy donors were treated with H_2O_2 and R837 (a Toll-like receptor 7 ligand), separately and in combination. Our results demonstrate that treatment with a low concentration (0.01 μM) of H_2O_2 resulted in only slight changes in the expression of CD40, CD80, CD86, and CD83; however, low-dose H_2O_2 markedly decreased the expression of HLA-DQ on pDCs. Exposure to H_2O_2 did not trigger the release of IL-6, TNF-α, IL-8, or IFN-α from pDCs. Although addition of H_2O_2 did not modify the capacity of pDCs to activate allogeneic IL-17- or IFN-γ-producing T cells, it significantly increased the ability of pDCs to stimulate IL-4-secreting T cells. Exposure of pDCs to H_2O_2 before cocultivation with naïve autologous T cells significantly lowered IL-10 production by T cells, but did not affect IL-17 release. It was also observed that H_2O_2-exposed pDCs provided stronger stimuli for Th2 than for Th1 differentiation upon autologous activation, compared to untreated pDCs, possibly because of elevated surface expression of OX40-L. Most importantly, when pDCs were stimulated with R837 in the presence of H_2O_2, decreased phenotypic activation, decreased chemokine and cytokine release, and impaired allo- and autostimulatory functions of pDCs were detected, indicating that pDCs exposed to oxidative stress in vivo may have an anti-inflammatory or tolerogenic role in regulating adaptive immune responses.

Plasmacytoid dendritic cells (pDCs) are a unique and rare cell population of the immune system. Immature pDCs exhibit a spherical shape and are referred to as plasmacytoid predendritic cells. They are specialized for the direct recognition of viral and microbial infections by their selectively expressed endosomal nucleic acid-sensing Toll-like receptors (TLRs) such as TLR7 and TLR9 [1]. After detection of pathogen-derived nucleic acids, plasmacytoid predendritic cells secrete large amounts of type I interferons (IFNs) and other inflammatory cytokines involved in innate immune responses [2]. Upon activation, the shape of plasmacytoid predendritic cells changes to dendritic cell morphology. In this state, the expression of MHC class II and costimulatory molecules is up-regulated and pDCs are able to prime naïve $CD4^+$ T lymphocytes [3]. Depending on the type of activation and maturation signals, pDCs have the ability to facilitate T helper (Th) 1, Th2, and regulatory T cell development [4–6]. Despite the fact that pDCs and conventional dendritic cells (DCs) may arise from common precursors through overlapping developmental pathways [7,8], pDCs display many features of lymphocytes and in their phenotypic and functional features they are explicitly distinct from conventional DCs. Plasmacytoid DCs lack expression of the myeloid markers such as CD11c, CD13, CD33, and mannose receptors; however, they express several lymphoid markers (CD2, CD5, and CD7), as well as transcripts for pre-T cell receptor α and immunoglobulin λ-like 14.2 [9,10]. Furthermore, expression of granzyme B and Spi-B, a lymphoid-restricted transcription factor, has also been reported in pDCs but not in conventional DCs [11]. Based on their tissue localization and migratory patterns, pDCs also appear different from conventional DCs but similar to lymphocytes [12]. After development in the bone marrow, pDCs are released into the blood circulation, and through high endothelial venules they can migrate from the bloodstream into secondary lymphoid organs and peripheral tissues [13]. Although it is difficult to detect pDCs under steady-state conditions in most peripheral tissues, their number increases dramatically in many tissues during inflammatory responses [14–16].

Abbreviations: 7-AAD, 7-aminoactinomycin-D; DC, dendritic cell; ELISPOT, enzyme-linked immunospot; FCS, fetal calf serum; H_2DCFDA, 2′,7′-dihydrodichlorofluorescein diacetate; ICOS-L, inducible costimulator ligand; IFN, interferon; mAb, monoclonal antibody; NAC, *N*-acetylcysteine, pDC, plasmacytoid dendritic cell; PBMC, peripheral blood mononuclear cell; PDL-1, programmed death ligand 1; ROS, reactive oxygen species; Th, helper T cell; TLR, Toll-like receptor.

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doi:10.1016/j.freeradbiomed.2011.11.022

Several lines of evidence indicate that inflammation is associated with oxidative stress. The mechanisms described for this phenomenon include generation of reactive oxygen species (ROS) by inflammatory cells recruited to the infected and/or damaged tissues and ROS induced by exposure to environmental factors such as ozone, cigarette smoke or pollen grains [17,18]. Elevated levels of ROS can cause cellular injury in various ways; membrane lipids can be attacked leading to the formation of peroxide derivatives, protein side chains can be modified, and even peptide backbones can be broken, and finally, DNA can be damaged resulting in strand breaks or nucleotide modifications. In contrast to these harmful events, low concentrations of ROS are being recognized as essential participants of several signal transduction pathways [19] and ROS have been demonstrated to induce phenotypic and functional maturation of human monocyte-derived DCs [20,21].

It has been recently demonstrated that in addition to detection of infection and induction of adaptive immunity, pDCs have an important function in sensing tissue damage and initiating tissue repair [22]. During infiltration into inflamed tissues and recognition of nucleic acids released by injured cells, pDCs can be exposed to elevated levels of ROS. We hypothesized that these stimuli could affect the cellular responses of pDCs to signals from the surrounding microenvironment. Thus, in this study we have investigated how oxidative stress conditions can change the phenotype of pDCs and modulate their cytokine production and T-cell-polarizing capacity.

Materials and methods

Cell purification

Human pDCs

Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll–Paque (GE Healthcare, Uppsala, Sweden) density gradient centrifugation of heparinized leukocyte-enriched buffy coats of healthy donors drawn at the Regional Blood Center of the Hungarian National Blood Transfusion Service (Debrecen, Hungary) in accordance with the written approval of the Director of the National Blood Transfusion Service and the Regional and Institutional Ethics Committee of the University of Debrecen, Medical and Health Science Center (Debrecen, Hungary). Plasmacytoid DCs were purified from PBMCs by negative selection using immunomagnetic cell separation kit (Miltenyi Biotec, Bergish Gladbach, Germany), according to the manufacturer's instructions. After separation on a VarioMACS magnet, the purity of $BDCA2^+BDCA4^+CD123^+$ pDCs was >98%, as confirmed by flow cytometry.

Human monocytes

$CD14^+$ monocytes were obtained from PBMCs by positive selection using immunomagnetic cell separation with anti-CD14 microbeads (Miltenyi Biotec), according to the manufacturer's instructions. After separation, 96 to 99% of the cells were $CD14^+$ monocytes as measured by flow cytometry.

Human $CD3^+$ pan-T cells

Anti-CD3 microbeads (Miltenyi Biotec) were used for the positive selection of $CD3^+$ T cells from PBMCs, according to the manufacturer's instructions. Freshly isolated $CD3^+$ T cells had a purity of at least 98% based on flow cytometric analysis.

Human naïve $CD4^+$ T cells

Naïve $CD4^+CD45RA^+CD45RO^-$ T cells were isolated from PBMCs by magnetic depletion of $CD4^+CD45RO^+$ memory T cells and non-$CD4^+$ T cells with the Naïve $CD4^+$ T Cells Isolation Kit II (Miltenyi Biotec), according to the manufacturer's instructions. The homogeneity of untouched naïve T cells was >97%, as measured by flow cytometry.

Generation of conventional DCs from human $CD14^+$ monocytes

Freshly isolated monocytes were cultured in 24-well tissue culture plates at a density of 2×10^6 cells/ml in RPMI 1640 medium (Sigma–Aldrich, St. Louis, MO, USA) supplemented with 2 mM L-glutamine (Sigma–Aldrich), 100 U/ml penicillin, 100 ng/ml streptomycin, 10% heat-inactivated fetal calf serum (FCS) (Invitrogen, Carlsbad, CA, USA), and 80 ng/ml granulocyte–macrophage colony-stimulating factor (Gentaur Molecular Products, Brussels, Belgium) and 100 ng/ml IL-4 (Peprotech EC, London, UK). On day 2, the same amounts of granulocyte–macrophage colony-stimulating factor and IL-4 were added to the cell cultures. More than 90% of the cells showed immature DC phenotype (DC-SIGN/$CD209^+$, $CD14^{low}$) and the percentage of $CD1a^+$ DCs varied among individuals (75–90%) on day 5 when they were used for experiments.

Assessment of cell viability

Isolated untouched pDCs and conventional DCs were seeded at a density of 5×10^5 cells/ml in RPMI 1640 medium containing 2 mM L-glutamine, 100 U/ml penicillin, 100 ng/ml streptomycin, and 10% heat-inactivated FCS (culture medium for pDCs was also supplemented with 50 ng/ml recombinant human IL-3; Peprotech) and treated with increasing concentrations (ranging from 0.01 to 10 μM) of H_2O_2 (Sigma–Aldrich) for 24 h. In control experiments, cells were pretreated with an antioxidant (30 mM *N*-acetylcysteine, NAC; Sigma–Aldrich) for 1 h and then cotreated with H_2O_2. Cell viability was determined by 7-aminoactinomycin-D (7-AAD; 10 μg/ml; Sigma–Aldrich) staining for 15 min immediately before flow cytometric analysis. Fluorescence intensities were measured by a FACSCalibur flow cytometer (BD Biosciences Immunocytometry Systems, Franklin Lakes, NJ, USA) and analysis of data was performed by FlowJo software (TreeStar, Ashland, OR, USA).

Measurement of intracellular ROS levels

Freshly isolated untouched pDCs and conventional DCs were loaded with 50 μM 2′,7′-dihydrodichlorofluorescein diacetate (H_2DCFDA; Invitrogen) at 37°C for 20 min. After excess fluorescent dye was removed, the cells were exposed to increasing concentrations of H_2O_2 for 2 h. Changes in DCF fluorescence intensity were detected on the FL1 (530 ± 15 nm) channel using a BD FACSCalibur flow cytometer. Data were analyzed by FlowJo software.

Consumption of H_2O_2 in cell culture medium of pDCs and conventional DCs

Freshly isolated untouched pDCs and conventional DCs were seeded at a density of 5×10^5 cells/ml in RPMI 1640 medium containing 10% heat-inactivated FCS, allowed to equilibrate for 30 min, and then treated with 0.01 μM H_2O_2. Samples were withdrawn every 5 min to assay H_2O_2 content. To measure H_2O_2 levels, 50 μl of cell-free supernatant was mixed with 50 μl H_2DCFDA (50 μM) and fluorescence intensity was assessed by a Synergy HT reader (Bio-Tek Instruments, Winooski, VT, USA) using a 485/20 excitation filter and a 528/20 emission filter. The level of H_2O_2 at time 0 was determined by taking a sample immediately after addition of H_2O_2 to the cells. For control experiments, H_2O_2 was added to cell-free medium and consumption of H_2O_2 was assayed as described above. The assay was linear over the concentration range from 20 nM to 156.25 pM ($r^2>0.99$) with a minimum detectable limit of quantitation of 312.5 pM H_2O_2.

Stimulation of the cells

Freshly isolated pDCs were seeded at 1×10^5 cells/well in 96-well flat-bottom plates in RPMI 1640 medium supplemented with 2 mM