Immunobiology xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Immunobiology

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

Research Paper

Indoxyl 3-sulfate inhibits maturation and activation of human monocyte-derived dendritic cells

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ARTICLE INFO

Keywords:
Indoxyl 3-sulfate
Tolerogenic dendritic cells
IL-12
T cells
IFNγ
TNF

ABSTRACT

Indole is produced from L-tryptophan by commensal bacteria and further metabolized to indoxyl 3-sulfate (I3S) in the liver. Physiologic concentrations of I3S are related to a lower risk to develop graft versus host disease in allogeneic stem cell transplanted patients pointing towards an immunoregulatory function of I3S. Here we investigated the impact of I3S on the maturation of human monocyte-derived dendritic cells (DCs). Even pathophysiologic concentrations of I3S did not affect viability of mature DCs, but I3S decreased the expression of co-stimulatory molecules such as CD80 and CD86 on mature DCs. Furthermore, I3S inhibited IL-12 and IL-6 secretion by mature DCs while IL-10 was significantly upregulated. Co-culture of I3S-treated mature DCs with allogeneic T cells revealed no alteration in T cell proliferation. However, interferon gamma and TNF production of T cells was suppressed. As I3S exerted no direct effect on T cells, the defect in T cell activation was mediated by I3S-treated mature DCs. Our study suggests an anti-inflammatory and tolerizing effect of I3S on human DCs.

1. Introduction

The interplay between the microbiome and the immune system has gained significant attention in recent years. It has been shown that immune-related diseases such as inflammatory bowel disease and graft versus host disease (GvHD) are linked to or the consequence of loss of microbial diversity in the gut (Holler et al., 2014; Ott et al., 2004; Weber et al., 2015). Several studies have investigated the immunomodulatory effects of metabolites produced by the microbiome and showed that a variety of metabolites induce a more tolerogenic phenotype in dendritic cells (DCs).

DCs are potent antigen presenting cells connecting innate and adaptive immunity. Immature DCs possess high phagocytic activity and take up soluble antigens or internalize apoptotic cells. DC maturation leads to a decreased ability to uptake antigens but results in increased stimulatory capacity as MHC molecules as well as CD40, CD80, CD83 and CD86 are upregulated providing the necessary equipment for T cell activation (e Sousa, 2006; Mellman and Steinman, 2001). Furthermore, interleukin-12 (IL-12 and NK cell) is secreted by mature DCs and is of special importance for T cell activation and interferon γ (IFNγ) production (Cella et al., 1996; Ferlazzo et al., 2004).

The bacterial metabolite, butyrate, a short chain fatty acid (SCFA) which is produced by clostridia species, has immunomodulatory effects on human monocyte-derived DCs (Nastasi et al., 2015).

Butyrate downregulates CD83 expression on the surface of mature DCs and suppresses the release of IL-6, IL-12 and pro-inflammatory chemokines in cell culture media (Nastasi et al., 2015), resulting in a more tolerogenic phenotype of DCs. Furthermore, butyrate can dampen intestinal epithelial cell damage and intestinal inflammation in an experimental model of GvHD (Mathewson et al., 2016).

The tryptophan metabolite indole, which is produced by species of clostridia, has a beneficial role on maintaining the epithelial barrier *in vivo* (Shimada et al., 2013). Indole derivatives such as indole 3-carbinol and indirubin-3′-oxime exhibit immunoregulatory and anti-inflammatory effects on bone marrow derived mature DCs in mice by suppressing DC surface molecules such as CD11c, CD40 and CD54 (Benson and Shepherd, 2011). Moreover, pro-inflammatory mediators like TNF, IL-1β, IL-6, IL-12 and nitric oxide were suppressed but IL-10 levels increased (Benson and Shepherd, 2011).

In addition, Weber et al. showed that a decrease in urinary indoxyl 3-sulfate (I3S), another indole derivative, was associated with poor outcome after allogeneic stem cell transplantation (ASCT). Low I3S levels were linked with a disrupted microbiome and low abundance of clostridia species (Weber et al., 2015). These data suggest that this

Abbreviations: GvHD, graft versus host disease; DCs, dendritic cells; MHC, major histocompatibility complex; IFNγ, interferon γ; I3S, indoxyl 3-sulfate; ASCT, allogeneic stem cell transplantation

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http://dx.doi.org/10.1016/j.imbio.2017.10.014
Received 24 May 2017; Received in revised form 19 September 2017; Accepted 3 October 2017

bacterial metabolite may not only reflect the disrupted microbiome but also act as an immunomodulator. Pathophysiologic high I3S concentrations up to 200 μM are detected in uremic patients due to decreased urinary excretion of I3S during renal failure (Niwa et al., 1988) compared to 1–10 μM in healthy individuals. Such high levels have been shown to exert toxic effects and are deleterious for epithelial and endothelial cells.

In the present study, we aimed to analyze effects of I3S on human monocyte-derived DCs by applying I3S in a concentration range reflecting physiologic to pathophysiologic levels. We investigated viability, maturation and function of human monocyte-derived DCs in the presence of I3S. Our results show that even a high concentration of I3S does not affect the viability of mature DCs. However, already low concentrations of I3S suppressed maturation and activation of DCs and thereby altered their capacity to stimulate cytokine production by T cells. Taken together, I3S may contribute to immune homeostasis by polarizing DC and T cell function.

2. Materials and methods

2.1. Monocyte separation and DC culture

The study was approved by the local ethical committee and all healthy volunteers gave written informed consent. Human T cells and monocytes were isolated from PBMC of healthy donors after leukapheresis followed by density gradient centrifugation over Ficoll/Hypaque as described previously (Andreesen et al., 1990). Immature DCs were differentiated from $7*10^5$/mL monocytes in RPMI-1640 (Biochrom, Berlin, Germany) supplemented with antibiotics (50 U/mL penicillin and 50 mg/mL streptomycin), L-glutamine (2 mM; Gibco, Carlsbad, CA), 10% fetal calf serum (FCS; BioWhittaker, Walkersville, MD), 144 U/mL IL-4 (Promokine, Heidelberg, Germany), and 225 IU/mL GM-CSF (Leukine/Sargramostim; Immunex, Seattle, WA). IL-4 and GM-CSF were not replenished during the culture. On day 5, 100 ng/mL LPS was added to induce maturation of DCs in the absence or presence of 1, 10 or 100 μM I3S, (Sigma-Aldrich. St Louis, MO) for another 48 h. In some experiments I3S was added directly to monocytes on day 0 as stated in the text. On day 7, cell number, viability, and cell size were determined using the CASY system (OLS, Germany). Left normalization cursor was set to 5.4 μm, right normalization cursor to 30 μm, left evaluation cursor to 9.6 μm and right evaluation cursor to 30 μm. Settings for determination of viability was determined by using CASY Blue. Cell culture supernatants were collected to determine cytokine production.

2.2. Mixed leukocyte reaction (MLR)

Total T lymphocytes were isolated from PBMC of healthy donors by elutriation as described earlier (Andreesen et al., 1990). T lymphocytes were frozen and stored at −80 °C or in liquid nitrogen until required. Mature DCs and allogeneic T cells were co-cultured in the ratio of 1:10 ($1*10^4$ mature DCs and $1*10^5$ T cells/200 μL) in RPMI containing 5% filtered AB serum for 6 days in 96 well plates. On day 5, cell supernatant was collected for cytokine analysis and on day 6 cells were counted and surface markers were analyzed by flow cytometry.

2.3. T cell stimulation with anti-CD3/CD28 dynabeads

To detect a direct impact of I3S on T lymphocytes, T cells were stimulated with anti-CD3/CD28 Dynabeads (Gibco, Life Technologies, Darmstadt, Germany) at a cell-to-bead ratio of 1:1 incubated with 0 μM, 1 μM, 10 μM, 100 μM I3S in RPMI supplemented with 5% AB serum and 25 IU/mL rhIL-2 (PeproTech, Hamburg, Germany). After 48 h, cytokines were analyzed in culture supernatants and surface marker expression was determined. Cells were splitted on day 3 and proliferation was measured on day 7.

2.4. Determination of surface marker expression by flow cytometry

Surface marker expression was determined by flow cytometry. Briefly, cells were washed twice with cold phosphate-buffered saline (PBS; Gibco) containing 0.1% sodium azide and 0.6 mg/mL immunoglobulin. Cells were incubated for 30 min at 4 °C with specific antibodies, washed two times, resuspended in buffer and analysed by flow cytometry (Calibur instrument BD, San Jose, CA). Twenty thousand events were acquired for mDCs and for allogeneic T cells. The following antibodies were used: CD86-FITC (clone 2331(FUN-1)), BD, Franklin Lakes, NJ), CD1a-PE (clone SFCI19Thy1A8, Beckman Coulter, CA), CD83-PE-Cy7 (clone HB15e, eBioscience, CA), CD80-APC (clone 2D10, Biolegend, CA) and HLA-DR-FITC (clone B8.12.2, Beckman Coulter, CA). Apoptosis was determined by Annexin V/7-AAD staining (BD Pharmingen, Heidelberg). Stimulated lymphocytes were stained with CD8-FITC (clone SK1), CD4-PE (clone RPA-T4), CD25-PE-Cy7 (clone M-A251), CD62L-APC (clone DREG-56), all purchased from BD Bioscience, Franklin Lakes, NJ, USA. Intracellular FoxP3 levels were determined according to the manufacturer's protocol (clone PCH101, eBioscience, San Diego, CA, USA). Data were analysed using the FlowJo software (Tree star, LLC, Ashland, Oregon, USA).

2.5. Determination of cytokines

Supernatants from dendritic cells and lymphocytes were stored at −20 °C and cytokines were analyzed using commercially available enzyme-linked immunosorbent assays (ELISA; R & D, Minneapolis, MN, USA) according to the manufacturer's protocol for the following cytokines: IL-6, IL-10, IL-12, TNF and IFNɣ.

2.6. Reverse transcription-quantitative real-time PCR (qPCR)

Mature dendritic cells were harvested and total RNA was extracted using RNeasy according to the manufacturer's protocol (Qiagen, Hilden, Germany). 1 μg RNA was reverse transcribed using M-MLV Reverse Transcriptase (Promega, Mannheim, Germany). Gene expression was quantified by using Mastercyler Ep Realplex (Eppendorf, Hamburg,Germany). For RT-qPCR 1 μL cDNA, 1 μL of specific primers (10 μM) and 5 μL QuantiFastSYBR Green PCR Kit (Qiagen, Hilden, Germany) in a total of 10 μL were applied. The following primers were used: *IL12A* sense: GAA GAT GTA CCA GGT GGA GTT CAA GAC, antisense: GCT CAT CAC TCT ATC AAT AGT CAC TGC C; *IL12B* sense: ACC AGC AGC TTC TTC ATC AGG GAC, antisense: ACG CAG AAT GTC AGG GAG AAG TAG GA; *18S* rRNA sense: ACC GAT TGG ATG GTT TAG TGA G, antisense:CCT ACG GAA ACC TTG TTA CGA C. All mRNAs were normalized to *18S* rRNA.

2.7. Western blot analysis

Immature DCs were stimulated with LPS in the presence or absence of I3S for 5, 30 and 60 min. Phospho-proteins were extracted as described earlier (Peter et al., 2015) and samples were separated on a denaturating 12% acrylamide gel. After western blotting, membranes were stained with rabbit-anti-ikB-β/(Santa Cruz, Dallas, TX)) in dry milk (5%), and detection was performed by chemoluminescence (ECL). Actin expression was used as loading control (Sigma Aldrich, St Louis, MO). Densitometric analyses were performed using Image Lab software where the volume of protein in each band was calculated.

2.8. Immunofluorescence analysis of NF-kB localisation

Immature DCs were stimulated with LPS in the presence or absence of I3S for 15 min. DCs were washed with PBS and transferred to adhesion slides (Marienfeld, Lauda Koenigshofen, Germany). Cells were fixed with 4% paraformaldehyde for 10 min and permeabilized with 0.2% Triton-X-100 for a minute. After blocking with 10% BSA for