

P. RADWAN¹, K. RADWAN-KWIATEK², J. TABARKIEWICZ², S. RADEJ², J. ROLINSKI²

ENHANCED PHENOTYPIC AND FUNCTIONAL MATURATION OF MONOCYTE-DERIVED DENDRITIC CELLS FROM PATIENTS WITH ACTIVE CROHN'S DISEASE AND ULCERATIVE COLITIS

¹Department of Gastroenterology, Medical University of Lublin, Lublin, Poland;

²Department of Clinical Immunology, Medical University of Lublin, Lublin, Poland

Disturbed immunoregulation and an inappropriate immune response to gut microflora is assumed to be involved in the pathogenesis of inflammatory bowel disease (IBD). Physiologically dendritic cells (DCs) as the professional antigen presenting cells play a crucial role in the control of intestinal inflammation and immune tolerance. In order to evaluate their role in the IBD we analyzed the phenotypic and functional properties of monocyte-derived DCs (MoDCs) generated from UC and CD patients following stimulation with TNF- α , lipopolisaccharide *E. coli* or hydrocortisone. Thirty seven patients with moderate to severe inflammation (19 UC, 18 CD) were recruited to the study. Monocyte-derived dendritic cell immunophenotypes and their endocytic ability were analysed by flow cytometry and confocal microscopy, IL-6, IL-10, IL-12 and IL-23 secretion were investigated by ELISA. Both unstimulated and stimulated MoDCs generated from IBD patients had more mature phenotype and secreted elevated concentrations of proinflammatory cytokines as compared to a control group. The addition of LPS *E. coli* to culture media was associated with enhanced dendritic cell activation and maturation as compared to DCs stimulated only with TNF- α . This may suggest altered dendritic cell interactions with intestinal microflora in inflammatory bowel disease. Hydrocortisone decreases the numbers of mature dendritic cells and the proinflammatory cytokine concentrations in all cell culture types that may explain the efficacy of steroid therapy in inflammatory bowel disease

Key words: *inflammatory bowel disease, dendritic cells, tumor necrosis factor-alpha, lipopolisaccharide, monocyte*

INTRODUCTION

Dendritic cells (DCs) play a central role in the normal immunoregulation of the gut associated lymphoid tissue (GALT). They can either initiate immune responses, or control intestinal inflammation and maintain tolerance (1). Disturbances of the balance between tolerance and an active immune response to luminal antigens of the gut (*e.g.* saprophytic microflora) is fundamental to the pathogenesis of inflammatory bowel disease (IBD) encompassing ulcerative colitis (UC) and Crohn's disease (CD) (2-4).

DCs generated from bone marrow circulate in the blood as immature cells and then locate in the areas, that are susceptible to pathogen infiltration, such as the gastrointestinal mucosa, monitoring the intestinal lumen by phagocytosing and processing luminal antigens (1, 5). Antigen captured in the presence of signals indicating danger or tissue damage induces DCs to undergo maturation. During maturation DCs cease phagocytosis, downregulate C-lectin receptors and upregulate their expression of MHC class II and costimulatory molecules, such as CD40, CD80, CD86. The process results in complete transition of DCs from antigen-capturing cells to antigen presenting cells (APC). Internalised antigens, processed in DC are finally exposed on the cellular membrane associated with MHC molecules. Antigen presentation to naive lymphocytes T

together with costimulatory molecules results in T-cell clonal expansion, and the adaptive immune response is initiated (1, 2, 5). On the other hand DCs play a key role in the development of oral tolerance. Immature DCs are implicated as promoting tolerance by inducing regulatory T-cells whereas fully mature DCs have the ability to initiate T-cell proliferation and effector function (2). Disturbances in the number and function of DCs may therefore lead to dysregulation of mucosal immune responses and the development of IBD (1-6).

In the human peripheral blood two subsets of DCs have been identified. Myeloid DCs expressing molecule CD1c⁺ and high levels of the granulocyte macrophage-colony stimulating factor (GM-CSF) and plasmacytoid DC (CD11c⁻), expressing high levels of IL-3 receptor (CD123) (2, 7).

Both human DCs subsets isolated directly from blood normally display immature phenotype with low levels of activation and costimulatory molecules (8). In the peripheral blood of patients with IBD significant decrease of the immature DCs of both myeloid and plasmacytoid origin was observed during acute exacerbation of the disease (9-11).

It was demonstrated recently that peripheral blood monocytes have the potential to differentiate into DCs *in vivo* during inflammation, and in the steady state (12, 13). Monocytes cultured *in vitro* with GM-CSF and IL-4 differentiate into immature DCs, characterized by their low expression of MHC

Class II and costimulatory molecules (14). This method has been proven to be a useful tool for studying DCs differentiation and maturation processes (12). It was found that DCs generated from blood monocytes of patients with UC show increased abilities to stimulate immune responses (15).

In the present study we investigated the phenotype and function of monocyte-derived dendritic cells (MoDCs) in patients with active phase of IBD.

MATERIAL AND METHODS

Human peripheral blood was obtained from 37 patients with active IBD (19 females and 18 males). UC was diagnosed in 19 patients (9 females and 10 males), aged from 20 to 56 years (mean 32 ± 7.13) and CD in 18 patients (10 females and 8 males), aged from 19 to 54 years (mean 38.16 ± 10.21). Control group included 12 healthy volunteers (6 females and 6 males), aged from 22 to 60 years (mean 35.6 ± 10.43). The diagnosis of IBD was made on the basis of standard clinical, radiologic, endoscopic and histologic criteria (16). Patients were newly diagnosed and they had not been treated with glucocorticosteroids, or immunomodulatory agents. The disease activity was evaluated according to the Crohn's disease activity index (CDAI) (17) and to the Modified Truelove-Witts Index (18). All patients had moderate to severe disease activity. No coincidental infections, allergy and any medication were reported in studied patients and the control subjects at the time of sample collection or within the month preceding the study. The study was approved by the Local Ethics Committee and all subjects gave informed consent.

In vitro generation of peripheral blood monocyte-derived DCs

Human peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood samples by density gradient centrifugation over Gradisol L (Aqua Medica, Poland) for 20 minutes and acceleration 700 xg. PBMC were collected, and washed twice in PBS without Ca^{2+} or Mg^{2+} (Biochrom AG, Germany). EasySep Human CD3 Positive Selection Cocktail and EasySep Magnetic Nanoparticles label CD3⁺ cells (StemCell Technologies, UK) were used for magnetic separation of PBMC. The separated fraction of CD3 negative cells was allowed to adhere to six-well culture plates (10×10^6 /well) (CellStar, Greiner Bio One, Germany) at 37°C in humidified atmosphere of 5% CO_2 for 90 min suspended in RPMI-1640 supplemented with 2% human albumin (Human Albumin Immuno 20%, Baxter AG, Austria) 100 IU/ml penicillin, 50 µg/ml streptomycin and 100 µg/ml neomycin (Sigma-Aldrich, Germany). Non-adherent cells (e.g. CD19⁺) were washed out with PBS without Ca^{2+} and Mg^{2+} (Biochrom AG, Germany). Adherent cells were grown in the appropriate culture medium as above for 5 days. For the induction of differentiation of monocytes into immature DCs culture medium was supplemented with recombinant human granulocyte macrophage-colony stimulating factor, (rhGM-CSF) at the dose of 1000 IU/ml (Gentaur, Belgium) and 500 IU/ml of recombinant human interleukin 4 (rhIL-4) (Strathmann, Germany). Cytokines were added to the cells on the 1, 3, and 5 day of the culture.

Endocytosis by MoDCs

Endocytosis of MoDCs was examined after 4 hours of incubation of the cells with molecules of Dextran-FITC (fast endocytosis) and after 24 hours (slow endocytosis).

To measure the endocytic activity of generated immature MoDCs, five-day cultures were carried out. After harvesting, the cells were resuspended in RPMI 1640 with 2% human albumin at

a concentration of 2 to 6×10^5 cells/mL medium. 40 000 kDa fluorescein isothiocyanate-conjugated dextran (FITC-Dx; Sigma-Aldrich, Germany) was then added to 2 mL of this suspension in a concentration 1 mg/ml of medium and the cells were incubated at 37°C or 4°C (internalization control) either for 4 hours (early endocytosis) or for 24 hours (late endocytosis). Incubations were stopped by adding cold RPMI 1640. Following 2 washes with 1 mL cold PBS, cells were immediately acquired on a FACS Calibur (Becton-Dickinson, USA) flow cytometer and analyzed using CellQuest software. The percentage of FITC-Dx Positive DCs and mean fluorescence intensity (MFI) of FITC-Dx were calculated.

Confocal microscopy visualization

Visualization of the FITC-Dx endocytosis was performed after 4 and 24 hours incubation with FITC-Dx with confocal microscopy. The DC suspension was cytospinned at 700 xg for 3 minutes to form a monolayer on the glass slide and immediately observed using the confocal laser scanning head LSM-5 Pascal on Axiovert 200 inverted microscope (Carl Zeiss, Canada). Microscopic visualization of the endocytosis was performed exclusively for the illustration of the process, while quantitative analysis was done by flow cytometry as described above.

Stimulation and maturation experiments

To induce maturation of the DCs recombinant human tumor necrosis factor- α (rhTNF- α) at the dose of 50 ng/ml (Strathmann, Germany) was added to each culture well on the sixth day of the culture. To evaluate immunomodulatory effects Mo-DCs were also treated with 100 ng/ml lipopolysaccharide *E. coli* - strain 055:B5 (LPS) (Sigma-Aldrich, Germany) and 5×10^{-6} mol/L of hydrocortisone hemisuccinate (HCT) (Jelfa, Poland) added simultaneously with TNF- α to the appropriate experimental wells.

Termination of the cultures

On the eighth day of the culture supernatants were collected and stored at -80°C for subsequent cytokine measurements. DCs were harvested using Trypsin/EDTA solution (Biochrom AG, Germany) for 10 mins in the atmosphere of 5% CO_2 . Detached cells were resuspended in the appropriate medium for future assays and counted in Neubauer chamber. Viability of the cells were estimated by Trypan Blue (Sigma-Aldrich, Germany) staining with light microscopy.

Flow cytometry analysis

The generated DCs were washed twice with PBS w/o Ca^{2+} or Mg^{2+} and stained with mouse anti-human monoclonal antibodies (mAbs). We used FITC-conjugated antibodies against CD83, CD80, and CD209, PE-conjugated antibodies against CD1a, CD86, and CD206, PeCy-5-conjugated antibodies against HLA-DR (all from Becton-Dickinson Pharmingen, USA), and the BD Simultest CD45-FITC/CD14-PE (Becton-Dickinson Pharmingen, USA). Immunophenotype of immature DCs was assessed on the sixth day of culture and the results were compared with the immunophenotype of the cells stimulated for 48 hours with rhTNF- α and LPS or rhTNF- α and HCT. We checked the co-expression of the DC markers in the following combinations: CD45/CD14, CD83/CD1a/HLA-DR, CD80/CD86/HLA-DR, and CD209/CD206/HLA-DR. 1×10^6 cells were incubated for 30 min at 4°C with each antibody. After immunofluorescent staining, the cells were analyzed by a

FACSCalibur using CellQuest software (Becton–Dickinson, USA). Monocyte-derived DCs were gated according to morphology on the FSC/SSC dot-plot and the expression of CD45 antigen. Samples were evaluated directly after the described protocol, without fixation.

Cytokine specific enzyme-linked immunosorbent assays (ELISAs)

Cytokine secretion by MoDCs at the immature stage of differentiation and after modulation by immunoregulatory agents rhTNF- α , LPS and HC were analyzed by enzyme-linked immunosorbent assays (ELISA). Interleukin 6 (IL-6) and interleukin 12 (IL-12) levels in supernatants from cell cultures were measured with the human IL-6 and human IL-12 kits (R&D Systems United Kingdom), while interleukin 10 (IL-10) and interleukin 23 (IL-23) were measured with human IL-10 and human IL-23 kits (Bender MedSystems, Austria) according to the manufacturer's instructions. The ELISA plates were read on an ELX-800 plate reader (BioTek Instruments Inc., USA).

Statistical analysis

Statistical significance was evaluated by nonparametric Mann-Whitney U-test (Wilcoxon-Kruskal Wallis) to analyze variables that were not normally distributed. Significance was defined at $p < 0.05$. Statistical calculations were performed with Statistica 7.1 PL software (StatSoft Inc., USA).

RESULTS

Phenotypic characterization of unstimulated MoDCs CD1a / CD83; CD80/ CD86 and HLA-DR expression on unstimulated MoDCs

The percentage of CD1a+/CD83+/HLA-DR+ MoDCs generated from patients with UC (22.1%, range 7.5-44.6) and

CD (23.3%, range 6.5-47.3) was significantly higher as compared to the control group (18.6%, range 5.3-43.3; $p < 0.05$; $p < 0.01$, respectively) (Fig. 1). The percentage of CD80+/CD86+ MoDCs generated from patients with CD (75.5%, range 28.8-98.1) was significantly higher ($p < 0.05$) as compared to controls (70.9%, range 25.9-94.3), while in the UC patients this difference was not significant. Unstimulated Mo-DCs from both groups of patients had significantly higher HLA-DR expression, expressed as the mean fluorescence intensity (MFI) indicating the density of expression of the molecules per cell (UC-1124.4, range 200.3-1432.1; CD-1041.7, range 189.1-1542.4), compared with the healthy subjects (624.34, range 285.32-1254.53; $p < 0.005$).

Endocytic activity of MoDCs

An unstimulated Mo-DC population was also tested in a functional assay to assess their capability to take-up antigen. Microscopic visualization of the cells loaded with FITC-dextran is presented on Fig. 2. Dextran endocytic ability of Mo-DCs, examined by their uptake of FITC-dextran was significantly reduced after 4-hour incubation in patients with UC (76.72%, range 35.43-91.40) and CD (75.21%, range 21.54-86.76) as compared to Mo-DCs generated from healthy subjects (82.94%, range 70.17-89.67; $p < 0.005$) (Fig. 3A). Mo-DCs from the control group were also significantly more endocytically active after 24-hour incubation (99.55%, range 81.72-99.96; $p < 0.05$) compared to with Mo-DCs from UC (92.43%, range 53.41-99.03) and CD patients (90.55, range 49.88-98.50) (Fig. 3B).

Phenotypic characterization of modulated Mo-DCs Mo-DCs exposed to TNF- α or to TNF- α and LPS *E. coli*

Under TNF- α and LPS culture conditions we found a lack of completely immature CD83-/CD1a+ DCs. All cells positive for CD1a co-expressed the maturation marker CD83 and a high expression of costimulatory molecules and HLA-DR, so we considered them mature DCs (Fig. 4). On day 7, most monocytes differentiated into DCs in UC, CD and control group

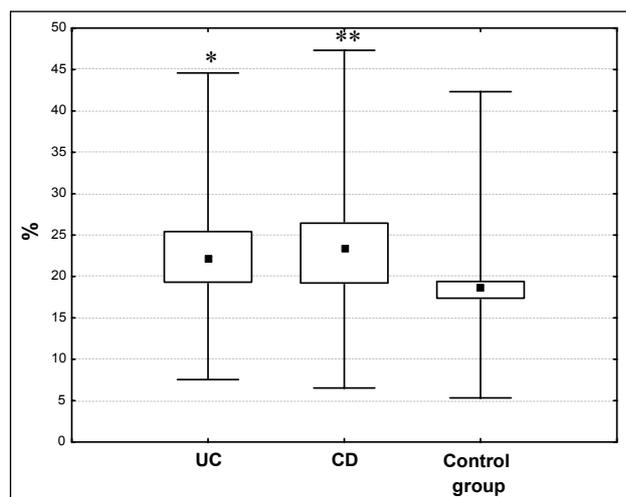


Fig. 1. Increased percentage of unstimulated Mo-DCs with the surface expression of maturation markers (CD1a+/CD83+) generated from inflammatory bowel disease patients. UC-ulcerative colitis, CD-Crohn's disease. Results are expressed as median (point), interquartile range (rectangles), and minimal and maximal values (whiskers) *Indicates a significant difference vs. control group $p < 0.05$, ** $p < 0.01$.

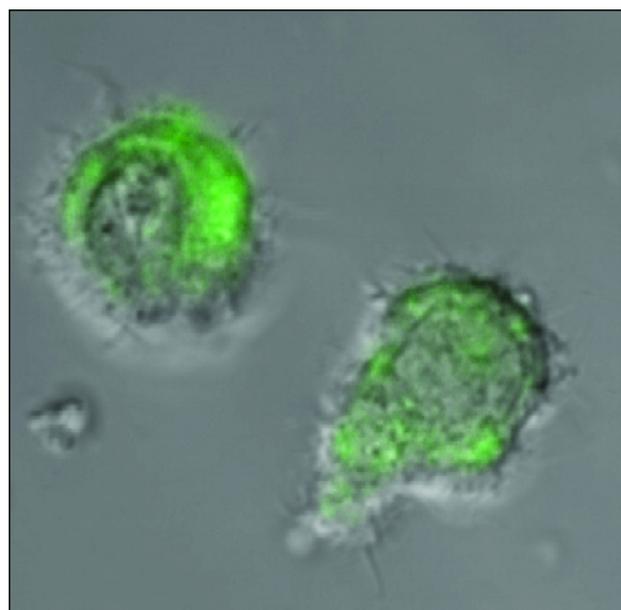


Fig. 2. Visualization of Dextran-FITC endocytosis (green) by unstimulated monocyte-derived dendritic cells after 4 h incubation. Confocal microscopic photography (100x).

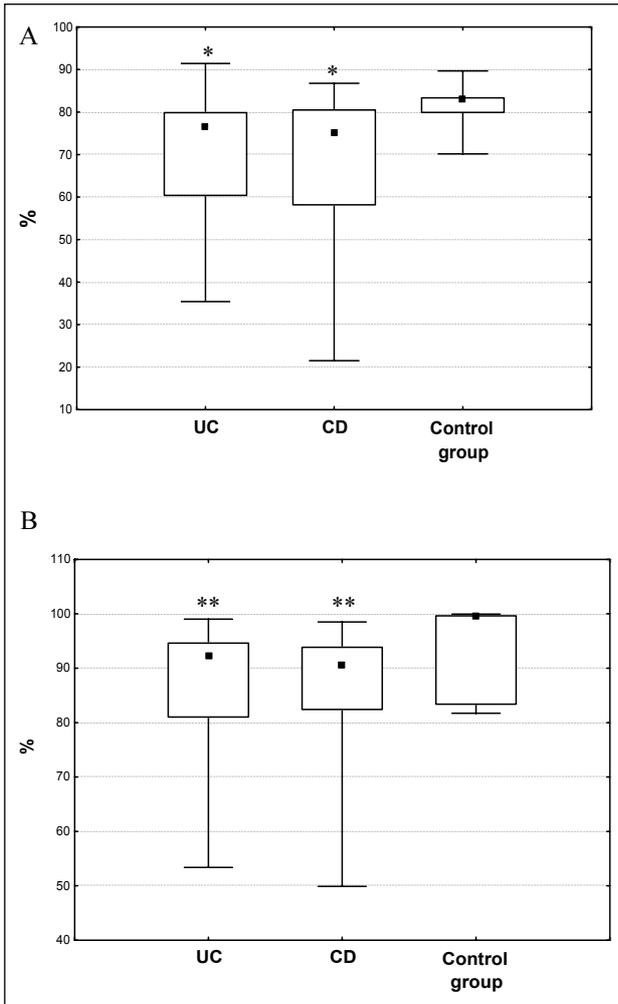


Fig. 3. Decreased endocytic activity of Mo-DCs generated from UC and CD patients after 4 (A) and 24 hour (B) incubation in comparison with the control group shown as percentage of Dextran-FITC positive cells. Results are expressed as median (point), interquartile range (rectangles), and minimal and maximal values (whiskers). */**Indicates a significant difference vs. control group with * $p < 0.005$, ** $p < 0.05$.

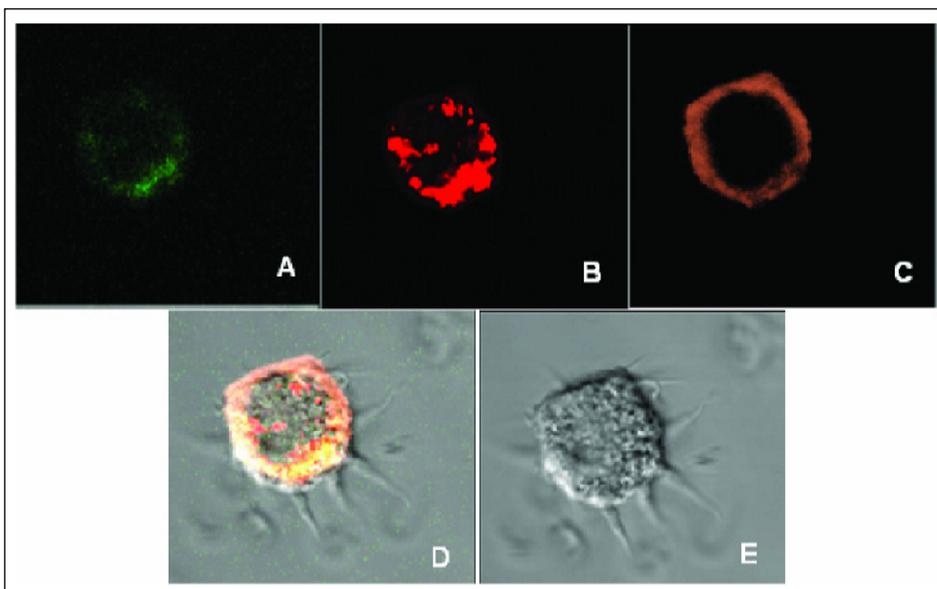


Fig. 4. Immunophenotype analysis of mature monocyte-derived dendritic cells. Confocal microscopy image (100x). Detection performed on a triple-stained immunofluorescence specimen – A) CD83-FITC (green), B) CD1a-PE (red), C) HLA-DR-PeCy5 (pink), D) CD1a+/CD83+/HLA-DR+ (triple stained), E) Nomarsky contrast.

cultures (>98.5%) and similar low percentages of CD14 positive cells were found in the cultures from UC, CD patients and control group (Table 1).

The percentage of CD1a+/CD83+ MoDCs generated from patients with UC or CD when stimulated with TNF- α or TNF- α and LPS, was significantly higher compared to controls ($p < 0.005$) (Table 1). Similarly, a significantly higher percentage of CD80+/CD86+ MoDCs from IBD was found in comparison with the healthy group (UC- $p < 0.05$, CD- $p < 0.005$) (Fig. 5). Moreover, the addition of LPS *E. coli* significantly changed the phenotype of Mo-DCs from UC and CD patients. Compared with the culture with TNF- α alone, the percentage of Mo-DCs expressing costimulatory molecules was significantly higher ($p < 0.05$) in UC patients (Fig. 6). This effect was absent in case of healthy subjects.

Similarly, in the presence of TNF- α MoDCs generated from UC and CD patients presented increased level of HLA-DR expression (MFI) and this difference was significant ($p < 0.05$). Exposure of Mo-DCs from IBD patients to LPS *E. coli* significantly up-regulated HLA-DR expression so that MFI level of HLA-DR on Mo-DCs from UC and CD patients was higher in comparison with control group ($p < 0.01$, $p < 0.005$, respectively) and with cells cultured with TNF- α alone ($p < 0.05$). Interestingly, there was no significant difference in the HLA-DR expression on Mo-DCs generated from healthy individuals whether cultured in the presence of TNF- α alone or TNF- α and LPS (Table 2).

On the other hand, following TNF- α stimulation the expression of C-lectin receptors (CD206, CD209) was similar and did not differ between the groups. However, incubation of DCs from IBD patients with LPS resulted in a significant decrease of CD206+/CD209+ expression as compared to control group under the same culture conditions ($p < 0.05$). Percentage of CD206+/CD209- DCs from IBD patients was higher as compared to the culture with TNF- α alone ($p < 0.05$) (Table 1).

These findings demonstrate striking differences in the expression of DC surface molecules between IBD patients and healthy individuals. After TNF- α or TNF- α and LPS stimulation, Mo-DCs from IBD patients displayed a more mature phenotype as compared to Mo-DCs from healthy subjects.

Mo-DCs exposed to hydrocortisone

No statistically significant differences in the percentage of Mo-DCs expressing CD14 were found following the

Table 1. Analysis of the immunophenotype of monocyte-derived DCs shown as percentage of positive cells. Results are expressed as the median with minimum and maximum values in parentheses.

	TNF- α			TNF- α +LPS			TNF- α +HCT		
	Control group	UC	CD	Control group	UC	CD	Control group	UC	CD
CD45+/CD14+	0.7 (0.21-3.54)	0.7 (0.1-3.9)	0.6 (0.2-2.2)	0.5 (0.3-1.2)	0.5 (0.1-3.4)	0.6 (0.1-1.1)	0.7 (0.0-3.1)	0.8 (0.1-3.7)	0.7 (0.1-11.32)
CD1a+/CD83+	91.6 (72.3-98.9)	93.9* (79.9-99.1)	94.4* (84.9-99.7)	91.9 (78.4-98.4)	94.2* (84.5-99.8)	94.6* (89.4-99.9)	78.05 ^{^d} (34.2-88.4)	78.5 ^{^c} (35.5-94.3)	79.4 ^{^c} (45.3-92.1)
CD1a-/CD83+	5.9 (0.6-20.1)	5.5 (0.8-19.2)	5.7 (0.3-14.5)	7.9+ (1.2-21.3)	5.7* (0.1-15.2)	5.6* (0.1-10.2)	7.6+ (1.3-10.2)	8.3 ^{^b} (1.1-16.4)	7.2 ^{^a} (2.8-12.4)
CD206+/CD209-	1.4 (0.1-2.4)	1.3 (0.2-2.2)	1.2 (0.1-2.5)	1.1 (0.1-3.4)	0.8+ (0.0-2.6)	0.7+ (0.1-2.9)	1.6 ^{^a} (0.7-1.9)	1.2 ^{^a} (0.3-1.8)	1.4 ^{^a} (0.3-1.9)
CD206+/CD209+	31.5 (14.6-52.7)	31.6 (18.3-51.9)	30.4 (13.9-51.3)	32.3 (11.6-55.2)	26.4# (10.3-44.1)	26.03# (10.1-52.3)	49.3 ^{^d} (23.5-80.9)	48.2 ^{^d} (17.4-68.4)	47.4 ^{^d} (16.4-74.1)
CD206-/CD209+	67.2 (47.4-82.8)	68.3 (49.8-72.3)	69.2 (45.5-80.3)	67.4 (43.2-83.4)	69.7 (58.4-87.6)	70.6# (48.6-89.4)	49.4 ^{^c} (18.3-63.4)	47.9 ^{^c} (21.3-63.6)	50.3 ^{^d} (19.3-76.6)

* #Indicates a significant difference vs. the control and #p<0.05. *p<0.005. + &^Indicates a significant difference vs. TNF- α -treated culture and + p<0.05. & p<0.0005. ^ p<0.0001. ^{abcd} Indicates a significant difference vs. LPS-treated culture and ^a p<0.05 ^b p<0.005. ^c p<0.0005. ^d p<0.0001.

Table 2. Analysis of the mean fluorescence intensity (MFI) of HLA-DR (MHC class II) indicating the density of expression of the molecules per cell. Results are expressed as the median with minimum and maximum values in parentheses.

Control group	TNF- α		TNF- α +LPS			TNF- α +HC		
	UC	CD	Control group	UC	CD	Control group	UC	CD
1457.5 (712.4-2543.2)	1658.6* (893.3-2567.6)	1706.4* (1243.6-2874.3)	1712.8 (965.5-2654.2)	2201.3##+ (1123.7-3245.9)	2290.8& (1234.2-4665.3)	1026.5 ^{^a} (533.6-1831.8)	1235.2 ^{^a} (493.2-2232.5)	1194.5 ^{^a} (589.6-1821.8)

* # & Indicates a significant difference vs. the control and p* < 0.05. # p < 0.01. & p < 0.005. +^ Indicates a significant difference vs. the TNF- α -treated culture and + p < 0.05. ^ p > 0.005. ^a Indicates a significant difference vs. the LPS-treated culture and ^a p > 0.001

Table 3. Cytokine production of MoDCs in different culture conditions. Results are expressed as the median with minimum and maximum values in parentheses.

pg/ml	TNF- α			TNF- α +LPS			TNF- α +HCT		
	Control group	UC	CD	Control group	UC	CD	Control group	UC	CD
IL-6	123.2 (12.2-241.4)	220.4* (19.9-261.5)	192.2* (26.0-472.6)	469.3++ (165.1-482.8)	490.3*++ (143.9-491.1)	481.1*+ (203.2-494.4)	100.4++ (24.4-408.9)	118.5 ^{^d} (35.9-388.9)	128.5 ^{^d} (13.8-469.9)
IL-12	5.9 (4.1-11.6)	8.7** (4.7-25.9)	8.1** (4.2-31.5)	9.4# (4.0-75.6)	16.7*## (5.4-52.6)	19.4*## (6.2-618.5)	3.8# ^a (0.5-9.1)	4.5* ^{^d} (1.7-12.3)	5.9* ^{^d} (2.0-16.2)
IL-23	22.2 (13.6-37.7)	25.4 (12.8-146.5)	29.6 (16.2-242.6)	90.5 ^{^^} (28.2-185.9)	112.3*++ (28.0-424.9)	162.5** ^{^A^} (14.4-723.9)	25.5 ^{^b} (14.4-63.6)	26.5 ^{^d} (19.5-61.4)	27.4 ^{^c} (16.2-77.9)

* /** Indicates a significant difference vs. the control and *p<0.05. **p<0.005. ^A Indicates a significant difference vs. the UC group and [^]p<0.05. # Indicates a significant difference vs. TNF- α -treated culture and #p<0.05. ##p<0.01. [^]p<0.005. ^{^^} p<0.0001. +p<0.0005. ++p<0.0001. ^{abcd} Indicates a significant difference vs. LPS-treated culture and ^a p<0.01. ^b p<0.001. ^c p<0.0005. ^d p<0.0001.

administration of hydrocortisone as compared to the culture with TNF- α alone (Table 1). However, the addition of HCT to MoDCs cultured with either TNF- α alone or with TNF- α and LPS resulted in a marked decrease in the expression of CD1a+/CD83+ in all groups (UC-p<0.0005, CD-p<0.0005, p<0.0001, CG-p<0.0001, respectively). Incubation of DCs from IBD patients with HCT down-regulated the expression of CD1a surface marker in comparison with TNF- α and TNF- α and LPS culture (Table 1).

Hydrocortisone added to MoDCs, stimulated with TNF- α caused a significant decrease of the percentage of

CD80+/CD86+MoDCs in all groups (UC, CD-p<0.0005, CG-p<0.0001) (Fig. 6). A substantial drop in the HLA-DR expression was also seen following HCT addition, which was statistically significant in all cultures performed (Table 2).

Administration of HC to all cell cultures was followed by highly significant changes in the C-lectin receptor expression on the Mo-DCs surface. The increase of CD206+/CD209+ DCs was shown in comparison to the culture with TNF- α alone (p<0.0005) (Table 1).

Our observations indicate that HC inhibits DC maturation and substantially reduces their immunostimulatory capacity.

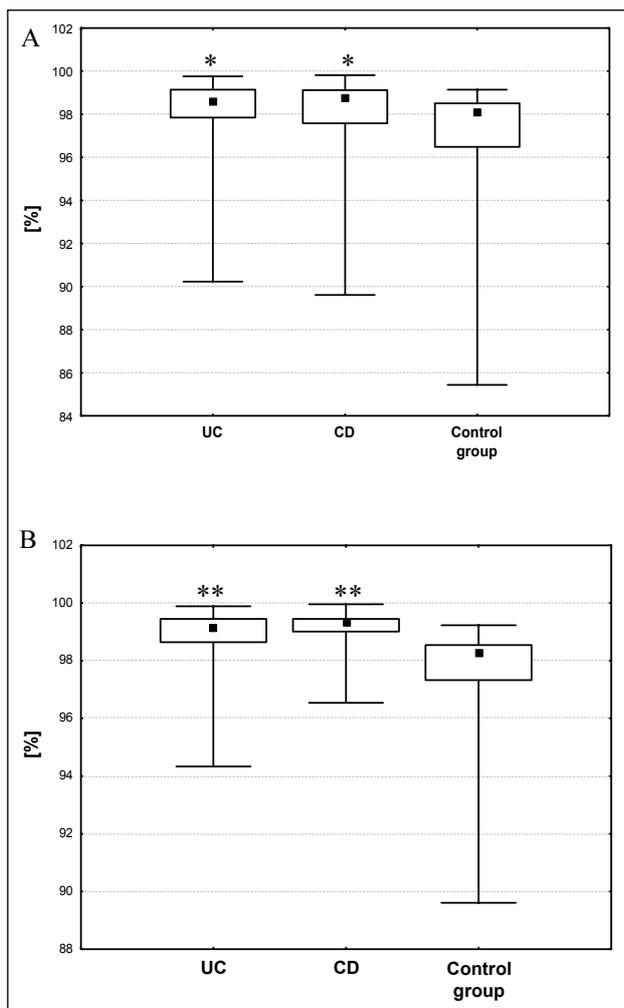


Fig. 5. Increased percentage of Mo-DCs with the surface expression of costimulatory molecules (CD80+/CD86+) stimulated with TNF-α (A) or TNF-α and LPS *E.coli* (B) generated from inflammatory bowel disease patients. UC - ulcerative colitis, CD - Crohn's disease. Results are expressed as median (point), interquartile range (rectangles), and minimal and maximal values (whiskers) *Indicates a significant difference vs. control group p<0.05, ** p<0.0005.

Cytokine production

To assess cytokine response of MoDCs from IBD patients in the immature state and in response to different immunomodulatory agents, concentrations of secreted IL-12, IL-6, IL-23 and IL-10 were measured.

Both unstimulated (data not shown) and stimulated MoDCs generated from IBD patients secreted higher levels of proinflammatory cytokines as compared to Mo-DCs from healthy individuals. The concentrations of IL-6 and IL-12 in the supernatants of MoDCs stimulated with TNF-α were significantly higher in the UC and CD patients than in the control group (IL-6: p<0.05, IL-12: p<0.005, in both groups). Stimulation with LPS resulted in a significant increase of the IL-6, IL-12 and IL-23 production in comparison with supernatants from cultures with TNF-α alone (Table 3). Furthermore, LPS-induced Mo-DCs from IBD patients were much more capable of secreting all three proinflammatory cytokines than MoDCs from the control group (Fig. 7). Interestingly, MoDCs from CD patients secreted more IL-23 than MoDCs generated from UC

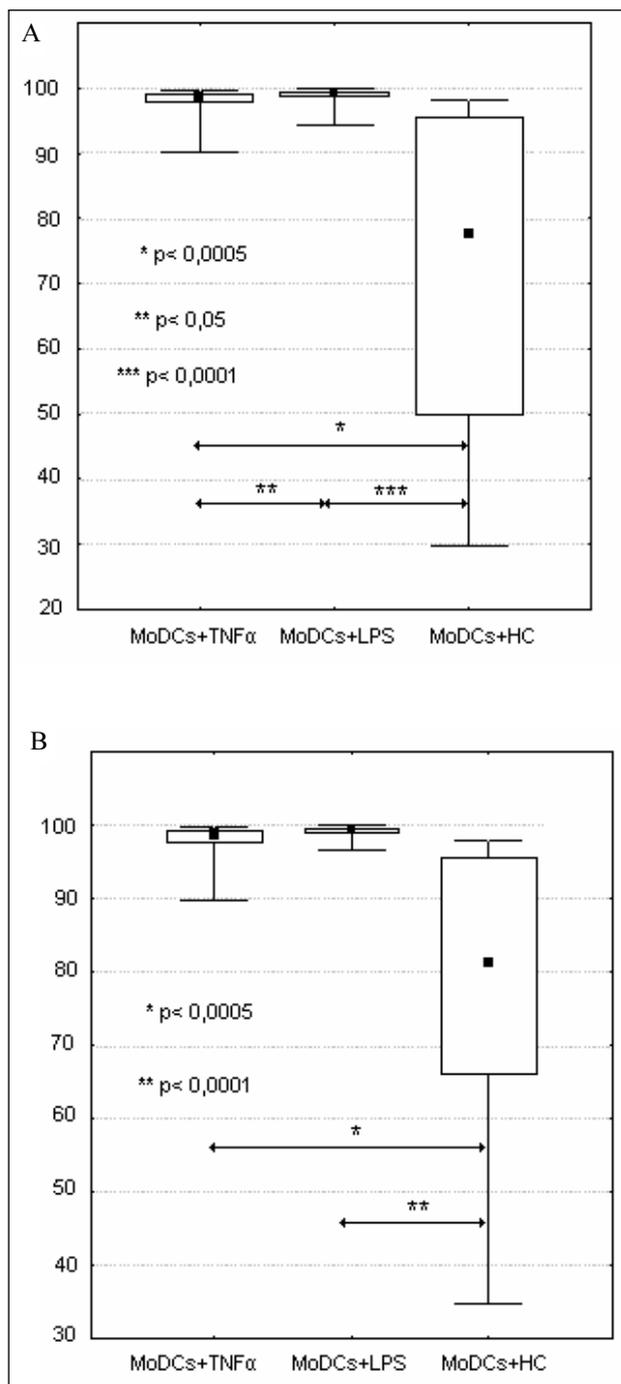


Fig. 6. Analysis of percentage of Mo-DCs with the surface expression of CD80 and CD86 costimulatory molecules generated from patients with ulcerative colitis (A) and Crohn's disease (B) in different culture conditions (TNF-α - tumor necrosis factor α, LPS - lipopolysaccharide *E. coli*, HC-hydrocortisone). Results are expressed as median (point), interquartile range (rectangles), and minimal and maximal values (whiskers).

patients (p<0.05). Consistent with our previous findings, addition of HC to the cultures caused diminished concentrations of these cytokines in the supernatants in all culture performed. There were no differences in the concentrations of IL-10 among patients and control group. Analysis of cytokine concentrations are presented in Table 3.

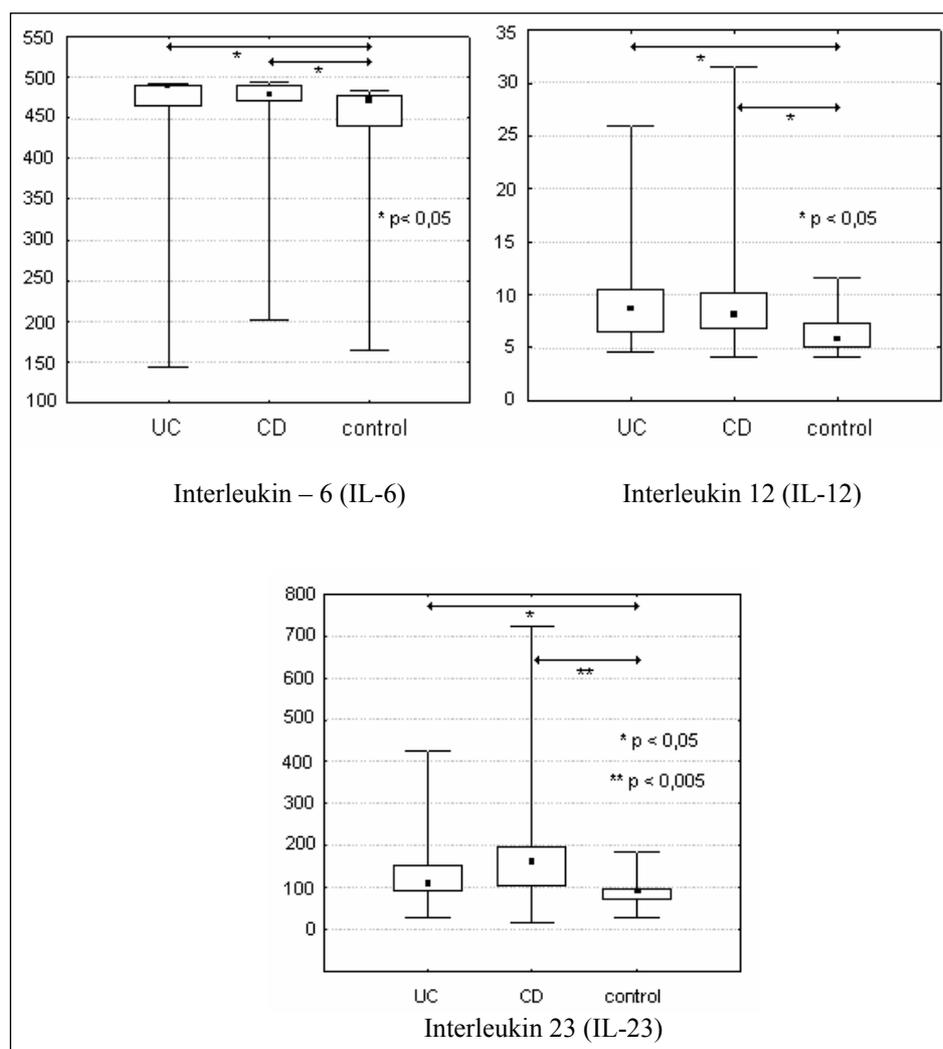


Fig. 7. Levels of proinflammatory cytokines (IL-6, IL-12, IL-23) in the supernatants of MoDCs cultures under LPS E.coli stimulation, generated from patients with ulcerative colitis (UC), Crohn's disease (CD) and control group (pg/ml). Results are expressed as median (point), interquartile range (rectangles), and minimal and maximal values (whiskers).

DISCUSSION

Dendritic cells and their functional properties have been extensively characterized in a variety of immunological disorders, but relatively few studies have been performed in IBD (3, 6, 9, 10, 19). Isolation of DCs *in vivo* is complicated, resulting in their limited availability for functional studies. In animal studies it was found that MoDCs may be compared to blood DCs in terms of antigen uptake, activation and maturation process (20, 21). Human peripheral blood monocytes can be differentiated into immature DCs by culturing with GM-CSF and interleukin IL-4. Immature DCs can be induced to mature by incubation with TNF- α . The changes in expression of cell surface markers such as decreased expression of the mannose receptor CD206, induction of the maturation marker CD83, upregulation of HLA-DR, CD40 and costimulatory molecules (CD80, CD86) and reduced abilities to take-up antigens represents a process commonly referred to as maturation (22).

Physiologically immature DCs display high endocytic/phagocytic capacity and are very efficient in antigen capture, but they are poor at presenting antigens because they express low levels of co-stimulatory molecules (5). Our data demonstrate a reduced endocytic function of MoDCs generated from patients with UC and CD, probably as a consequence of their maturation.

In this study we observed that unstimulated MoDCs of IBD patients displayed a more mature phenotype compared to

controls. The percentages of immature MoDCs expressing maturation antigens such as CD1a/CD83, CD80/CD86 and the expression of HLA-DR were significantly higher in patients with UC and CD compared to healthy subjects. In other words, MoDCs from patients with UC and CD seem to be "spontaneously" activated even before their *in vitro* stimulation. Enhanced maturational process of unstimulated Mo-DCs from IBD patients may also reflect an impaired promotion of tolerogenic DC pathway. Vuckovic *et al.* also observed higher expression of CD86 and CD40 in freshly isolated blood DC from patients with UC and CD (8).

Gut mucosa is the main site of inflammatory process in IBD. The population of gut DCs is a dynamic one, with cells constantly trafficking into and out of the gut compartment (2). Immunological disturbances observed in the peripheral blood may, at least in part reflect immunopathological mechanisms (events) responsible for intestinal tissue injury and clinical symptoms of IBD. Activated DCs in the blood are either recirculating from peripheral tissue or are being activated by systemic influences as a result of the disease process in the gastrointestinal disease (8).

Several studies have shown a significant decrease in immature DCs in the peripheral blood of patients with IBD and this drop correlated with the severity and the extent of the inflammatory process (9-11). It was suggested that the decrease of DCs in the blood resulted from a migration of the cells to the inflamed tissue of the gut. This is consistent with

immunohistochemical studies showing increased numbers of DCs expressing costimulatory molecules such as CD40, CD80, CD83 and CD86 in mucosal tissue from CD and UC (6, 16). Baumgart *et al.* reported that in patients with IBD expression of co-stimulatory molecules (CD 86) was low or even absent (CD83) on peripheral blood DCs. However these studies were performed on DCs, obtained directly from fresh blood with no stimulation, which may at least in part explain the discrepancies with our results (9).

The addition of substances with immunomodulatory properties to the culture is likely to substantially change both the differentiation of monocytes and the maturation of monocyte-derived DCs. In humans some studies revealed that DCs of IBD patients show increased abilities to stimulate immune responses (15). It was also observed that potentially probiotic bacteria induce MoDCs maturation as efficiently as pathogenic bacteria (23, 24).

In our study, TNF- α , LPS *E. coli* and hydrocortisone were added to the culture medium. TNF- α constitutes an activating cytokine and a maturation factor of DCs as well as a pivotal immune mediator in the pathogenesis of IBD (16). Although the pathogenesis of IBD is multifactorial, it is generally assumed that inappropriate stimulation of the immune system by the endogenous intestinal microflora results in a chronic inflammatory process. Thus, we used LPS *E. coli* to assess its immunomodulatory impact on DC function (5). We observed significant differences in its influence on DC differentiation in UC and CD patients in comparison with healthy control. Both TNF- α and LPS were found to significantly increase the percentage of CD1a+/CD83+, CD80+/CD86+ DCs and to markedly upregulate the expression of MHC class II molecule (HLA-DR) in IBD patients. The phenotypic DC changes in the cultures from UC and CD patients suggest their enhanced immunostimulatory potential in comparison with DCs from healthy subjects. This is in agreement with data of resident intestinal DCs from CD patients that show increased cell surface activation markers (19). We also found that phenotypically activated MoDCs generated from UC and CD patients also displayed a dramatically increased release of proinflammatory interleukins (IL-6, IL-12 and IL-23) as compared to controls. Our findings correspond to the observations of increased IL-6 and IL-12 production by colonic DCs in patients with Crohn's disease (19, 25).

Stimulation with LPS resulted in a further significant rise in the percentages of CD1a/CD83, CD80/CD86 positive MoDCs, HLA-DR expression and enhanced cytokines production, which was much higher in UC and CD than in controls. LPS *E. coli* is a microbial cell wall component of gram-negative bacteria which belongs to structures, designated PAMPs (pathogen-associated molecular patterns). In previous studies it was observed that microbial products such as LPS stimulate the maturation and cytokine production of DCs, both in animals and humans (2, 20, 24). Enhanced responses of MoDCs to LPS in terms of maturation and cytokine production, observed in our patients with UC and CD strongly suggest inappropriate reactivity to bacterial microflora in these inflammatory disorders of the intestines. DCs play a crucial role in maintaining intestinal homeostasis. DCs can either initiate immune responses or control intestinal inflammation and maintain tolerance. The loss of tolerance to resident intestinal bacteria is assumed to result in IBD (24-27). Salucci *et al.* showed normal activation and maturation, but impaired proinflammatory cytokine production of Mo-DCs in response to *Salmonella typhimurium*, generated from Crohn's disease patients with a L1007fs mutation in NOD2 gene (28), but the pathophysiological significance of this observation is not clear.

In LPS culture from IBD patients we observed decrease in the percentage of CD1a negative DCs. Another study

demonstrated that proinflammatory cytokines may inhibit the CD1a expression during DC differentiation, but promoting maturation of both DC populations (29). However, CD1a+ DCs are generally considered to be more potent at activating T cells CD4+ (30). Another study carried out in healthy individuals showed that CD1a+ DCs strongly stimulate proliferation and the IL-12 production in Th cells (31).

In our study, a low percentage of CD206+DC was observed after stimulation. According to Kato *et al.* the expression of the mannose receptor CD206 is down-regulated during the DC maturation process (32). Furthermore, the percentage of CD206+/CD209+ Mo-DCs generated from IBD patients under LPS stimulation was lower than in the control group suggesting their more mature phenotype, which is consistent with our previous findings. Other studies suggest that C-lectin receptor-mediated endocytosis (especially CD209) may be involved in the development of immune tolerance (33). Therefore, according to results of our study it may be assumed that altered DC phenotype is associated with impaired mechanisms of tolerance to gut microflora in IBD. However, as far as we know our study is the first attempt to assess C-lectin receptor expression on MoDCs in IBD patients.

HC added to MoDCs stimulated with TNF- α resulted not only in a significant fall in the percentage of MoDCs expressing costimulatory and maturation molecules, but also significant decrease in the concentrations of IL-6, IL-12 and IL-23 in the supernatants of the MoDCs cultures. Our findings are similar to other authors' observations (34). Recent studies performed in healthy subjects showed that corticoids may induce the differentiation of tolerogenic DCs capable of stimulating IL-10-secreting T cells (35), which could be one of the numerous therapeutic mechanisms of glucocorticosteroids in IBD. This is extremely relevant since the degree of DC maturation determines the generation and regulation of interactions between DCs and T cells. Mature DCs induce T cells and initiate Th-cell antigen-specific activation, while immature DCs have tolerogenic properties.

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Author's address: Prof. Piotr Radwan, Department of Gastroenterology, Medical University of Lublin, 8 Jaczewskiego Street, 20-850 Lublin, Poland; Phone: 603 79 49 44; Fax: 81 72 44 535; E-mail: piotr_radwan@wp.pl