

# Increased Expression of Inducible Nitric Oxide Synthase in Circulating Monocytes from Patients with Active Inflammatory Bowel Disease

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**Background:** Inducible nitric oxide synthase (iNOS) expression and nitric oxide (NO) synthesis are increased in epithelial cells and in tissue macrophages of the inflamed mucosa from patients with inflammatory bowel disease (IBD). Since tissue macrophages are derived from circulating monocytes, we studied iNOS expression in circulating monocytes and related this expression to disease activity. In view of the possible role of NO in monocyte function, we also studied iNOS expression in relation to markers of monocyte activation. **Methods:** The expression of iNOS in circulating monocytes from 15 patients with active IBD, 6 patients who went into remission and 18 healthy controls was quantified by flow cytometry and correlated with surface markers (CD63, CD11b, HLA-DR) for monocyte activation. In addition, iNOS expression in circulating monocytes was assessed by Western blotting, immunocytochemistry and measurement of the NO metabolites nitrite and nitrate in plasma. **Results:** The expression of iNOS in circulating monocytes and the percentage of iNOS-positive monocytes were increased in patients with active IBD compared to healthy controls (fluorescence index: 1.3 (0.1–6.3) versus 0.8 (0.0–1.8);  $P < 0.05$ ; percentage of iNOS positive monocytes: 37.3 (1.0–77.0)% versus 5.3 (0.0–43.3)%;  $P < 0.01$ ). The six patients who went into remission all had a marked reduction of iNOS expression. iNOS expression was confirmed by Western blotting and immunocytochemistry. Plasma nitrite and nitrate levels were elevated in three patients with active IBD. The surface markers for monocyte activation, CD63 and CD11b, were not elevated. HLA-DR expression was decreased on circulating monocytes from patients with active ulcerative colitis. **Conclusions:** iNOS is increased in circulating monocytes from patients with active IBD and this increased expression correlates with disease activity. Considering the decreased HLA-DR expression and absence of monocyte activation markers, NO produced by iNOS may have a function in suppressing systemic monocyte activation.

**Key words:** Crohn disease; inducible nitric oxide synthase; inflammatory bowel disease; monocytes; neopterin; nitric oxide; NO; ulcerative colitis

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Conflicting data have been reported concerning activation of circulating monocytes from patients with inflammatory bowel disease (IBD). Increased numbers (1) and increased turnover (2) of circulating monocytes were found in patients with active IBD. Evidence of monocyte activation was found in their increased motility, phagocytosis, spontaneous giant cell formation, superoxide production and release of lysosomal enzymes (3, 4). In contrast, a deficient spontaneous cell-mediated cytotoxicity, antibody-dependent cellular cytotoxicity (ADCC) and hyporesponsiveness to activation with interferon-gamma (IFN- $\gamma$ ) and mitogenic lectins have also been reported (5). Nitric oxide

(NO) may be involved in the regulation of monocyte function. Both NO-mediated induction (6) and NO-mediated suppression (7) of lipopolysaccharide (LPS) induced tumour necrosis factor alpha (TNF- $\alpha$ ) production by human macrophages have been described.

Nitric oxide synthase (NOS) is an enzyme that catalyses the synthesis of NO and L-citrulline using oxygen and L-arginine as substrates. The enzyme exists in three isoforms encoded by distinct genes. Two constitutively expressed isoforms, neuronal NOS (nNOS, type I) and endothelial NOS (eNOS, type III), produce small amounts of NO (picomolar range) for neuronal and cardiovascular regulatory processes. The third,

Table I. Patient and control characteristics

	Crohn disease	Ulcerative colitis	Healthy controls
Number	4	11	18
Age*	38 (20–61)	50 (28–71)	31 (19–36)
Male/female	1/3	5/6	11/7
Smoking	2	0	4
Localization	Ileum (1) Colon + ileum (3)	Proctitis (1) proctosigmoiditis (3) left-sided (3) pancolitis (4)	
Medication			
a. Mesalazine (range)	3–5 g/day	2–4 g/day	
b. Prednisolone (mg/day)	20 (1 pt), 30 (1pt)	5 (1 pt), 20 (2 pts), 40 (1 pt) and 60 (1 pt)	
Disease index*	CDAI 258 (207–320)	7 (5–12)	
CRP*	26 (0–201) mg/l	20 (0–192) mg/l	
ESR*	35 (1–67)	28 (3–92)	
Monocytes*	0.68 (0.3–1.1) 10 <sup>9</sup> /l	0.6 (0.2–6.6) 10 <sup>9</sup> /l	

\* Median and range.

calcium- and calmodulin-independent isoform, inducible NOS (iNOS, type II), is induced by the cytokines TNF- $\alpha$ , interleukin-1 (IL-1), IFN- $\gamma$  and by LPS in a variety of cell types. Physiologic expression of iNOS has been detected in airway (8) and colonic epithelial cells (9). iNOS produces large amounts (micromolar range) of NO. Induction of iNOS is suppressed by the interleukins IL-4,-8,-10, -13, transforming growth factor beta (TGF- $\beta$ ) and by steroids.

In IBD, iNOS is expressed in epithelial cells and in tissue macrophages of the inflamed mucosa (10–12). iNOS-positive tissue macrophages have also been detected in a-septic bone necrosis (13), breast tumours (14) and tuberculosis (15). Tissue macrophages are derived from circulating monocytes. In healthy persons, circulating monocytes contain no functional iNOS (16). In HIV infection (17), tuberculosis (18), Leishmania infection (19), sepsis (20), alcoholic hepatitis/cirrhosis (21, 22), rheumatoid arthritis (23) and Graves' disease (24), circulating monocytes were found to be iNOS positive.

The purpose of this study was to determine whether circulating monocytes of patients with IBD express iNOS and, if so, whether this expression is related to disease activity. Considering the possible role of NO in monocyte activation we also investigated the expression of monocyte activation markers in relation to iNOS expression.

## Patients and Methods

### Study groups

Thirty-three persons were included in this study; 18 healthy controls and 15 patients with active IBD: 4 patients with active Crohn disease (CD) and 11 with active ulcerative colitis (UC). Active disease was defined as endoscopic and histologic inflamed mucosa and an increased activity score. For CD, disease activity was assessed using the Crohn Disease Activity Index (active disease was defined as CDAI >150) (25). For UC, disease activity was assessed using a clinical activity score, which is based on the presence and intensity of eight key symptoms of UC, i.e. blood loss, mucoid discharge, frequency

of defecation, consistency of faeces, tenesmus, abdominal pain, rectal pain and nausea/vomiting. Each symptom is scored from 0 to 2 points, resulting in a total score ranging from 0 (no symptoms) to 16 (all symptoms present, most of severe degree). A score of >4 was considered as active disease. All patients had endoscopic and histologic inflamed mucosa at the time of the first measurement. Because iNOS expression in circulating monocytes was studied in relation to gut inflammation, patients with extraintestinal manifestations were excluded. Furthermore, none of the patients was pregnant or had cirrhosis, malignancy or rheumatic disease and none of them used azathioprine or cyclosporin A at the time of inclusion. The patient characteristics are summarized in Table I. A second measurement was performed in all four patients with active CD and two of the patients with UC who went into remission as judged by their disease activity score. At the time of the second measurement, the medication was unchanged in three patients; in one patient the prednisolone was stopped, in one patient the prednisolone was reduced and azathioprine was added, and one patient had received prednisolone and azathioprine.

### Monocyte isolation and stimulation

Circulating blood monocytes were isolated from patients and control subjects using a non-activating method as described by Freundlich (26). Briefly, peripheral blood mononuclear cells (PBMNC) were isolated by density gradient (Lymphoprep, Nycomed Pharma AS, Oslo, Norway) centrifugation (20 min at 992g) of ethylenediaminetetraacetic acid (EDTA) anticoagulated blood. The PBMNC were washed twice and resuspended at a concentration of  $2-4 \times 10^6$  cells/ml in RPMI 1640 medium (Boehringer Ingelheim, Verviers, Belgium) containing 10% v/v fetal calf serum (FCS) (Gibco BRL Life Technologies, Breda, The Netherlands). The cells were allowed to adhere to plasma-treated gelatine-coated culture flasks for 40 min at 37 °C, washed to remove non-adherent cells, and subsequently incubated with 10mM EDTA in phosphate buffered saline (PBS), pH 7.4, to

recover adherent monocytes (>90% purity as checked by May-Grunwald-Giemsa staining).

For stimulation experiments, the monocytes were diluted to  $1 \times 10^6$  cells/ml in RPMI 1640 containing 10% v/v FCS and 0.06 mg/ml Gentamycin (Gibco BRL Life Technologies, Breda, The Netherlands). Stimulation was performed in polystyrene tubes with 500 U/ml IFN- $\gamma$  (Boehringer Mannheim, Almere, The Netherlands) for 3 days at 37 °C in 5% CO<sub>2</sub> humidified air. The mouse macrophage cell line RAW 264.7 (ATTC, Manassas, Va., USA) was used as a positive control for iNOS protein detection. This cell line was stimulated for 24 h with 1  $\mu$ g/ml LPS (*E. coli*, serotype 055:B5, Bio Whittaker, Walkersville, Md., USA) and 200 U/ml IFN- $\gamma$  (Boehringer Mannheim).

#### Immunocytochemistry

Cytospots of monocytes ( $1 \times 10^5$  cells/ml) were made by centrifugation (Shandon Cytospin 2) at 32g for 5 min. Cytospots were fixed in acetone for 10 min at room temperature. For iNOS detection, a mouse IgG1 monoclonal antibody (diluted 1/100) against amino acids 961-1144 of the C-terminus of mouse iNOS (cat. no. N39120, Transduction Laboratories, Lexington, Ky., USA) was used as primary antibody. This antibody also recognizes human iNOS. Endogenous peroxidase activity was blocked by treatment with 0.075% v/v H<sub>2</sub>O<sub>2</sub> in PBS for 30 min. A mouse IgG1 monoclonal antibody against Amyloid P Protein, diluted 1/100 (Novocastra Laboratories, Newcastle-upon-Tyne, UK) served as negative control antibody. Peroxidase-conjugated rabbit anti-mouse IgG was used as secondary antibody. Peroxidase activity was developed with 0.2 mg/ml 3-amino-9-ethyl-carbazole (AEC) in sodium acetate buffer containing 0.03% v/v H<sub>2</sub>O<sub>2</sub> for 10 min. Cytospots were counterstained with haematoxylin for 1 min, washed with tap water and covered with Kaiser's glycerin-gelatin.

#### Western blotting

Monocyte pellets were homogenized in a buffer containing 10 mmol/l Tris-HCl (pH 7.2), 0.2 mmol/l phenylmethylsulfonylfluoride (PMSF), 1 mmol/l EDTA and 1 mmol/l dithiothreitol. The protein concentration in the supernatant (crude lysate) was determined by a Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, Calif., USA) using bovine serum albumin as protein standard. Aliquots of crude lysates (100  $\mu$ g) were boiled for 5 min, and separated on a 10% w/v sodium dodecylsulphate polyacrylamide gel and transferred to nitrocellulose (Amersham International plc, Buckinghamshire, UK), using a semi-dry blotting system in accordance with the manufacturer's instructions (Pharmacia, Uppsala, Sweden). Prestained molecular weight standards (Bio-Rad) were used as marker proteins. The blots were incubated with the monoclonal iNOS antibody diluted 1/100 in PBS containing 4% v/v skim milk powder and 0.1% v/v Tween-20, consecutively followed by incubation with horseradish peroxidase labelled rabbit anti-mouse IgG (dilution 1/2000,

DAKO A/S, Glostrup, Denmark) and horseradish peroxidase labelled swine anti-rabbit IgG (dilution 1/2000, DAKO) and finally developed using an enhanced chemoluminescence (ECL) Western blotting system (Amersham).

#### Flowcytometry

For flowcytometric analysis of circulating monocytes we used a whole blood method in which in vitro activation of leukocytes is minimized (27). Because iNOS is associated with the peripheral cell membrane (28, 29) this protocol, which is associated with a mild permeabilization, could detect iNOS expression in monocytes. Using this protocol, iNOS expression was only slightly lower (data not shown) than results obtained with a fixation and permeabilization protocol (Fix & Perm<sup>®</sup>, An der Grub Bioresearch GmbH; Kaumberg, Austria). EDTA anticoagulated blood was obtained and kept on ice until sample preparation. Sample preparation was always started within 10 min after blood sampling. All steps were performed in Hanks' Balanced Salt Solution (HBSS) without calcium and magnesium (Gibco BRL, Life Technologies Ltd, Breda, The Netherlands), supplemented with 1% v/v Bovine Serum Albumin (BSA, Boseral, Organon Teknika, Boxtel, The Netherlands). Cells were fixed with 1% w/v paraformaldehyde in PBS for 10 min on ice, washed and subjected twice to erythrocyte lysis with lysis buffer (155 mM NH<sub>4</sub>Cl, 10 mM KHCO<sub>3</sub>, 0.1 mM Na<sub>2</sub>EDTA.H<sub>2</sub>O) for 5 min at 37 °C. For monocyte activation studies, IgG1 antibodies against CD11b (dilution 1/10) (Dako, Glostrup, Denmark), CD63 (dilution 1/10) (CLB-gran/12.435, CLB, Amsterdam, The Netherlands) and a phycoerythrin labelled antibody against HLA-DR (dilution 1/10) (IgG2a antibody from Becton-Dickinson, San Jose, Calif., USA) were used. For iNOS detection, a IgG1 monoclonal antibody, diluted 1/100, against iNOS (Transduction Laboratories) was used. A mouse IgG1 monoclonal antibody (dilution 1/100) against Amyloid P Protein served as isotypical control antibody. The optimal concentration (1/50; 1/100; 1/200) of the primary antibody was determined together with a concentration matched isotypic control antibody in the first patients. Thereafter the optimal concentration (1/100) of the iNOS antibody was used along with the same concentration of the isotypical control antibody. A sample without any antibody, and a sample with only the second antibody, served as negative controls. The first antibody was incubated for 1 h at 4 °C. After washing, the cells conjugated with primary antibodies were incubated with a goat anti-mouse Ig polyclonal antibody conjugated with Phycoerythrin (Southern Biotechnology Associates Inc., Birmingham, Al., USA), diluted 1/20 in PBS supplemented with 5% v/v normal goat serum and 5% v/v normal human serum (in order to prevent binding to the Fc receptor), for 30 min at 4 °C in the dark. Subsequently, cells were washed and flowcytometric analysis was performed on a Coulter Epics ELITE flowcytometer (Coulter, Hialeah, Fl., USA). In order to quantify the fluorescence intensity of samples in terms of molecules of equivalent soluble fluorochrome units

(MESF), Quantum 1000 beads (Flow Cytometry Standards Corp, San Juan, Pr., USA) were used to calibrate the flowcytometer. Data were analysed using WinList software (Verity Software House Inc, Topsham, Me., USA). The expression of surface markers was calculated as a fluorescence index (FI):

$$\text{FI} = \frac{\text{MESF sample} - \text{MESF irrelevant antibody}}{\text{MESF irrelevant antibody}}$$

The percentage of positive cells was calculated as the percentage positive cells minus percentage positive cells incubated with irrelevant antibody.

#### *Neopterin and nitrite/nitrate assays*

**Neopterin ELISA:** Levels of neopterin, a plasma marker for monocyte activation, were measured using a commercially available ELISA (Brahms Diagnostica, Berlin, Germany).

**Nitrite/nitrate levels:** The stable end products of NO, nitrite and nitrate (NOx) were measured in plasma according to the method described by Moshage et al. (30).

#### *Statistical analysis*

Data are represented as the median + range. Differences between groups were analysed using the Mann-Whitney U test. A two-tailed *P*-value <0.05 was considered to indicate statistical significance. Differences between paired observations were analysed using the paired Wilcoxon test. Correlation between parameters was analysed by the Spearman rank correlation test.

## **Results**

### *iNOS protein expression in isolated monocytes*

As detected by immunocytochemistry, unstimulated macrophages from the mouse RAW 264.7 cell line and unstimulated human monocytes from healthy controls showed no iNOS expression after 1 and 3 days in culture (Fig. 1A and C, respectively). Mouse macrophages showed intense iNOS staining after 1 day of stimulation with IFN- $\gamma$  and LPS (Fig. 1B). Isolated human monocytes from healthy controls showed iNOS expression after 48 h stimulation (data not shown) and was clearly demonstrated after 3 days of stimulation with IFN- $\gamma$  (Fig. 1D). Freshly isolated monocytes from patients with active IBD showed a weak expression of iNOS (Fig. 1E). No staining was observed after incubation with an irrelevant antibody (Fig. 1F). Culturing these cells for 24 h without stimulation enhanced the expression of iNOS (Fig. 1G). Staining was absent when the irrelevant antibody was used as primary antibody (Fig. 1H).

The expression of iNOS protein was confirmed by Western blot analysis. The mouse IgG1 monoclonal antibody recognized a single protein of approximately 130 kDa in crude lysates of LPS/IFN- $\gamma$  stimulated RAW 264.7 mouse macrophages (Fig. 2, lane 1) and in monocytes from healthy controls after 3 days stimulation with IFN- $\gamma$  (Fig. 2, lane 3).

Human monocytes from healthy controls showed no iNOS expression after 3 days of culture without IFN- $\gamma$  stimulation (Fig. 2, lane 2). However, freshly isolated monocytes from a patient with active CD showed iNOS expression (Fig. 2, lane 4).

### *Flowcytometric detection of iNOS*

Flowcytometric analysis of circulating monocytes from patients with active IBD revealed a significant increase ( $P = 0.02$ ) in iNOS expression (FI = 1.3, range 0.1–6.3) compared to healthy controls (FI = 0.8, range 0.0–1.8 (Fig. 3A)). The percentage of iNOS positive monocytes (37.3%, range 1.0–77.0) was increased ( $P = 0.005$ ) in patients with active IBD compared to healthy controls (5.3%, range 0.0–43.3) (Fig. 3B). Analysis of the CD patients showed that both the iNOS expression (FI = 3.1, range 1.1–6.3 versus 0.8, range 0.0–1.8,  $P = 0.009$ ) and percentage of iNOS positive monocytes (62.0%, range 37.3–76.4 versus 5.3%, range 0.0–43.3,  $P = 0.004$ ) were increased compared to controls. In CD patients, neither the fluorescence index nor the percentage of iNOS positive monocytes correlated with the CDAI score. For UC patients, the percentage of positive monocytes was higher than in controls (17.5%, range 1–77.0 versus 5.3%, range 0.0–43.3,  $P = 0.04$ ). For this subgroup, iNOS expression was not different compared to controls (FI = 1.2, range 0.2–3.0 versus 0.8, range 0.0–1.8,  $P = 0.14$ ). In UC patients, neither the fluorescence index nor the percentage of iNOS positive monocytes correlated with the disease activity score. There was no significant difference in iNOS expression between CD (Fig. 3A and B black circles) or UC (Fig. 3A and B open circles) patients. In addition, there was no difference between patients taking steroids (FI = 1.1, range 0.2–2.0; % iNOS positive cells: 49.7%, range 1–74.4) or not taking steroids (FI = 2.5, range 0.1–6.3; % iNOS positive cells: 36.3%, range 1.0–77.0). No iNOS expression was detected in circulating lymphocytes or granulocytes from IBD patients or controls.

Analysis of paired data showed that both the iNOS expression ( $P = 0.03$ ) and the percentage of iNOS positive monocytes ( $P = 0.03$ ) were higher in IBD patients with active disease than in the same patients after they went into remission (Fig. 4).

### *Flowcytometric analysis of monocyte activation*

Surface markers for monocyte activation CD63 and CD11b were not elevated on circulating monocytes of patients with active IBD (Table II). Analysis of paired data showed that the CD63 and CD11b expression in patients with active disease was not different from expression in the same patients after achieving remission.

The percentage of HLA-DR positive monocytes was decreased in IBD patients compared to healthy controls (Table II). Subgroup analysis showed that this decrease was mainly due to a decreased percentage of HLA-DR in UC patients. Analysis of paired data showed that neither the

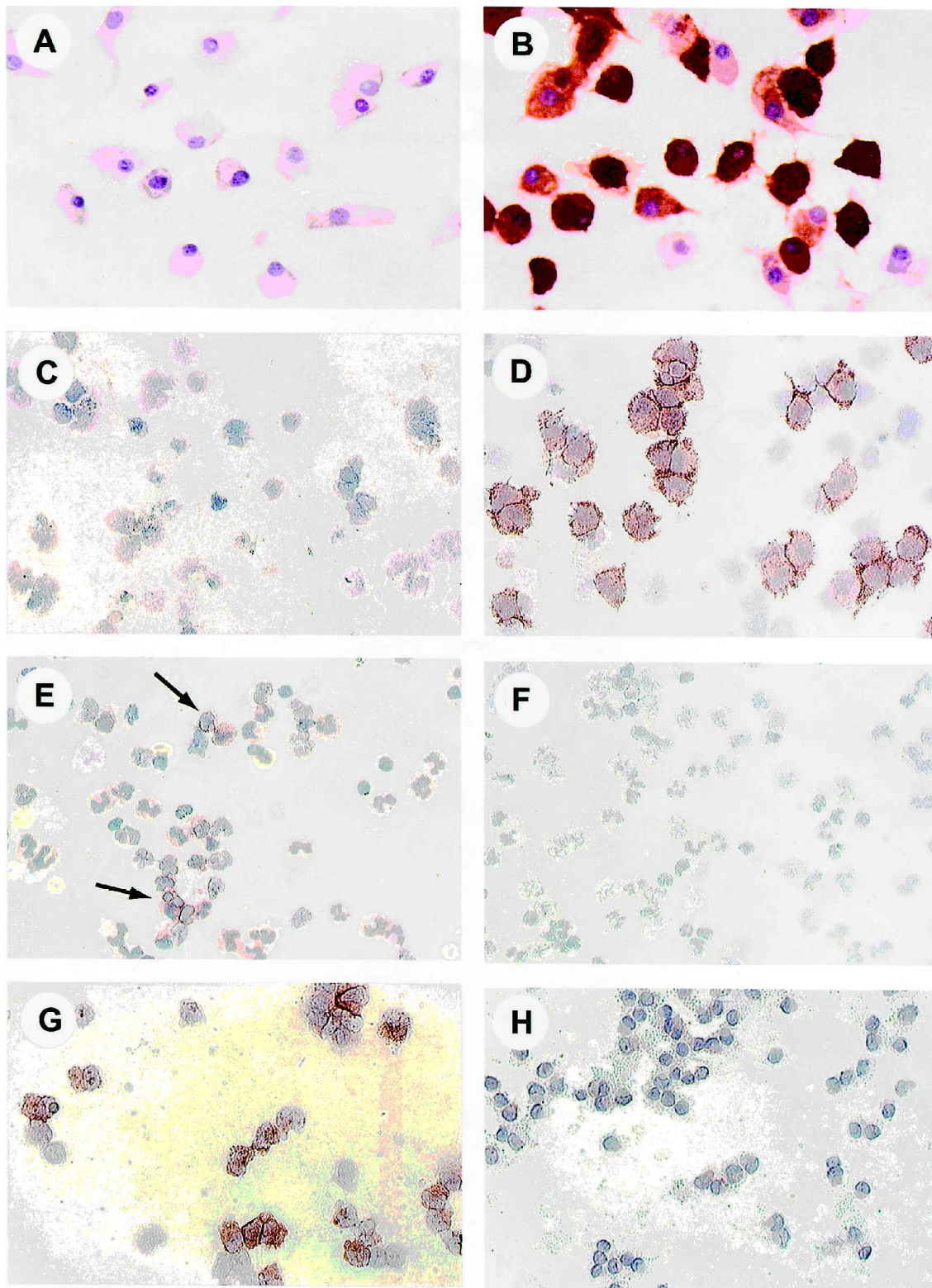


Fig. 1. Detection of iNOS expression by immunocytochemistry on cytospts of stimulated and unstimulated monocytes. A. Unstimulated mouse macrophages showed no iNOS expression. B. Mouse macrophages stimulated with IFN- $\gamma$  and LPS for 1 day showed intense iNOS staining. C. Unstimulated human monocytes isolated from a healthy control subject showed no iNOS staining after 3 days of culture. D. Human monocytes isolated from a healthy control subject stimulated with IFN- $\gamma$  (500 U/ml) for 3 days showed iNOS staining. E. Freshly isolated monocytes from a patient with active CD showed weak iNOS staining (arrows). F. Staining was absent when an irrelevant antibody was used as primary antibody. G. Monocytes isolated from a patient with active CD and cultured for 1 day without stimulation showed clear iNOS staining. H. Staining was absent when an irrelevant antibody was used as primary antibody.

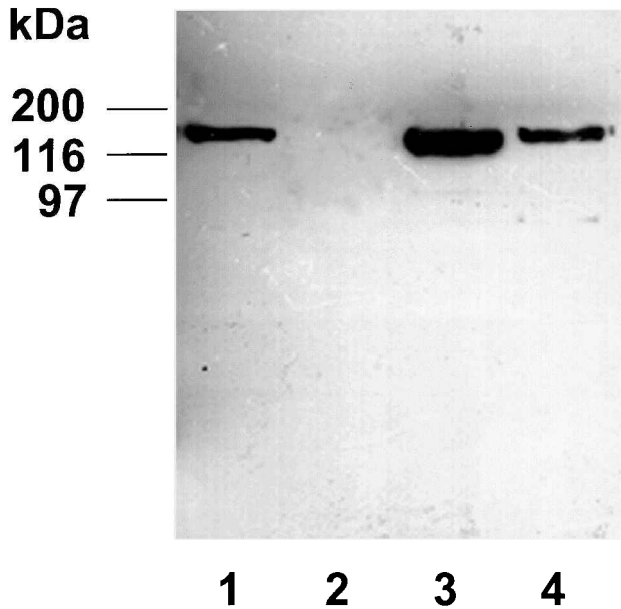


Fig. 2. Immunodetection on Western blots of crude lysates from monocytes. Lane 1: 50  $\mu$ g protein of mouse macrophages stimulated for 1 day with IFN- $\gamma$  and LPS. Lane 2: 100  $\mu$ g protein of unstimulated monocytes from a healthy control subject after 3 days culture. Lane 3: 100  $\mu$ g protein of monocytes from a healthy control subject after 3 days stimulation with IFN- $\gamma$ . Lane 4: 100  $\mu$ g protein from freshly isolated monocytes from a patient with active Crohn disease.

fluorescence index nor the percentage of HLA-DR positive monocytes was different between patients with active disease and the same patients in remission.

#### Correlation with plasma C-reactive protein, neopterin and nitrite/nitrate levels

The iNOS fluorescence index ( $r=0.6$ ,  $P=0.03$ ) and percentage of iNOS positive monocytes showed a weak correlation with C-reactive protein levels ( $r=0.5$ ,  $P=0.04$ ). The levels of neopterin in IBD patients (12.6, range 5.2–23.8 nmol/l) were significantly higher ( $P=0.002$ ) than in healthy controls (6.3, range 4.7–12.7 nmol/l), but neopterin levels were not correlated with the iNOS expression in circulating monocytes. Although 3 patients with IBD had elevated plasma nitrite/nitrate levels ( $>50$   $\mu$ mol/l) there was no significant difference between levels in IBD patients (37, range 23–104  $\mu$ mol/l) and controls (31, range 18–58  $\mu$ mol/l).

#### Discussion

Tissue macrophages in inflamed mucosa from patients with active IBD are activated and express iNOS (10, 11). Tissue macrophages are derived from circulating monocytes. In this study, we provide evidence that circulating monocytes from patients with active IBD express iNOS but are not otherwise activated. Furthermore, we found that the iNOS expression in circulating monocytes is related to disease activity.

There is increasing evidence that iNOS can be induced in human macrophages/monocytes (31–33), but the induction occurs slower and the amount of NO produced is lower than in rodent macrophages/monocytes (34). Indeed, we could detect iNOS in a large percentage of circulating monocytes from patients with active IBD; however, the amount of iNOS per cell (fluorescence index) was low. The end products of NO synthesis, nitrite and nitrate, were elevated in only 3 out of 15 patients with active IBD and no correlation between iNOS expression in circulating monocytes and plasma nitrite/nitrate levels was found. Therefore, iNOS in circulating monocytes probably does not produce high amounts of NO. It is possible that human monocytes are not able to produce large amounts of NO because they produce not enough 5,6,7,8-tetrahydro-L-biopterin (BH<sub>4</sub>), an essential co-factor for iNOS activity (35). Alternatively, although the mouse and human iNOS gene share 80% homology in their coding sequences, homology in their promoter regions is only 60% (36, 37). This difference may account for the less rapid induction of iNOS in human monocytes/monocytes.

Interestingly, the expression of iNOS was reduced in

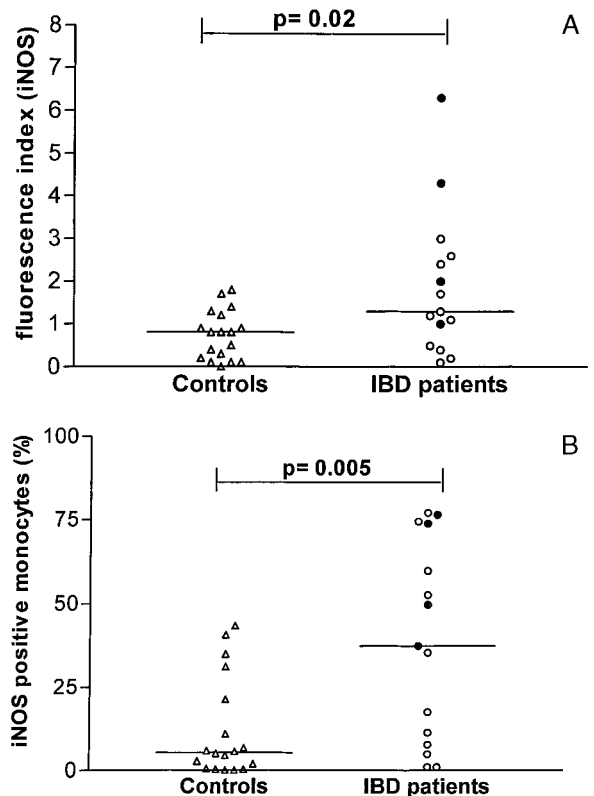


Fig. 3. A. Difference in iNOS fluorescence index (FI) of monocytes from 18 healthy controls ( $\Delta$  = median FI 0.8, range 0.0–1.8) and 15 patients with IBD (Crohn disease ( $\bullet$ ), ulcerative colitis ( $\circ$ ), median FI 1.3, range 0.1–6.3) as detected by flowcytometry. B. Difference in percentage of iNOS positive monocytes from 18 healthy controls ( $\Delta$  = median 5.3, range 0.0–43.3) and 15 patients with IBD (Crohn disease ( $\bullet$ ), ulcerative colitis ( $\circ$ ), median 37.3, range 1.0–77.0) as detected by flowcytometry.

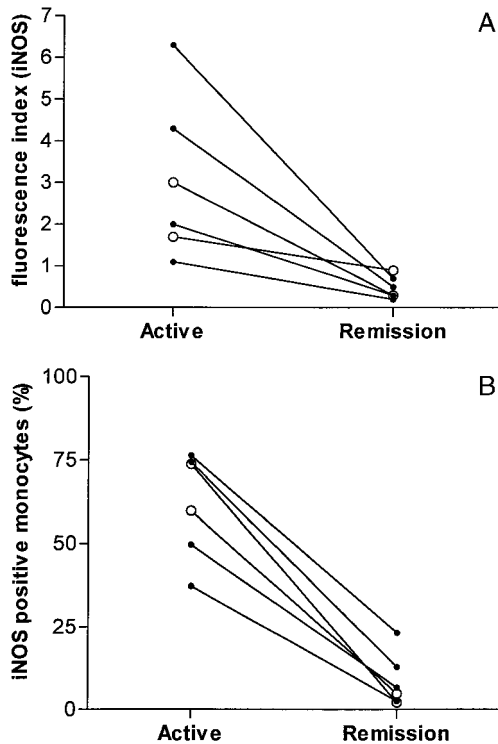


Fig. 4. A. Fluorescence index for iNOS expression as detected by flowcytometry in monocytes from 4 patients with Crohn disease (●) and 2 patients with ulcerative colitis (○) during active disease and during remission. B. Percentage of iNOS positive monocytes as detected by flowcytometry from 4 patients with Crohn disease (●) and 2 patients with ulcerative colitis (○) during active disease and during remission.

patients who went into remission. Patients in remission had lower levels of CRP and neopterin. The proinflammatory cytokines TNF- $\alpha$ , IFN- $\gamma$  and IL-1 are the main inducers of iNOS, whereas the anti-inflammatory cytokines IL-4, IL-10, IL-13 and TGF- $\beta$  are the main suppressor cytokines of iNOS induction (38). It is known that proinflammatory cytokines are downregulated and anti-inflammatory cytokines are upregulated during remission (39). A relation between the presence of proinflammatory cytokines and iNOS in circulating monocytes is supported by a correlation between iNOS expression and CRP levels. Although the group of patients who went into remission was small, the significantly reduced expression of iNOS in circulating monocytes indicates that it perhaps can be used as a disease activity marker.

Recruitment of circulating monocytes and T cells to the inflamed mucosa plays a key role in the pathogenesis of IBD (40, 41). The data on the functional changes of circulating monocytes in IBD are conflicting. Isolated monocytes from patients showed an increased production of cytokines (42) and reactive oxygen species (3) upon stimulation in vitro. We showed here that isolated monocytes from patients with active IBD, in contrast to monocytes from healthy controls, spontaneously express iNOS after 1 day of culture, without stimulation. However, we found no evidence for activation of circulating monocytes from patients with active IBD. For example, the CD63 molecule, a marker for monocyte and neutrophil activation (43, 44) was not increased on circulating monocytes and, in agreement with Greenfield (45), we found no upregulation of the CD11b adhesion molecule on circulating monocytes from patients with active IBD.

Although these findings support the idea that circulating monocytes in active IBD are not activated, we did find an increase in plasma neopterin levels. Neopterin is produced by both circulating monocytes and activated tissue macrophages. Therefore, the increased levels of neopterin may have been derived from activated tissue macrophages. The expression of surface markers of monocyte activation reflects the state of activation of circulating monocytes only.

What could be the role of NO production in circulating monocytes from patients with active IBD? It is clear that in rodents NO is involved in macrophage mediated killing of (intracellular) microorganisms and tumour cells (32). The course of an infection or inflammation in mice lacking the iNOS gene is aggravated compared to the wild type mice (46). Apart from its role in macrophage cytotoxicity, there is evidence that NO is involved in immunoregulation. For example, production of NO is one mechanism by which activated mouse macrophages can restrict T cell expansion (47) and IFN- $\gamma$  production by human T-lymphocytes (48). A differential susceptibility to NO has been described for murine Th1 and Th2 cells (49), but a preferential inhibition of Th1 cytokine secretion by NO was not found in human T cells (50). NO may inhibit monocyte/macrophage function by inhibiting cytokine (7, 51) and chemokine (52, 53) production, phagocytic activity (54), and respiratory burst (55). Interestingly, NO can also downregulate the HLA-DR expression on monocytes/macrophages (56). In the present study, we found a decreased HLA-DR expression on circulating monocytes from patients with active UC, as was

Table II. Monocyte activation markers

Marker	Fluorescence index		Percentage of positive monocytes	
	IBD patients <i>n</i> = 15	Controls <i>n</i> = 18	IBD patients <i>n</i> = 15	Controls <i>n</i> = 18
CD 63	8.3 (3.4–44.3)	7.5 (2.6–20.5)	94.3 (39.3–97.6)	92.7 (67.7–96.5)
CD 11b	35.6 (22.1–174.2)	38.3 (20.1–83.8)	95.1 (39.9–98.7)	95.3 (90.4–98.7)
HLA-DR	14.7 (3.7–86.3)	17.4 (6.8–75.4)	89.5 (67.3–95.8)	92.2 (81.3–95.8)*

\* *P* < 0.05 control versus IBD.

also previously reported (57). Therefore, NO may perhaps inhibit monocyte activation.

Recently, a frameshift mutation in the *NOD2* gene was associated with susceptibility to CD but not to UC (58, 59). *NOD2* is restricted to monocytes and the frameshift mutation can lead to a truncated protein unable to activate NF- $\kappa$ B in monocytes in response to bacterial lipopolysaccharides (60). Because iNOS is a NF- $\kappa$ B mediated protein, patients with CD who do not express iNOS could be the patients with this mutation. Furthermore, it would be interesting to relate this mutation and the iNOS expression in peripheral monocytes to the occurrence of extraintestinal manifestations.

In conclusion, this study shows that iNOS is expressed in circulating monocytes from patients with active IBD and is related to disease activity. Considering the absence of systemic monocyte activation markers, NO may have a function in preventing systemic monocyte activation and thus may modulate disease activity in extra-intestinal sites in patients with active IBD.

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