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Co-expression of inducible nitric oxide synthase and arginases in different human monocyte subsets. Apoptosis regulated by endogenous NO

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Abstract

Human monocyte subsets, isolated from cultures of mononuclear cells, or freshly obtained from patients with multiple sclerosis, Graves' disease or pemphigus vulgaris, differed in phenotype, apoptotic features, mRNA levels of arginase II (A-II) and the inducible form of nitric oxide synthase (iNOS). Liver-type arginase I mRNA was present in all subsets. Apoptosis was followed by the expression of T cell intracellular antigen (TIA) and the simultaneous detection of DNA stainability by propidium iodine and annexin V binding. Apoptosis was practically absent both in activated $CD14^{++}CD33^{++}DR^{++}CD25^{++}CD69^{++}CD71^{++/+}CD16^{-}$ cells, expressing A-II mRNA and having arginase activity, but not iNOS mRNA, and in not fully mature large $CD14^{++}CD16^{+}CD23^{+}DR^{++}$ monocytes, expressing simultaneously both mRNAs and having both enzyme activities. However, differentiated small $CD14^{+/++}CD16^{+}CD69^{+}$-$CD25^{+/-}CD71^{++}CD23^{+}DR^{++}$ monocytes, expressing high levels of iNOS mRNA, exhibited apoptotic signs. Amounts of NO synthesised by monocytes co-expressing iNOS and arginase changed with the addition of arginine or an iNOS inhibitor; in that case a correlation of NO production and apoptotic features was observed. Data suggest a regulatory role for endogenous NO in apoptosis of stimulated and differentiated monocytes, and also that iNOS and A-II, when simultaneously present, could control the production of NO as a consequence of their competition for arginine.

Keywords: Nitric oxide synthase; Arginase; Human monocyte; Apoptosis

1. Introduction

Nitric oxide synthase (iNOS) and arginases are expressed in murine macrophages [1–3]. Arginase I (A-I) plays a critical role in liver metabolism as a key component of the urea cycle; however, this enzyme is also present in tissues where the urea cycle is not operating. On the other hand many extrahepatic tissues contain a second form of arginase, arginase II (A-II), involved in the metabolism of nitric oxide [4,5]. It has been reported that LPS induces rat peri-

Abbreviations: PBMC, peripheral blood mononuclear cells; iNOS, inducible nitric oxide synthase; A-I, arginase I; A-II, arginase II; NMLA, *N*-methyl-L-arginine; PE, phycoerythrin; PI, propidium iodine; TIA, T cell intracellular antigen

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toneal macrophage expression of iNOS and liver-type A-I [1]; and A-II and iNOS mRNAs are co-induced in murine macrophage-like RAW 264.7 cells by LPS [2,6]. Arginases and iNOS are controlled in macrophages by Th1/Th2-dependent cytokines [7,8]. Arginase expression in murine bone marrow-derived macrophages is specifically triggered by PGE2 and Th2 cytokines IL-4, IL-10; in contrast, Th1 cytokine IFN-γ induces NO synthesis, but arginase is not expressed under those conditions [7]. The simultaneous presence of both types of mediators leads to a reduced expression of iNOS and arginase, enzymes which seem to define alternate functional states of macrophages induced by Th1 and Th2 cytokines respectively [8].

A human-type A-II arginase is expressed in extrahepatic tissues and regulated both tissue specifically and developmentally [5]; it has been suggested that this enzyme plays some important role in the arginine regulatory system encompassing several enzymes, among them nitric oxide synthase [9]. We have described iNOS induction in human monocytes, when PBMC from healthy donors are incubated in the presence of an immunomodulating 15 amino acid peptide, peptide Pa, (NVLGAPKKLNESQAV) [10] capable of activating monocytes [11], and inducing Th1 cytokine liberation [12]. We have also reported that monocytes, freshly obtained from patients with multiple sclerosis [13] or Graves' disease [14], have iNOS already expressed in a monocyte subset with high levels of HLA-DR molecule expression and exhibiting a $CD16^+$ phenotype characteristic of a state of differentiation [13]; this iNOS expression depends on the liberation of Th1 cytokines, IFN-γ and IL-2 [14].

It has been described that the simultaneous presence of iNOS and arginases, enzymes implicated in the metabolism of arginine, may play an important regulatory role, through the control of NO production, in LPS-activated J774 mouse macrophages [15]. After initiating the production of NO, macrophages can suffer on themselves the toxicity of this radical; it has been reported that NO induces monocyte/macrophage apoptosis [16,17], through multiple non-redundant signaling pathways [18], where NO is necessary, but insufficient. The existence of specific down-modulatory mechanisms related to NO-induced apoptotic DNA fragmentation in murine RAW 264.7 macrophages has been described [19]; and it has also been observed that cells belonging to this line can be made resistant to the toxic effects of NO [20], after treatment with cycles of non-lethal doses of LPS and INF-γ. This macrophage resistance toward NO-mediated apoptosis is due, at least in part, to an increased superoxide formation [21]. The apoptotic signaling cascades converge in the regulation of caspases; the endogenous NO is capable of down-regulating caspase-3 by S-nitrosation and oxidation of critical thiol groups [22]; but the induced apoptosis on resistant cells, via exogenous NO donors, has been found to be caspase-3-independent [23].

We report now that monocytes, entering a process of activation and differentiation, responded with a differential expression of mRNAs corresponding to iNOS and A-II and showed different arginase and NOS enzyme activities. An increase in A-II expression seemed to rescue monocytes from apoptosis, whereas the down-regulation of A-II, concomitant with an up-regulation of iNOS, led to apoptosis. Changes in endogenous NO production induced by the addition of L-arginine or NMLA resulted in changes in apoptosis of $iNOS^+A\text{-}II^+$ monocyte subsets. Results suggest that endogenous NO production by iNOS, and its down-regulation by A-II in monocyte subsets containing both enzymes, might be involved in the regulation of maturation and auto-apoptosis in mature hyperstimulated cells.

2. Materials and methods

2.1. Peptide synthesis

Peptide NVLGAPKKLNESQAV, known to have immunomodulating properties [11,12], and capable of inducing iNOS in human monocytes [10], was synthesised by the solid phase method of Merrifield [24].

2.2. Cell sources and cultures

PBMC were obtained from healthy donors, or from patients with multiple sclerosis or Graves' disease or pemphigus vulgaris following the technique described by Bøyum [25]. Cells were incubated at 1×10^6 per ml in RPMI-1640 medium supplemented

with 10% FCS, 2 mM L-glutamine, penicillin (100 U/ml) and streptomycin (100 μg/ml) in a 5% CO_2 humidified atmosphere. Isolation and purification of monocytes were carried out taking advantage of their adherence to culture plates. After a period of 4 h at 37°C non-adherent cells were removed with the supernatant followed by three washes with saline solution. Adherent cells, consisting mainly of monocytes, were then carefully detached from the surface of the culture plates by gentle friction and resuspended in a small volume of saline. We considered as basal state of the monocytes that of adherent cells after 4 h of culture of freshly isolated PBMC from patients or from healthy donors before the addition of peptide Pa. After the first 24 h of culture, peptide Pa was added (30 μg per ml), and 4 h later the phenotype of the monocytes in the whole PBMC population, as well as mRNA levels of the enzymes in the adherent cells were determined; addition of Pa was reiterated 24 h later and the same analyses repeated after additional periods of 24, 48 and 72 h.

In another series of experiments we isolated different subsets of monocytes from adherent cells belonging to PBMC incubated for 72 h in the presence of peptide Pa. Adherent cells were resuspended in medium culture and then $CD14^{++}$ and $CD14^{-/+}$ monocytes were separated with magnetisable polystyrene beads coated with a primary mononuclear antibody for CD14 membrane antigen (Dynal, Oslo, Norway). 10^7/ml adherent cells were incubated for 30 min with 2×10^6/ml magnetisable polystyrene beads at 4°C and then centrifugated on a magnetic camp; the subset having a high CD14 expression remained on the tube wall forming cell-polystyrene bead complexes, and the $CD14^{-/+}$ monocytes could be recovered in the supernatant.

2.3. Flow cytometry analysis

PBMC were suspended in PBS containing 0.1% sodium azide at a density of 10^7 cells per ml. Cells were incubated with monoclonal antibodies labeled with FITC or PE: CD14 antibody (Leu-M3)-FITC, anti-CD16 (Leu-11a)-PE, anti-CD25 (anti-IL-2R)-PE, anti-CD69 (Leu-23)-PE, anti-HLA-DR-PE, anti-CD71 (Leu-3a), anti-CD33 (Leu-M9) or anti-CD23 (Leu-20). Monoclonal antibodies were from Becton Dickinson. Incubations were performed in the dark at 4°C for 30 min. Cells were washed twice with cold PBS containing 0.1% sodium azide, fixed with 1% paraformaldehyde and stored in the dark at 4°C until analysis. Fixed cells (20 000 per sample) were analysed by two-colour flow cytometry with a Becton Dickinson FAC Scan flow-cytometer. Monocytes were gated according to their light scattering properties; some monocyte subsets exhibited an increased size and granularity, features being reflected in the flow cytometry analysis by their forward and side light scattering properties. Isotype antibodies of irrelevant specificity were used as negative control. Specific fluorescence intensity represents the difference between the mean channel of the specific antibody and the mean channel of the negative control antibody expressed on a logarithmic scale. The percentage of positive cells was calculated from specific and non-specific staining. For intracellular iNOS or TIA labeling, 5×10^5 PBMC were previously fixed with 4% *p*-formaldehyde in the dark at 4°C for 20 min, and then washed with PBS containing 0.1% sodium azide and suspended in 50 μl of permeabilisation buffer (PBS with 0.1% sodium azide, 1% heat-inactivated FCS and 0.1% saponin). Cells were incubated with anti-TIA-FITC or with anti-iNOS antibody (Transduction Laboratories, Lexington, KY, USA), 1:10 diluted, or IgG-FITC (Becton Dickinson) as control at 4°C for 45 min, washed with permeabilisation buffer, suspended in PBS and analysed by flow cytometry, the small and large monocyte areas gated according to the pattern of light scatter. Expression of markers and iNOS or TIA was classified as high (++), low (+), absent (−), or intermediate (++/+ and +/−), according to fluorescence intensity related to protein expression.

The simultaneous detection of DNA stainability by PI and annexin V binding was analysed by two-colour flow cytometry, using an apoptosis detection kit (R and D Systems, Minneapolis, MN, USA). Cells (10^6/ml) were resuspended in 100 μl of binding buffer (HEPES buffered saline solution supplemented with 2.5 mM Ca_2Cl) and labeled with 10 μl of annexin V-FITC and 10 μl of PI for 15 min to determine phosphatidyl serine exposure to the outer face of the cell membrane. Cells were then diluted in 600 μl of binding buffer and immediately analysed by flow cytometry gating on the monocyte popula-

tion. This method permits to distinguish three types of cells: live cells, not staining with either fluorochrome, necrotic cells, staining with both, and cells undergoing apoptosis, staining only with the annexin V-FITC. Values of log of fluorescence intensity less than 0.5×10^2 were considered to represent a low annexin V binding, and values of log of fluorescence intensity less than 10^1 were considered to be low for DNA stainability by PI.

2.4. mRNA analysis of iNOS and type I and II arginases

For the preparation of RNA, adherent cells were collected by centrifugation, pelleted cells immediately disrupted, and RNA extracted using the commercial kit 'Total RNA Isolation Reagent' (Biotecx Laboratories, Houston, USA) with the lysis buffer 'Chasolv' containing guanidinium isothiocyanate following the manufacturer's instructions. Total RNA recovered was treated with RNase-free DNase by standard methods [26], precipitated with absolute ethanol and resuspended in water. For cDNA synthesis 1 μg of RNA was incubated at 90°C for 3 min and chilled on ice for 3 min. Samples were then incubated at 42°C for 1 h after adding 10 U of RNAsin, 1 mM deoxynucleoside triphosphates, 200 U M-MLV reverse transcriptase (Gibco BRL, UK), 100 pmol of random hexamers as primers and 4 μl of 5× reverse transcriptase buffer (125 mM Tris-HCl, pH 8.5, 15 mM $MgCl_2$, 325 mM KCl) to give a final volume of 20 μl. After the incubation, the reaction was heat-inactivated at 75°C for 5 min, and cDNA frozen at −20°C until use. Dilutions (1:10 to 1:1000) of each cDNA sample were prepared and a PCR carried out from each dilution in order to determine and select the exponential phase conditions for the amplification: conditions where the reaction proceeds linearly before reaching the plateau, so that the amount of PCR product obtained would reflect the amount of starting cDNA, which in turn reflects the amount of mRNA in the original sample [27]. All PCR reactions were carried out in 0.2 ml thin wall tubes with an OmniGene Thermal Cycler (Hybaid) at a final volume of 20 μl. The reaction mixture contained 1 μl of the cDNA dilution, 20 pmol of each specific primer, 0.2 mM deoxynucleotide phosphates, 2 μl of 10× PCR buffer (100 mM Tris-HCl, pH 9.0, 500 mM KCl, 1% Triton X-100) and 1 unit of DNA polymerase from *Thermus brockianus* 'Dynazime' (Finnzymes). After an initial period of 5 min at 95°C, 35 cycles of denaturation (95°C for 30 s), annealing (T_a 60°C for 30 s) and extension (72°C for 30 s) were performed, ending with a single final incubation of 5 min at 72°C. Electrophoresis of PCR products was performed on 1% agarose gels containing 1 μg/ml ethidium bromide. Stained gels were photographed and optical density (O.D.) of each band measured by densitometric scanning, subtracting the background from an equivalent empty area of the gel picture. A fragment of β-actin mRNA was amplified in each sample as an internal normalisation standard and linear conditions were also determined for this reaction. The relative amount of each mRNA was expressed as the O.D. value obtained in linear phase conditions divided by the O.D. value obtained for the β-actin mRNA in the same sample, determined also in linear phase conditions. For iNOS amplification a commercially available amplimer set (Clontech, Palo Alto, CA, USA) giving a 259 bp fragment was used. Arginase I primers (sense: 5′-CTTGTTTCGGACTTGCTCGG-3′; antisense: 5′-CACTCTATGTATGGGGGCTTA-3′) were designed from the described sequence [28] giving a 330 bp product. Arginase II primers (sense: 5′-TCTATGACCAACTTCCTACTC-3′; antisense: 5′-CTTCTGACTACTCCCCACTT-3′) were designed from the described sequence [29] giving a 660 bp product. These primers were designed from non-homologous region between both isoforms. Sequencing of the PCR products was conducted to confirm the identity of each mRNA in an ALF automatic laser fluorescent apparatus (Pharmacia).

2.5. Measurement of arginase activity

Arginase activity was measured in monocyte lysates as described [30]; 10^6 adherent cells from PBMC cultures in the presence of Pa were lysed with 0.5 ml of 0.1% Triton X-100, and after 30 min 0.5 ml of a 25 mM Tris-HCl and 5 mM $MnCl_2$, pH 7.4 buffer added. The arginase activity was activated by heating for 10 min at 56°C; 25 μl of the activated lysate (containing 2 a 10 μg of protein) were incubated with 25 μl of 0.5 M arginine, pH 9.7 at 37°C for 1 h. The reaction was stopped with 400 μl of an

acidic mixture (H_2SO_4, H_3PO_4 and H_2O, 1:3:7 v/v) and the urea measured at 540 nm after addition of 25 μl of 9% α-isonitrosopropiophenone, dissolved in 100% ethanol, and then heating at 100°C for 45 min. One unit of arginase activity was defined as the amount of enzyme catalysing the formation of 1 μmol urea/min. A calibration curve for urea was used in the range of 0.05 to 1 μmol.

2.6. Determination of nitrite/nitrate and NO synthase assay

For the determination of nitrites and nitrates, spontaneous degradation products of nitric oxide, a commercial kit was used (Alexis Corporation, Läufelfingen, Switzerland). Photometric measurement was carried out at 540 nm. Assays were performed on samples containing 2×10^6 lymphomononuclear cells previously disrupted by means of a Parr bomb. An aliquot of the incubation medium was used as reaction blank.

NOS activity was determined by measuring the formation of ^{3}H-L-citrulline from ^{3}H-L-arginine as described [31]. Reactions were conducted at 37°C in 50 mM Tris-HCl, pH 7.4, containing 50 μM L-arginine (approximately 300 000 cpm of [2,3,4,5-^{3}H]-L-arginine HCl; 70 Ci/mmol; Amersham Corp. IL, USA), 100 μM NADPH, 10 μM tetrahydrobiopterin and 2–10 μg protein from monocytes. Enzymatic reaction was initiated by the addition of the protein extract and after 1 h finished with the addition of 2 ml of ice-cold buffer (20 nM sodium acetate, pH 5.5, containing 1 mM L-citrulline, 2 mM EDTA and 2.0 mM EGTA). L-citrulline was determined by monitoring the formation of ^{3}H-L-citrulline from ^{3}H-L-arginine; samples of 2 ml were applied to 1 cm diameter columns containing 1 ml of Dower AG50W-X8, Na^+ form; the fluid eluted was collected in a liquid scintillation vial and columns washed with 2 ml of water and collected in another vial. Ten ml of Aquasol-2 were added to each vial and samples were counted.

2.7. Statistical analysis

Data were analysed using ANOVA and Fisher's PLsD tests. A P value less than 0.05 was considered to be significant.

3. Results

3.1. Phenotype of monocytes

An analysis of monocytes was carried out after incubating separately PBMC belonging to three different healthy subjects in the presence of peptide Pa for up to 5 days. Samples were analysed every 24 h and two additions of Pa were made at 24 and 48 h after initiating the culture. Adherent cells, mainly monocytes, recovered from the incubated PBMC from healthy donors, or fresh cells from five patients with multiple sclerosis, four with Graves' disease, and three with pemphigus vulgaris were detached by gentle friction, after removing the supernatant containing non-adherent cells. We observed the co-expression of CD14 and specific activation or differentiation surface markers, CD25 (IL-2 receptor), CD69 (signal transducing receptor), CD71 (transferrin receptor), or CD23 (low-affinity receptor for IgE), as well as the co-expression of the early myelomonocytic antigens CD33 and CD71, using two-colour immunofluorescence analysis. The higher or lower proportion of monocytes (CD14 expressing cells) co-expressing the different markers, as well as the density of marker per cell, obtained for each sample during the incubation, are a reflection of the different subpopulations present, i.e. of the evolution of the monocytes. The 30 samples studied were grouped into four states (A through D) according to the characteristic profile of the subpopulations present. In each panel of Fig. 1 (A, B, C, D) the characteristics of the group are indicated, giving the mean of the percentage of the different monocyte subsets (CD14 expressing cells), co-expressing HLA-DR or a surface marker. In some samples we observed the existence of two subpopulations differing in size and granularity, features being reflected in flow cytometry analysis by their forward and side light scattering properties.

The predominant basal non-activated population, state A (Fig. 1A), was $CD14^{++}DR^+$ (approximately 50% of all monocytes) and 38% of the monocytes belonged to the $CD14^{++}DR^{++}$subset, whereas the $CD14^+DR^{++}$ and $CD14^+DR^+$ subsets were practically absent. The number of monocytes co-expressing CD16 was very low. The majority of immature $CD14^{++}$ monocytes exhibited a low expression of

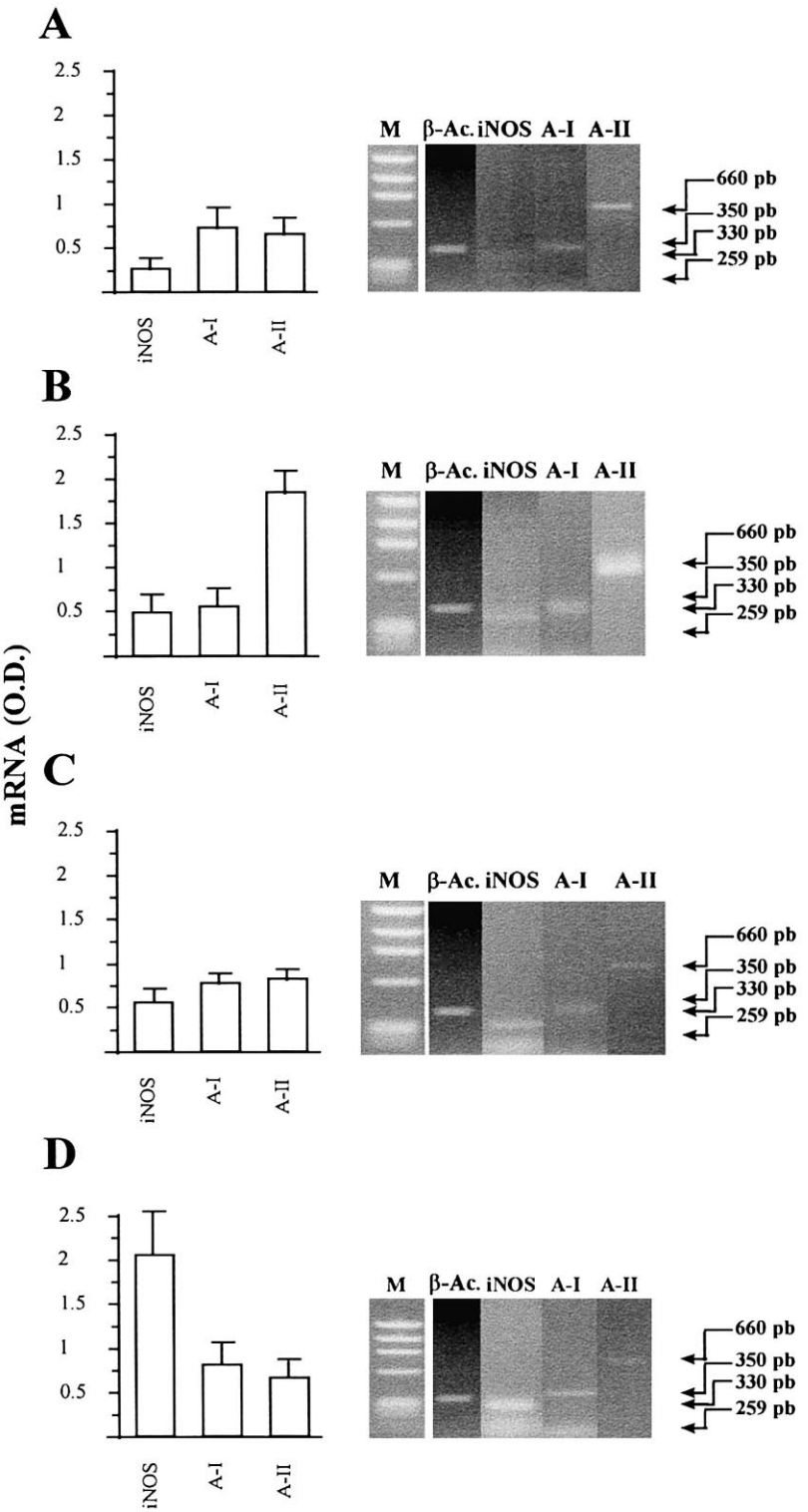

Fig. 1. RT-PCR analysis of A-I, A-II and iNOS gene expression on monocytes of 30 samples grouped into phenotypes (A–D), as shown in Fig. 1. Graphics represent the mean of the normalised O.D. for each mRNA band obtained by densitometric analysis (mean ± S.D.; $n = 6$ for panel A; $n = 8$ for panels B, C and D). Normalisation was done dividing each O.D. value by the value of the β-actin band in the same sample. All PCR reactions were performed in exponential phase conditions. Photographs show a representative example of the PCR product of each gene in each monocyte state. M, molecular weight marker (ϕX-174 DNA digested with *Hae*III), iNOS (259 bp), A-I (330 bp) and A-II (660 bp) and β-actin (350 bp).

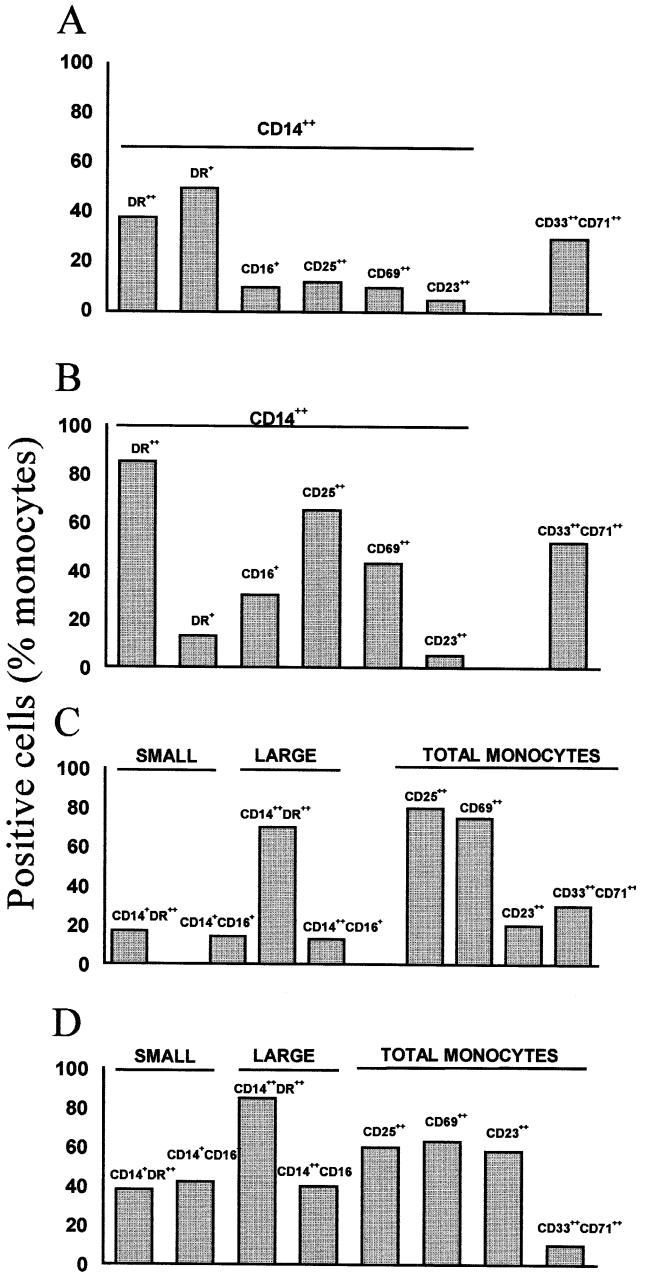

Fig. 2. Phenotypes of human monocytes in different states of activation and differentiation. Fresh monocytes were obtained from 12 different patients with autoimmune diseases. Monocytes were also obtained after incubation of PBMC from three different healthy subjects; in this latter case the incubation was carried out in the presence of immunomodulating peptide Pa, withdrawing samples after different periods of incubation (see details in Section 2). Panels A–D group the phenotypes into four monocyte states. Data represent the mean of the percentage of monocytes co-expressing CD14 and CD16, CD25, CD69, CD23 or HLA-DR; and that of those co-expressing CD33 and CD71 ($n=6$ for panel A; $n=8$ for panels B, C and D).

the marker of early stimulation CD25, and very few monocytes expressed CD69; CD23 expression was very low, so that only 3% of the monocytes had the $CD14^{++}CD23^{++}$ phenotype. No $CD14^{++}$-$CD71^{++}$ monocytes (data not shown) were found. A large number of monocytes (85% of all monocytes) presented a high expression of CD33, and most of them (70%) co-expressed low levels of CD71, their phenotype being represented by $CD33^{++}CD71^{+}$. These results indicate that monocytes in state A belonged to an immature, non-activated population (Fig. 1A).

After activating the cells by incubation in the presence of peptide Pa, five samples obtained on the second or third day of culture of PBMC from healthy donors and other five samples from patients (state B) presented a phenotype corresponding to activated monocytes (Fig. 1B). In this state the predominant subset was $CD14^{++}DR^{++}$ (80–90%); the number of monocytes expressing CD16 increased slightly with respect to state A. Similarly, the number of cells expressing CD25 and CD69 increased, making the most abundant subsets those with phenotypes $CD14^{++}CD25^{++}$ (65%) and $CD14^{++}CD69^{++}$ (43%). In contrast, only a slight expression of CD23 could be detected. About 50% of monocytes expressing CD33 were $CD71^{++}CD33^{++}$, indicating that in the evolution from state A to state B a high proportion of $CD33^{++}$ monocytes changed from $CD71^{+}$ into $CD71^{++}$ monocytes.

In more advanced states in the evolution of monocytes induced by peptide Pa (state C, corresponding to samples taken after 2–4 days of incubation, and state D, corresponding to samples taken after 3–5 days), and on fresh monocytes from patients, we observed the existence of two subpopulations differing in size and granularity, features being reflected in flow cytometry analysis by their forward and side light scattering properties. Small monocytes were more abundant in state D than in state C, and both had a similar phenotype (Fig. 1C and D): they expressed CD16 on $CD14^{+}$ monocytes, and high levels of HLA-DR both on $CD14^{+}$ and $CD14^{++}$ cells. The small monocytes with a $CD14^{+}CD16^{+}DR^{++}$ phenotype co-expressed high levels of CD25 and CD69 in state C, and low levels of both markers in state D. Within this population of small monocytes we found a subpopulation consist-

ing of $CD14^{++}DR^{++}$, which co-expressed high levels of CD25 and CD69 in states C and D, and only co-expressing CD16 and CD23 in state D. However, large and granular monocytes were almost exclusively $CD14^{++}$ and co-expressed CD16, CD25 and CD69 and very high levels of HLA-DR. $CD14^{++}$ monocytes co-expressed higher levels of CD16 and CD23 in state D than in state C. The total number of monocytes expressing high levels of CD71 increased from about 25% in state A or B to about 50% in state C, and 85% in state D, whereas the number of monocytes expressing high levels of marker CD33 decreased when passing from state A to state D (data not shown). Fig. 1 shows also that a small percentage of monocytes in state C had a $CD71^{++}CD33^{++}$ phenotype, a phenotype practically absent in state D.

3.2. *iNOS, A-I and A-II mRNA expression, arginase and NOS activities, and iNOS protein detection on activated and mature monocytes*

To find out if iNOS and arginases were selectively, or preferentially, associated with certain monocyte subsets, adherent cells were analysed for iNOS, A-I and A-II mRNA expression by RT-PCR in each of the 30 samples, with the characteristic phenotype of states A, B, C and D, after linear conditions for PCR were determined. A fragment of β-actin mRNA was amplified in each of the 30 samples as an internal normalisation control; also for this reaction linear conditions were determined. Fig. 2 shows a representative example of RT-PCR gels of the mRNAs analysed, together with the mean of the O.D. values for each mRNA, normalised with respect to that of β-actin amplified for the same sample. No significant differences in A-I mRNA were observed on monocytes in any of the four states, indicating that the expression remained constant during the incubation period, in contrast with the variations observed for the mRNA corresponding to either A-II or iNOS. Table 1 shows the changes in expression of these two latter mRNAs with respect to A-I mRNA in the monocytes analysed. In state A, A-I and A-II mRNA had a similar expression, and iNOS mRNA expression was practically non-existent (Fig. 2A). A-II mRNA reached its maximum value in state B (Fig. 2B). Monocytes in state C maintained the expression levels of mRNA corresponding to A-I and iNOS, already detected in state B; however, a significant fall in the level of A-II mRNA (Fig. 2C and Table 1) took place. Strikingly, monocytes in state D expressed iNOS mRNA in high levels (Fig. 2D and Table 1).

We looked also for possible positive or negative correlations between each mRNA and the proportion of the different monocyte subsets; r values are given in Table 2. A-I seemed to be associated (Table 2) with the immature $CD14^{++}$ monocyte subset, which practically did not express CD71 nor CD23; whereas A-II would be associated with the more activated $CD14^{++}$ monocytes, co-expressing $CD33^{++}CD71^{++}$ ($r=0.5$). Taken together, these data permit to associate the induction of the expression of A-II with the early activation of monocytes. Mature monocyte subsets, characterised by the co-expression of CD16 and DR, could be associated with the expression of iNOS, in agreement with data in Table 2.

We determined also the arginase and NOS activities on 5×10^6 monocytes belonging to samples in states A and B obtained after incubation of PBMC from two new donors. Adherent cells were obtained and lysed, and formation of urea determined. A higher arginase activity was detected on samples in state B than in samples in state A (Table 3); a slight basal arginase activity was detected in samples lacking A-II mRNA, probably due to the activity of constitutive A-I.

To ascertain if iNOS was present in both subpopulations of $CD16^{+}$ mature monocytes (small and large) in state D we looked for iNOS protein using

Table 1
mRNA values of A-II and iNOS relative to A-I mRNA in states A, B, C and D in the evolution of monocyte subpopulations

mRNA	State A	State B	State C	State D
A-II/A-I	0.9 ± 0.2	3.4 ± 0.8	1.1 ± 0.3	0.82 ± 0.3
iNOS/A-I	0.2 ± 0.1	0.9 ± 0.2	0.75 ± 0.2	2.6 ± 0.9

Table 2
Relationship between the percentage of different monocyte subsets and the expression of A-I, A-II and iNOS mRNA in total monocytes

Positive cells (% of total monocytes)							
mRNA	$CD14^{++}DR^{+}$	$CD14^{++}CD25^{++}$	$CD14^{++}CD69^{++}$	$CD33^{++}CD71^{+/-}$	$CD14^{+}DR^{++}$	$CD14^{+}CD16^{+}$	$CD14^{++}CD16^{++}$
iNOS	$r=-0.32$	$r=0.14$	$r=0.12$	$r=-0.61$	$r=0.51$*	$R=0.50$*	$r=0.50$
A-I	$r=0.74$*	$r=0.43$	$r=0.29$	$r=0.72$*	$r=-0.60$	$R=-0.64$	$r=-0.34$
A-II	$r=-0.31$	$r=0.65$*	$r=0.70$*	$r=-0.39$	$r=0.21$	$R=0.32$	$r=0.56$

mRNA of the different enzymes was evaluated as O.D. normalised with respect to O.D. corresponding to β-actin mRNA in the same sample. Results indicate the values of the Spearman's r for positive or negative relationships.
*$P<0.05$.

flow cytometry. We carried out a series of experiments incubating PBMC, from other three healthy donors, for 5 days in the presence of peptide Pa and also we analysed fresh monocytes from three patients. Fig. 3 shows the histograms of a representative sample; total monocytes co-expressing CD16 were either $CD14^{+}$ or $CD14^{++}$ and they were also $iNOS^{+}$ or $iNOS^{++}$, as the existence of two areas with different fluorescence intensities indicated; when the largest ones were gated it was seen that the predominant subpopulation of monocytes were $CD14^{++}CD16^{+}$ and contained high levels of iNOS protein; the subpopulation of small monocytes, which contained all the $CD14^{+}CD16^{+}$ cells, besides some $CD14^{++}CD16^{+}$ cells, co-expressed also iNOS protein. The relative proportion of both monocyte subpopulations in the six samples analysed was 30–35% of large $CD14^{++}$ $iNOS^{++}$ cells and 70–65% of small $CD14^{+/++}$ $iNOS^{+}$ cells.

We selected and analysed comparatively ten samples (five from healthy donors, subjected to the required incubations, and other five from patients), all of them characterised by the absence of the $CD14^{+}CD16^{+}$ subset, abundance of $CD14^{++}$ monocytes co-expressing CD16 and DR, together with the simultaneous presence of iNOS and A-II mRNAs. In five samples iNOS mRNA level was higher than A-II mRNA (the ratio iNOS mRNA/A-II mRNA was approximately 2.5) and $CD14^{++}CD16^{++}DR^{++/+}$ was the most abundant phenotype; in the other five samples the iNOS mRNA level was similar to that of A-II (the mean of the iNOS mRNA/A-II mRNA ratio was 0.7) and the most abundant subset was $CD14^{++}CD16^{+}DR^{++}$. These data confirm that the decrease in the expression of A-II mRNA concomitant with an increase in iNOS mRNA expression was associated with an increase in CD16 expression on $CD14^{++}DR^{++}$ monocytes.

Another series of experiments was carried out to ascertain if A-II mRNA and arginase activity were simultaneously present in the iNOS positive, $CD14^{++}CD16^{++}$ subset, and absent in the iNOS

Table 3
Arginase and nitric oxide synthase activities of monocyte subsets in different states of activation and differentiation

	Enzyme activities	
Predominant monocyte subset	Arginase (mU/10^6 cells)	NOS (pmol/min/mg prot.)
$CD14^{++}DR^{+}$ (state A)	20–30	not detected
$CD14^{++}DR^{++}$ (state B)	85–95	<20
Total $CD16^{+}DR^{++}$ (state D)	45–53	90–120
Large $CD14^{++}$ (state D)	60–70	75–100
Small $CD14^{+/-}$ (state D)	28–35	100–130

Arginase and NOS activities were determined on monocytes belonging to samples obtained after incubation of PBMC. $CD14^{++}$ and $CD14^{+/-}$ monocytes from samples on state D were separated by their binding to anti-CD14 antibody magnetic balls. 2–10 μg of monocyte protein were used in each determination. Activities were measured in triplicates of each sample. Two different experiments were carried out to obtain cells in states A and B, and other two for state D, using four different donors. A basal arginase activity (approximately 15–25 mU/10^6 cells) was detected in samples lacking A-II mRNA probably corresponding to A-I.

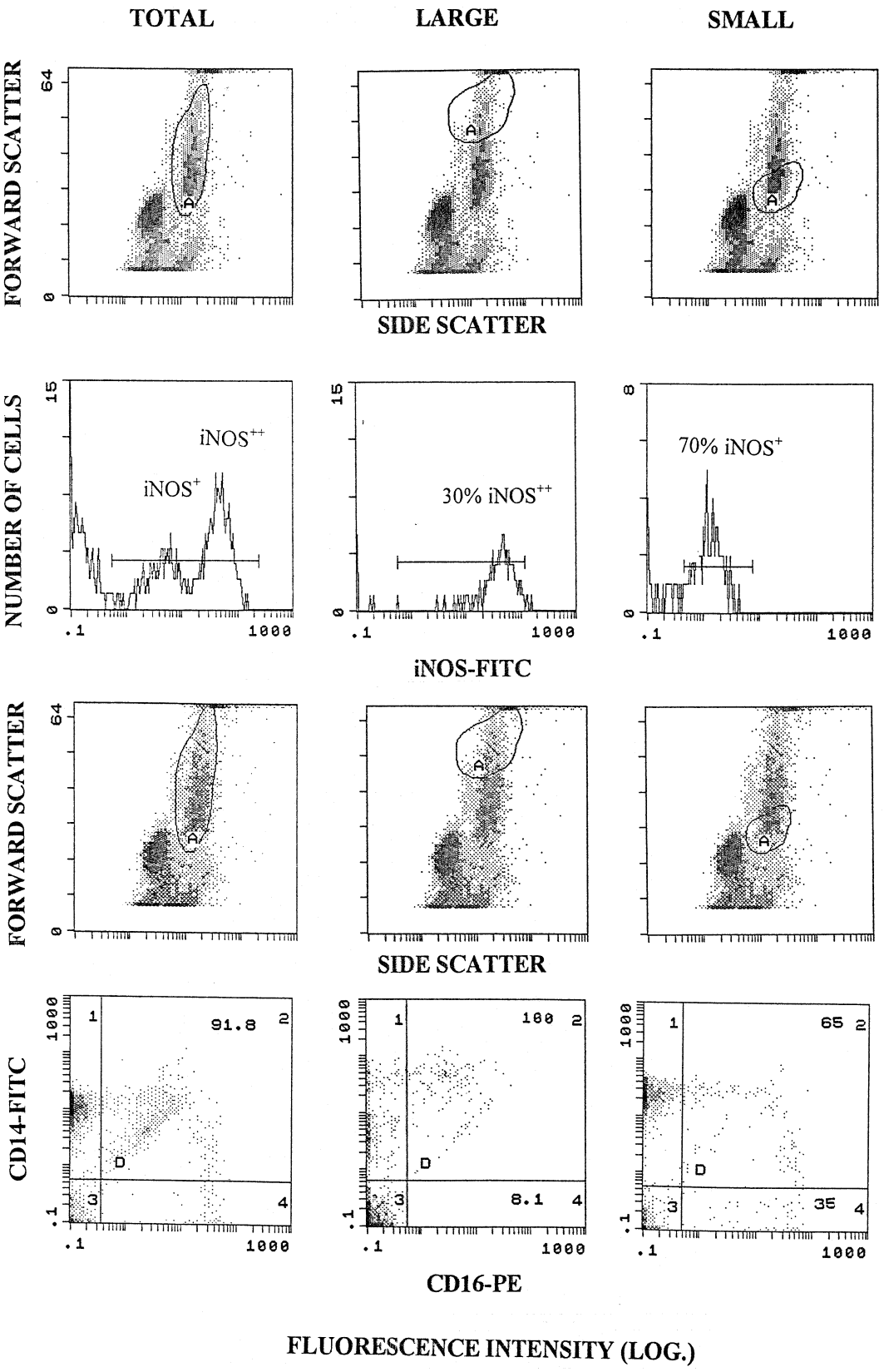
TOTAL
LARGE
SMALL
FORWARD SCATTER
SIDE SCATTER
NUMBER OF CELLS
iNOS++
iNOS+
30% iNOS++
70% iNOS+
iNOS-FITC
CD14-FITC
91.8
100
65
8.1
35
CD16-PE
FLUORESCENCE INTENSITY (LOG.)

Fig. 3. Flow cytometry analysis of cytoplasmic iNOS expression by large and small monocytes, co-expressing CD14 and CD16 markers. 5×10^5 PBMC were fixed, permeabilised and stained using FITC mouse anti-iNOS. 20 000 cells were used in each cytometry analysis of CD14/CD16 or for iNOS expression on parallel samples. Monocytes were gated into two areas according to forward and side scatter properties to analyse the large or the small cells. The relative proportions of $CD14^{++}CD16^{+}$ and $CD14^{+}CD16^{+}$ monocytes (large or small) are indicated.
←

positive, $CD14^{+/-}CD16^{+}$ subset. Taking advantage of the different capacity of $CD14^{-/+}$ and $CD14^{++}$ cells to adhere to anti-CD14 antibody magnetic balls we separated both monocyte subsets from approximately 10^7 cells adhered obtained from 9.5×10^7 PBMC. A-II mRNA, arginase and NOS activities were measured in the samples of both monocyte subsets. Cells complexing with the magnetic balls were predominantly $CD14^{++}$, contained practically all A-II mRNA and, as shown in Table 3, the arginase activity present in the entire population of the monocytes of that sample. However, isolated $CD14^{+/-}$ monocytes did not contain any A-II mRNA, and had a very low arginase activity (Table 3). Both monocyte subsets had a very high NOS activity; and higher in $CD14^{+/-}$ than in $CD14^{++}$ monocytes. Thus the $CD14^{++}$ monocytes had a higher arginase/NOS ratio than the $CD14^{+/-}$ cells.

In summary, we identified a subpopulation of monocytes in state B, expressing arginase; another subpopulation formed by large granular monocytes in state D, expressing high levels of CD14, co-ex-

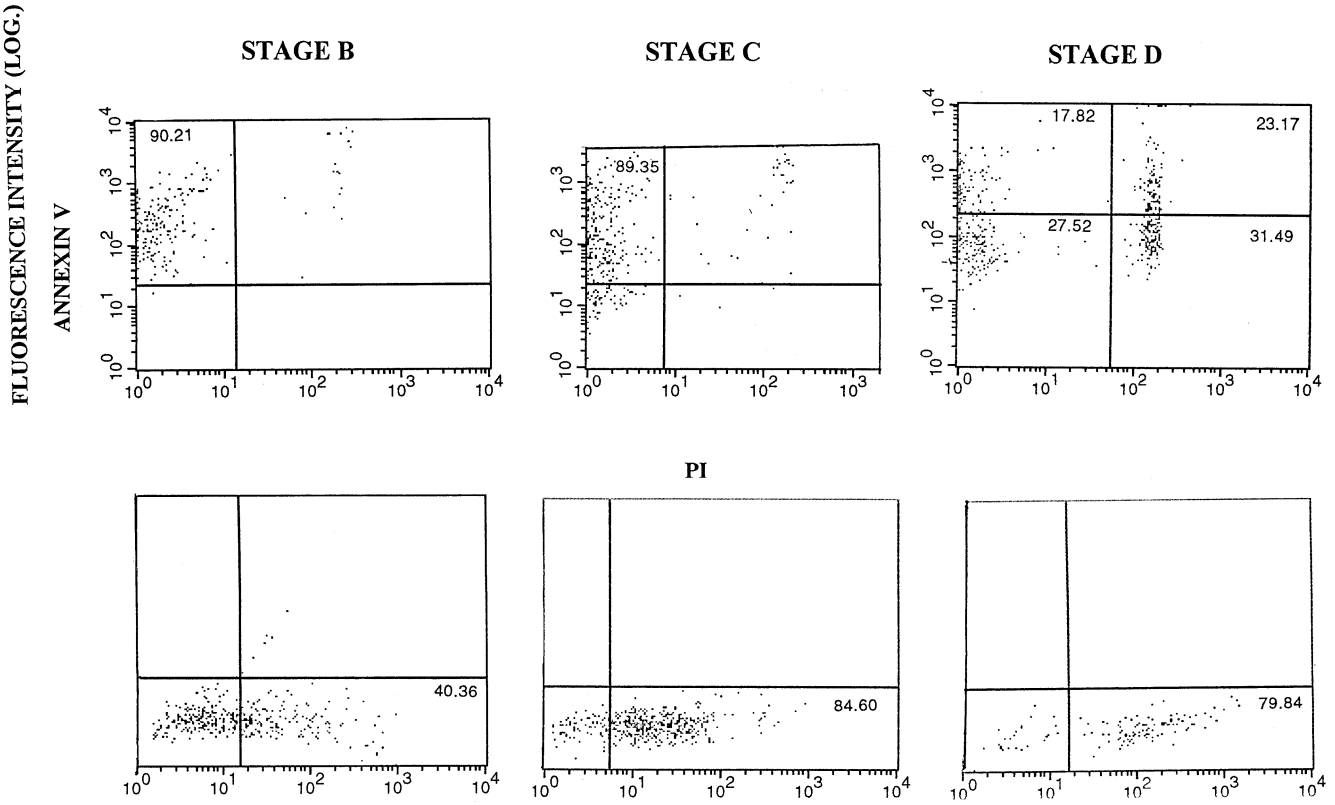

Fig. 4. Flow cytometry analysis of intracellular TIA and detection of apoptotic or necrotic monocytes by simultaneous reduced DNA stainability with PI and enhanced annexin V binding on monocytes. Samples are representatives of monocytes on different states as grouped in Fig. 1, panels B–D. Sample B: $CD14^{++}DR^{++}A\text{-}II^{++}iNOS^{+}$ subset. Sample C: $CD14^{++/+}DR^{++}A\text{-}II^{+}iNOS^{++/+}$ subset. Sample D: $CD14^{++/+}DR^{++}A\text{-}II^{+/-}iNOS^{++}$ monocyte subsets. Values of iNOS mRNA/A-II mRNA ratios were 0.3, 0.7 and 2.7 respectively. 5×10^5 PBMC were fixed, permeabilised and stained using FITC anti-TIA. 20 000 cells were used in each cytometry analysis of annexin V/PI or for TIA expression on parallel samples. The relative proportions of cells with low or high annexin V binding and DNA stainability with PI or with high TIA expression are indicated.

Table 4
Endogenous NO production and induction of apoptosis and necrosis on monocytes

Treatment (48 h culture)	Nitrite/nitrate (nmol/10^5 cells)		TIA^{++} cells (% monocytes)		Annexin V^{++} PI^{+} (Annexin V^{++} PI^{++}) (% monocytes)	
	High iNOS/A-II	Low iNOS/A-II	High iNOS/A-II	Low iNOS/A-II	High iNOS/A-II	Low iNOS/A-II
None	4.5 ± 0.6	1.8 ± 0.4	16–26	2–10	20–30 (23–29)	10–15 (0–1)
0.5 mM L-arginine	10 ± 1	2.1 ± 0.2	40–50	3–9	45–50 (35–40)	10–17 (8–15)
1 mM L-arginine	12 ± 2	2.2 ± 0.4	42–50	8–12	50–58 (38–45)	8–16 (10–14)
1 mM arginine+1 mM NMLA	5.2 ± 0.5	2.1 ± 0.3	28–33	8–10	35–40 (30–40)	8–15 (8–13)

Monocytes from three selected patients with autoimmune disease had a high iNOS/A-II mRNA ratio (1.8 ± 0.4), other monocytes from three other patients had a low ratio (0.5 ± 0.1). PBMC were incubated for 48 h in the absence or in the presence of L-arginine, or L-arginine together with NMLA.

pressing simultaneously arginase and iNOS; and a further differentiated subpopulation of small monocytes, in which the decrease in the expression of arginase and the increase in the expression of iNOS would be accompanied by a decrease in CD14. The enzyme activities correlated with the levels of mRNA expression.

3.3. *Apoptosis of different monocyte subsets with different iNOS and arginase expression levels*

We analysed TIA expression and annexin V binding, together with DNA accessibility to PI, on six samples from patients with autoimmune disease, already co-expressing iNOS and A-II on freshly obtained monocytes and on ten samples of monocytes from cultures of PBMC. Fig. 4 shows the histograms of representative samples of cells on states B, C and D with different iNOS mRNA/A-II mRNA ratios (values of this ratio were 0.3, 0.7 and 2.7 respectively). Monocytes in state B, with low iNOS mRNA expression and high level of A-II mRNA, show a low proportion of total cells stained; approximately 20% of total monocytes had a high TIA expression and high annexin V binding and low DNA accessibility to PI. Activated monocytes in state C, with a higher proportion of iNOS mRNA with respect to A-II, were in a more advanced stage of apoptosis, manifested by a high TIA expression and annexin V binding, together with low DNA accessibility to PI. Monocytes in state D, exhibiting a high expression of iNOS mRNA and a very low expression of A-II mRNA, presented a large proportion of necrotic monocytes (annexin V binding and high DNA accessibility to PI) (Fig. 4), co-expressing very high levels of HLA-II (data not shown), and also a large proportion of apoptotic cells (annexin V binding and low DNA accessibility to PI); a reduced number of monocytes in this state D had a high expression of apoptotic marker TIA (Fig. 4). A number of large $CD14^{++}CD16^{+}$ in state D monocytes, co-expressing both enzyme mRNAs did not show signs of apoptosis (data not shown). A positive correlation was found between the number of TIA^{++} monocytes and apoptotic cells.

Finally, we studied also on several samples, with different iNOS mRNA/A-II mRNA ratios, the endogenous production of NO, measured as nitrate/nitrite, necrosis and apoptosis, determined as annexin V binding and DNA accessibility to PI and also TIA expression, when PBMC were incubated for 48 h in the absence or in the presence of L-arginine, or L-arginine together with NMLA. Table 4 shows that incubation induced an increase in NO degradation products and the number of cells with features of apoptotic and necrotic cells, more markedly when L-arginine (0.5 or 1 mM) was added to the incubation medium of cells with high iNOS mRNA/A-II mRNA ratio. However, NMLA, an inhibitor of iNOS, reduced slightly the number of apoptotic and necrotic cells.

4. Discussion

The results now reported (Figs. 1–3 and Tables 1–3) suggest that the evolution of monocytes implicated changes in the expression of at least two enzymes

involved in arginine metabolism, A-II and iNOS; A-I remained practically constant. The more immature, but activated, $CD14^{++}DR^{++}CD16^{-}$ monocyte subset expressed arginase A-II mRNA and had a high arginase activity. Two $CD14^{++}$ subpopulations of large and small monocytes, co-expressing CD16 and HLA-DR, contained simultaneously arginase and NOS activities. Finally, a population of small differentiated monocytes expressing CD16 and characterised by low CD14 and low arginase activity, together with a high NOS activity, was also found. These changes are consistent with reports showing that expression of iNOS [32] is mainly regulated at the transcriptional level, and also that this enzyme, as well as arginase II, can be induced with suitable agents, such as LPS or cytokines [2,6–8]. The increase in the proportion of $CD14^{+}CD16^{+}DR^{++}$ monocytes, as well as the decrease in the proportion of $CD14^{++}$ monocytes are in agreement with observations reported by others [33–35], who consider the $CD14^{+}$ cells as small mature blood monocytes, and seemingly having their origin in the maturation of $CD14^{++}$ monocytes. An evolution of monocytes towards macrophages, similar to that induced by peptide Pa, has also been described as being operative in patients with multiple sclerosis [13], or Graves' disease [36]. Furthermore, a correlation of iNOS, CD16 and HLA-DR has already been described by us [13]. On the other hand, it has been shown that CD69, a member of the natural killer cell gene complex family of signal transducing receptors, is constitutively expressed on human peripheral blood monocytes [37], and also that a CD69 cross-linking induces strong nitric oxide production by monocytes [38]. It has also been demonstrated that the expression of both transferrin receptor (CD71) and low-affinity type II membrane receptor for IgE (CD23) increase on monocytes after stimulation with LPS [39]; in vitro ligand binding to CD23 induces iNOS expression [40] and activation of monocytes ensued by NO production [41]. Furthermore, it has been reported that nitric oxide is an important mediator in the monocytic differentiation of U937 cells induced by vitamin E-succinate, or fibronectin [42].

Fig. 4 shows that apoptosis was favoured in some mature monocyte subsets and necrosis in hyperstimulated cells. Two different methods, based on flow cytometry, were used to determine the proportion of apoptotic monocytes: an assessment of the expression of TIA, which has been associated with cytotoxic T lymphocytes and responsible for triggering apoptosis [43], and the simultaneous PI uptake and annexin V binding [44], signs indicative of the initiation of apoptosis. The observed correlation between both methods suggests that the quantification of TIA expression is a valid way to assess the apoptotic state of monocytes. Differences in the proportion of monocytes undergoing apoptosis seemed to be due, at least in part, to the amount of endogenous NO produced, which in turn seemed to depend on the balance of iNOS and A-II, associated with the state of activation and differentiation of monocytes (Tables 3 and 4). Apoptosis of $CD14^{++}$ monocytes correlated with the decrease in the A-II mRNA/iNOS mRNA ratio (Fig. 3). The more immature, but activated monocytes, with high expression of HLA-DR, lacking CD16 and expressing arginase, were non-apoptotic cells. However, the more differentiated monocytes, with low levels of CD14 and expressing CD16, CD23, HLA-DR, as well as iNOS, showed signs of apoptosis. The degree of apoptosis could be related, in turn, to the stage of maturation manifested by the proportion of monocytes with low expression of CD14, in agreement with the observation that the down-regulation of CD14 triggers apoptosis, whereas its up-regulation promotes survival [43]. $CD14^{++}CD16^{++}DR^{++}$ monocytes co-expressing both enzyme mRNAs seemed to be protected from apoptosis. Data of Table 4 indicate that the simultaneous presence of iNOS and A-II, associated with this subset in an early state of differentiation and activation, led to a low production of endogenous NO. That would be a consequence of the competition of these enzymes for the same substrate, in agreement with reports showing that arginase modulates NO production in macrophages [15]. Under that circumstance NO-triggered apoptosis would be kept at bay, in contrast with the situation in which iNOS was present, and A-II absent. These data agree with reports of others stating that apoptosis is triggered by NO [16,17,45] and regulated by the formation of superoxides [46], related, in turn, to the state of maturation of the monocytes.

The results now presented suggest a physiological role for the dual pathway of arginine metabolism in human monocytes: the control of endogenous NO

production, which would permit the evolution from immature to differentiated cells, preserving them from a premature NO-induced apoptosis.

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