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# Maturation-Dependent Expression and Function of the CD49d Integrin on Monocyte-Derived Human Dendritic Cells<sup>1</sup>

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Dendritic cells (DC) are highly specialized APC that are critical for the initiation of T cell-dependent immune responses. DC exert a sentinel function while immature and, after activation by inflammatory stimuli or infectious agents, mature and migrate into lymphoid organs to prime T cells. We have analyzed integrin expression on monocyte-derived DC (MDDC) and found that expression of CD49d integrins (CD49d/CD29 and CD49d/ $\beta$ 7) was induced/up-regulated during TNF- $\alpha$ - or LPS-initiated MDDC maturation, reflecting the induction/up-regulation of CD49d and  $\beta$ 7 mRNA. CD49d mRNA steady-state level increased more than 10 times during maturation, with the highest levels observed 24 h after TNF- $\alpha$  treatment. CD49d integrin expression conferred mature MDDC with an elevated capacity to adhere to the CS-1 fragment of fibronectin, and also mediated transendothelial migration of mature MDDC. Up-regulation of CD49d integrin expression closely paralleled that of the mature DC marker CD83. CD49d integrin expression was dependent on cell maturation, as its induction was abrogated by *N*-acetylcysteine, which inhibits NF- $\kappa$ B activation and the functional and phenotypic maturation of MDDC. Moreover, CD49d integrin up-regulation and MDDC maturation were prevented by SB203580, a specific inhibitor of p38 mitogen-activated protein kinase, but were almost unaffected by the mitogen-activated protein/extracellular signal-related kinase kinase 1/2 inhibitor PD98059. Our results support the existence of a link between functional and phenotypic maturation of MDDC and CD49d integrin expression, thus establishing CD49d as a maturation marker for MDDC. The differential expression of CD49d on immature and mature MDDC might contribute to their distinct motility capabilities and mediate mature DC migration into lymphoid organs. *The Journal of Immunology*, 2000, 165: 4338–4345.

Dendritic cells (DC)<sup>3</sup> are professional APCs that play a pivotal role in the initiation of T cell-dependent immune responses as a consequence of their high expression of MHC and costimulatory molecules (1). DC are sparsely distributed throughout the body and, in most tissues, DC are present in an immature state, exhibiting a high capacity for Ag uptake and processing, but unable to stimulate T cells (1, 2). Once activated by inflammatory stimuli or infectious agents, DC undergo a maturation process whose hallmarks are increased expression of costimulatory (CD40, CD80, CD86) and adhesion (CD54, CD58) molecules, migration into lymphoid organs, and subsequently, acquisition of the capacity to activate quiescent, naive, and memory lymphocytes (1, 2).

Several pathways for DC generation have been demonstrated (1–4). In vitro, DC can be derived from either precursor cells or peripheral blood monocytes (5–8) when the appropriate cytokine

signals are provided, raising the possibility of using DC therapeutically as adjuvants for immunization (9, 10). Immature monocyte-derived DC (MDDC) can be obtained by culturing peripheral blood monocytes in the presence of GM-CSF and IL-4. The further addition of LPS or TNF- $\alpha$  leads to the appearance of MDDC with all the morphological, phenotypic, and functional characteristics of mature DC (5, 6, 11). Thus, in vitro MDDC maturation represents a useful system to analyze the molecular and functional changes that take place during acquisition of the optimal T cell-stimulating activity by DC.

One of the most relevant attributes of DC is their motility and migratory capacity (1–3). DC migration is a consequence of DC maturation and contributes to the acquisition of all the functional features of mature DC. The interdependence of DC maturation and DC migration is best exemplified by the capacity of peripheral blood monocytes to differentiate into either macrophages or DC depending on their pattern of transendothelial migration (12). Moreover, extracellular matrix components can alter the differentiation pathway of DC from peripheral blood monocytes (13).

The CD49d/CD29 integrin is preferentially expressed in cells of the hemopoietic lineage and mediates both cell-cell and cell-extracellular matrix interactions through its specific interactions with VCAM-1 or the alternatively spliced CS-1 sequence in fibronectin (FN) (14). In addition, the heterodimer CD49d/ $\beta$ 7 is capable of interacting with mucosal addressin cell adhesion molecule 1 (15). Secondary to these interactions, CD49d integrins are key players in the process of leukocyte transendothelial migration as they participate in both the rolling and tight adhesion steps of extravasation (16). In the present manuscript, we have analyzed the changes in the pattern of integrin expression during MDDC maturation. The expression of the CD49d integrins was found to be either induced or greatly up-regulated during MDDC maturation, reflecting the

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<sup>3</sup> Abbreviations used in this paper: DC, dendritic cell; BCECF, 2',7'-bis-(2-carboxyethyl)-5-(and -6)-carboxyfluorescein acetoxymethyl ester; ERK, extracellular signal-related kinase; FN, fibronectin; MAPK, mitogen-activated protein kinase; MDDC, monocyte-derived DC; MFI, mean fluorescence intensity; NAC, *N*-acetylcysteine; SDF, stromal cell-derived factor.

increase in CD49d and  $\beta 7$  mRNA steady-state levels from immature to mature MDDC, and conveying mature MDDC with an elevated capacity to adhere to the FN CS-1 fragment. The regulated expression of CD49d integrins from immature to mature MDDC might contribute to the distinct motility properties of both cell populations, including the maturation-dependent migration of DC into lymphoid organs.

## Materials and Methods

### Cytokines and reagents

GM-CSF (Leucomax) was purchased from Schering-Plough (Madison, NJ) and used at 1000 U/ml. TNF- $\alpha$  and IL-4 were obtained from PeproTech (Rocky Hill, NJ) and used at 100 and 1000 U/ml, respectively. Stromal cell-derived factor 1 $\alpha$  (SDF-1 $\alpha$ ) was obtained from PeproTech and used at 100 ng/ml. LPS from *Escherichia coli* 055:B5 was obtained from Sigma (St. Louis, MO) and used at 10 ng/ml. *N*-acetylcysteine (NAC) was obtained from Sigma and dissolved in RPMI. SB203580 and PD98059 were obtained from Calbiochem (La Jolla, CA) and used at 13 and 40  $\mu$ M in DMSO, respectively.

### Cells

Human PBMC were isolated from buffy coats from normal donors over a Lymphoprep (Nycomed, Oslo, Norway) gradient according to standard procedures. Monocytes were purified from PBMC by a 1-h adherence step at 37°C in complete medium. Nonadherent cells were washed off by extensive washing with PBS, and the remaining adherent cells were >90% monocytes, as determined by flow cytometric analysis of forward scatter/side scatter, CD14, and CD11c staining. Monocytes were immediately subjected to the DC differentiation protocol, as previously described (5, 6). Briefly, monocytes were resuspended to 0.5–1  $\times 10^6$  cells/ml and cultured in RPMI supplemented with 10% FCS, 25 mM HEPES, and 2 mM glutamine (complete medium) containing 1000 U/ml GM-CSF and 1000 U/ml IL-4. Cells were cultured for 5–7 days, with cytokine addition every second day, to obtain a population of immature MDDC. MDDC maturation was induced by treatment with 100 U/ml TNF- $\alpha$  for 5 days or, alternatively, by treatment with LPS at 10 ng/ml. In experiments using SB203580 and PD98059 mitogen-activated protein kinase (MAPK) inhibitors, cells were cultured for 1 h in the presence of each inhibitor before TNF- $\alpha$  addition. Control cells were treated with an identical amount of DMSO.

Human endothelial cells (HUVEC) were obtained from umbilical veins and cultured following standard procedures. Cells were seeded on tissue culture flasks coated with 0.5% gelatin and grown in 199 medium (BioWhittaker, Verviers, Belgium) supplemented with 20% FCS (Life Technologies, Gaithersburg, MD), 50 IU/ml penicillin, 50  $\mu$ g/ml streptomycin, 250  $\mu$ g/ml fungizone, 50  $\mu$ g/ml endothelial cell growth supplement (prepared from bovine brain), and 100  $\mu$ g/ml heparin, and used up to the third passage. The human cell line U937 (histiocytic lymphoma) was cultured in complete medium, at 37°C in a humidified atmosphere with 5% CO<sub>2</sub>. Induction of differentiation of U937 was conducted with PMA at 10 ng/ml for 24–48 h and at a density of 5  $\times 10^5$  cells/ml.

### Flow cytometry and Abs

Phenotypic analysis of the distinct cell populations was conducted by indirect immunofluorescence. mAbs used for cell surface staining included T3b (anti-CD3), TS1/11 (anti-CD11a), Bear-1 (anti-CD11b), HC1/1 (anti-CD11c), UCH-M1 (anti-CD14, from Santa Cruz Biotechnology, Santa Cruz, CA), TS1/18 (anti-CD18), P5D2 and TS2/16 (anti-CD29), TS2/7 (anti-CD49a), HP1/2, HP2/1, HP2/4 (all anti-CD49d), Lia 1/2 (anti-CD29), HP2/9 (anti-CD44) (all of them kindly provided by Dr. F. Sánchez-Madrid, Hospital Universitario de La Princesa, Madrid, Spain), P1/D6 (anti-CD49e, Telios), RR1/1 (anti-CD54; provided by Dr. R. Rothlein, Boehringer Ingelheim Pharmaceuticals, Ridgefield, CT), P2W7 (anti-CD51; provided by Dr. F. Watt, Imperial Cancer Research Fund, London, U.K.), HB1/5 (anti-CD83; Immunotech, Marseille, France), and Act-1 (anti-CD49d/ $\beta 7$ ; provided by Dr. A. I. Lazarovits, Robarts Research Institute, Ontario, Canada). For double immunofluorescence experiments, cells were serially incubated with anti-CD49d, fluorescein-labeled rabbit anti-mouse Abs, and PE-labeled anti-CD83 Ab in the presence of normal mouse serum. In all indirect immunofluorescence experiments, the supernatant from the myeloma P3X63 was included as negative control. All incubations were done in the presence of 50  $\mu$ g/ml of human IgG to prevent binding through the Fc portion of the Abs. Flow cytometry analysis was performed with an EPICS-CS (Coulter Científica, Madrid, Spain) using log amplifiers.

### Adhesion and transmigration assays

CD49d-dependent adhesion to the CS-1 fragment of FN was evaluated using the recombinant FN-H89 peptide. Ninety-six-well microtiter EIA II-Linbro plates were coated overnight with FN-H89 at 3  $\mu$ g/ml in 100 mM NaHCO<sub>3</sub>, pH 8.8, and at 4°C, and the remaining sites were blocked with 0.4% BSA in the same solution for 2 h at 37°C. MDDC were labeled in complete medium with the fluorescent dye 2',7'-bis-(2-carboxyethyl)-5-(and -6)-carboxyfluorescein, acetoxymethyl ester (BCECF; Molecular Probes, Leiden, The Netherlands), and then preincubated in RPMI containing 0.4% BSA and the distinct Abs for 20 min at 37°C. Cells were then allowed to adhere to each well for 15 min at 37°C. Unbound cells were removed by three washes with warm RPMI, and adhered cells were quantified using a fluorescent analyzer. MDDC adhesion to total FN was performed following the same protocol and coating the plates with 100  $\mu$ g/ml FN (Life Technologies) in 100 mM NaHCO<sub>3</sub>, pH 8.8. Specificity of the interactions was analyzed by performing the adhesion assays in the presence of function-blocking Abs against CD49d (HP1/2), CD49e (P1D6), CD29 (P5D2 or Lia1/2), CD11c (HC1/1), CD49d/ $\beta 7$  (Act-1), or the myeloma P3X63 culture supernatant as negative control. Transendothelial migration assay for DC was performed in polycarbonate transwell inserts (5  $\mu$ m pore; Corning, Costar, Cambridge, MA), as previously described (17). Briefly, inserts were coated with HUVEC, grown as monolayer for 24 h, and then treated with 10 ng/ml human rTNF- $\alpha$  for 10 h. DC were cytoplasmically labeled with BCECF and labeled DC were preincubated with Abs for 20 min before plating. Typically, 1.25  $\times 10^5$  BCECF-labeled DC were seeded in the upper compartment, and SDF-1 $\alpha$  (100 ng/ml) was placed in the lower compartment. After 24 h at 37°C, the number of fluorescent labeled DC that had migrated through the monolayer was determined by direct counting on a FACScan (Becton Dickinson Immunocytometry Systems, Mountain View, CA) using CellQuest software.

### Northern blot

After extensive washing in PBS, cells were harvested and total cellular RNA was isolated using RNeasy columns (Qiagen, Chatsworth, CA) following the manufacturer's recommendations. RNA integrity was initially confirmed in formaldehyde-containing agarose gels. Denatured RNA (10  $\mu$ g) was size fractionated on formaldehyde-containing 1% agarose gels in the presence of ethidium bromide. After electrophoresis, RNA was transferred overnight onto nitrocellulose membranes with 20 $\times$  SSC. Prehybridization was conducted overnight at 42°C in 50% formamide, 5 $\times$  SSC, 5 $\times$  Denhardt's, 50 mM sodium phosphate, pH 6.5, and 250  $\mu$ g/ml denatured salmon sperm DNA, and membranes were hybridized for 16 h at 42°C in the same solution containing 10<sup>6</sup> cpm/ml of oligo-labeled probe. Blots were sequentially washed in 2 $\times$  SSC, 0.5% SDS at room temperature, in 0.3 $\times$  SSC, 0.5% SDS at 65°C, and exposed to x-ray film at -70°C. Detection of CD49d mRNA was accomplished with a 1.8-kbp *EcoRI* fragment of the CD49d cDNA (18), while the 1.8-kbp insert from the  $\beta 7$  cDNA clone P9 (19) was used to detect  $\beta 7$  mRNA. For comparative purposes, all filters were subsequently hybridized with a 417-bp fragment from the GAPDH cDNA (20).

### Polymerase chain reaction

Determination of the level of CD49d mRNA along the MDDC differentiation/maturation pathway was accomplished by relative PCR. To that end, 2  $\mu$ g of RNA from either immature or mature MDDC was reverse transcribed in a total volume of 20  $\mu$ l of the amplification buffer (50 mM Tris-HCl, pH 8.2, 5 mM MgCl<sub>2</sub>, 10 mM DTT, 50 mM KCl, 1 mM of each deoxynucleotide, 0.5  $\mu$ M random hexamers) including RNAsin and AMV reverse transcriptase at 1 U/ $\mu$ l. The mixture was incubated at 42°C for 60 min, followed by a 30-min incubation at 52°C, and the final volume was taken to 100  $\mu$ l with water.

Amplification of the CD49d mRNA was conducted on 5  $\mu$ l of each cDNA synthesis reaction in 50  $\mu$ l of a solution containing 0.2 mM of each deoxynucleotide, 1  $\mu$ M of each oligonucleotide primer, and 2.5 U of *Pfu* DNA polymerase (Stratagene, La Jolla, CA). Preliminary experiments indicated that the CD49d mRNA was optimally amplified after 35 cycles of denaturation (95°C, 45 s), annealing (62°C, 45 s), and extension (72°C, 1 min), followed by a 10-min extension step at 72°C. Oligonucleotides used for CD49d mRNA amplification (VLA4-III, 5'-GCTGATTACAG GTTTCTGC-3'; VLA4-IV, 5'-ACTTCTGACGTGATTACAGGAAGC-3') flank a 286-bp fragment from the CD49d mRNA (between nucleotides 1946 and 1660), and their sequences are derived from separate exons in the CD49d gene (21). As an internal control, each PCR also included oligonucleotides 5'-GGCTGAGAACGGGAAGCTTGCA-3' and 5'-CGGCC ATCACGCCACAGT TTC-3' (1  $\mu$ M), which together amplify a 417-bp fragment from the GAPDH mRNA (20). Analysis of the relative levels of

CD49d mRNA in immature and mature MDDC was done by removing 15- $\mu$ l aliquots of the PCR during the log phase of the amplification (after 22, 24, 27, and 30 cycles), and the amplified fragments were detected by agarose gel electrophoresis.

#### Western blot

Total cell lysates were obtained in 50 mM HEPES, pH 7.5, 250 mM NaCl, 1 mM EDTA, 0.5% Triton X-100, 0.5 mM DTT, 10 mM NaF, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 20 mM Pefabloc, and 2  $\mu$ g/ml of aprotinin, antipain, leupeptin, and pepstatin. A total of 10  $\mu$ g of each lysate was subjected to SDS-PAGE under reducing conditions and transferred onto an Immobilon polyvinylidene difluoride membrane (Millipore, Bedford, MA). After blocking of the unoccupied sites with 5% BSA in 50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 0.1% Tween-20, protein detection was performed using the Supersignal West Pico Chemiluminescent system (Pierce, Rockford, IL), according to the manufacturer's instructions. For reprobing, membranes were incubated in stripping buffer (62.5 mM Tris-HCl, pH 6.7, 100 mM 2-ME, 2% SDS) for 30 min at 50°C with occasional agitation. Detection of extracellular signal-related kinase (ERK)1/2, p38, phospho-ERK1/2 (pERK1/2), and phospho-p38 (pp38) was conducted using specific polyclonal Abs from New England Biolabs (Beverly, MA).

## Results

### CD49d integrin expression upon activation of MDDC

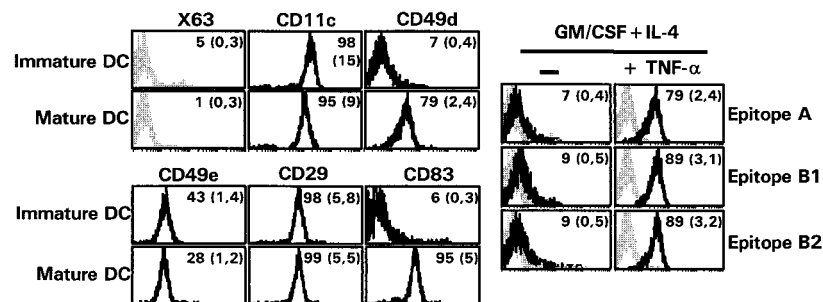
The pattern of integrin expression changes during differentiation in numerous cell lineages (22, 23). Conversely, integrin molecules directly affect the differentiation program of hemopoietic cells (24, 25). Because the functional activity of DC is dependent on their adhesive and migratory capabilities, the levels of integrin expression were analyzed during DC differentiation/maturation from peripheral blood monocytes. Flow cytometry analysis revealed that the expression of several  $\alpha$  subunit integrins greatly differed between immature and mature MDDC. Immature MDDC expressed high levels of CD49e/CD29, CD11a-c/CD18, moderate levels of CD51, and very low (or absent) levels of CD49d (Fig. 1, *left*). The low expression of CD49d on immature MDDC is in agreement with our previous results, showing that its expression on monocytes is down-regulated upon *in vitro* culture (26). TNF- $\alpha$ -induced MDDC maturation led to induction of CD83 expression, without affecting the expression of the CD11a-c/CD18 integrins (Fig. 1). By contrast, the expression of CD49d was up-regulated during MDDC maturation. Analysis of MDDC from more than 30 donors revealed that CD49d was either absent or extremely low on immature MDDC, while TNF- $\alpha$  maturation either induced or greatly up-regulated the expression of CD49d (Fig. 1, *left*). To exclude a conformational change as the basis for the differential CD49d expression, both cell types were analyzed with Abs recognizing distinct functional epitopes on the CD49d molecule (27). mAbs

against epitopes A, B1, and B2 similarly detected the changes in CD49d expression between immature and mature MDDC (Fig. 1, *right*), thus ruling out an epitope-specific effect and confirming that CD49d expression is greatly increased by TNF- $\alpha$  on MDDC. Furthermore, LPS treatment of MDDC up-regulated CD49d (see below, Fig. 3), indicating that maturation-inducing agents other than TNF- $\alpha$  are also capable of increasing the expression of CD49d-containing integrins.

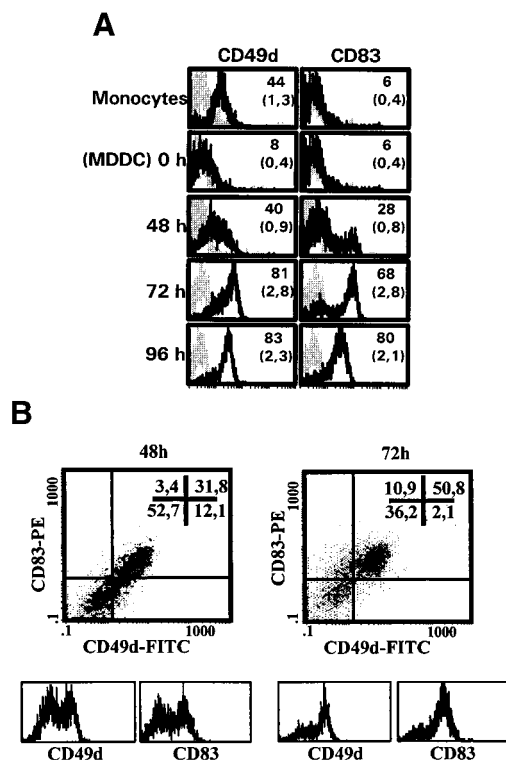
### Kinetics of CD49d integrin acquisition during MDDC maturation

Maturation of MDDC by TNF- $\alpha$  increases the expression of costimulatory molecules (CD80, CD86) and induces the expression of the mature DC marker CD83 after 2–4 days of treatment (3, 11). To determine the kinetics of the TNF- $\alpha$ -mediated up-regulation of CD49d, integrin expression was measured at different time points during MDDC differentiation/maturation and compared with CD83. Forty-eight hours after TNF- $\alpha$  addition, a time point at which a CD83-expressing subset had already appeared, CD49d expression had also started to increase (Fig. 2, *A* and *B*). The proportion of cells with high CD49d expression steadily increased during MDDC maturation, and the whole cell population had acquired CD83 expression and exhibited high levels of CD49d 96 h after TNF- $\alpha$  addition (Fig. 2, *A* and *B*). Therefore, expression of the CD49d integrins is up-regulated concomitantly with CD83 induction, suggesting that a high expression of CD49d can be considered as a maturation marker on MDDC. To determine whether CD49d expression was associated with specific cell subsets in mature MDDC, double-labeling experiments were performed with anti-CD83 and anti-CD49d Abs on TNF- $\alpha$ -treated MDDC. Two days after initiation of the maturation program, all CD83-positive cells exhibited high expression of CD49d (Fig. 2*B*), and a similar result was observed 72 h after TNF- $\alpha$  addition (Fig. 2*B*). Therefore, CD83 and CD49d expression are almost simultaneously acquired by maturing MDDC, indicating that CD49d integrin expression can be considered a marker for mature MDDC.

MDDC maturation can be initiated by either proinflammatory cytokines or bacterial agents like LPS. To determine whether CD49d induction represents a general phenomenon upon DC maturation, CD49d expression was analyzed on MDDC treated with LPS. As shown in Fig. 3, CD49d integrin expression was induced upon LPS-triggered maturation, emphasizing the importance of the CD49d expression along MDDC maturation. Moreover, because CD49d integrins include the heterodimers CD49d/CD29 and



**FIGURE 1.** Phenotypic changes during TNF- $\alpha$ -induced MDDC maturation. *Left*, Cell surface expression of CD11c, CD49d, CD49e, CD29 integrins and the maturation marker CD83, as determined by indirect immunofluorescence. Immature MDDC were obtained as described in *Materials and Methods*, and either treated with TNF- $\alpha$  (mature DC) or left untreated (immature DC) for 4 days. Two independent sets of experiments were performed, of which one is shown. *Right*, Cell surface expression of CD49d on mature (TNF- $\alpha$ -treated) and immature (untreated) DC, as determined by indirect immunofluorescence with mAbs recognizing the three distinct functional epitopes on CD49d. The percentage of positive cells and MFI (between parenthesis) are shown in the upper right corner. Gray profiles indicate the fluorescence produced by an irrelevant Ab (X63).

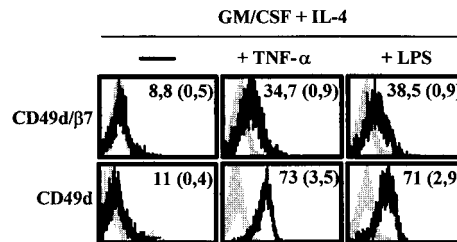


**FIGURE 2.** Kinetics of CD49d integrin acquisition during MDDC maturation. *A*, Cell surface expression of the CD49d integrins and the maturation marker CD83 along MDDC maturation, determined at the indicated time points before (monocytes, MDDC 0 h) or after (48, 72, and 96 h) TNF- $\alpha$  addition. Gray profiles indicate the fluorescence yielded by a negative control Ab (X63). The percentage of positive cells and MFI (between parenthesis) are shown. *B*, Double immunofluorescence analysis of CD49d and CD83 expression after 48 and 72 h of TNF- $\alpha$  treatment. The percentage of cells in each region is indicated. The corresponding staining profiles for each individual marker are shown in the lower panels. The experiment was performed three times on distinct donors, and a representative experiment is shown.

CD49d/ $\beta$ 7, flow cytometry experiments were performed to determine the identity of the induced molecules. CD49d/ $\beta$ 7 cell surface expression was not detected on immature MDDC, as analyzed with the CD49d/ $\beta$ 7-specific mAb Act-1 (Fig. 3). However, TNF- $\alpha$  or LPS induced the expression of CD49d/ $\beta$ 7 on mature MDDC (Fig. 3). The Act-1 epitope appeared at lower levels (Fig. 3) and with slower kinetics (data not shown) than the CD49d-specific epitopes. Therefore, while TNF- $\alpha$ - or LPS-triggered MDDC maturation induces expression of both CD49d integrins, CD49d/CD29 appears to be the predominant CD49d integrin on mature MDDC.

#### Agents blocking MDDC maturation inhibit CD49d integrin up-regulation

DC constitutively express NF- $\kappa$ B (2), and DC maturation is dependent on the functional activity of the NF- $\kappa$ B family of transcription factors (2, 28). In fact, NAC, an antioxidant agent that inhibits activation of the transcription factor NF- $\kappa$ B (29), inhibits the phenotypic and functional maturation of DC (28). To analyze whether CD49d integrin up-regulation requires NF- $\kappa$ B-mediated MDDC maturation, immature MDDC were treated with NAC before exposure to maturation-inducing agents. As shown in Fig. 4, NAC abrogated the TNF- $\alpha$ -dependent up-regulation of CD49d expression and, in agreement with previous data (28), prevented CD83 induction and CD54 up-regulation. By contrast, CD11c ex-



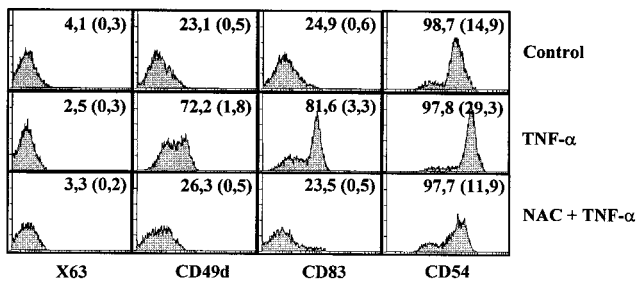
**FIGURE 3.** Acquisition of CD49d integrins during MDDC maturation. Cell surface expression of CD49d and CD49d/ $\beta$ 7 integrins determined by indirect immunofluorescence on MDDC either untreated (immature DC) or treated with LPS or TNF- $\alpha$  for 4 days (mature DC). Gray profiles indicate the fluorescence produced by an irrelevant Ab (X63). The percentage of positive cells and the MFI (between parenthesis) are indicated. The experiment was performed three times on cells from different donors, and a representative experiment is shown.

pression was unaffected by NAC treatment (data not shown). The inhibition could be observed 24 and 48 h after TNF- $\alpha$  addition, and similar results were observed upon LPS-induced MDDC maturation (data not shown). Therefore, CD49d induction appears to be dependent on the NF- $\kappa$ B activation initiated by MDDC maturation-inducing agents.

TNF- $\alpha$  stimulation of immature MDDC also initiates activation of several MAPK, including ERK2, stress-activated protein kinase/c-Jun N-terminal kinase, and p38 MAPK (30). To determine the influence of these signaling pathways on the maturation-dependent CD49d integrin up-regulation, immature MDDC were treated with TNF- $\alpha$  in the presence of either PD98059 (31), an inhibitor of mitogen-activated protein/ERK kinase 1/2 in the ERK2 activation pathway, or SB203580 (32), which specifically inhibits p38 MAPK. Inhibition of the ERK signaling pathway slightly increased the TNF- $\alpha$ -triggered up-regulation of either CD83 or CD49d (Fig. 5A). By contrast, the p38 MAPK inhibitor SB203580 reduced to one-third the percentage of cells that had acquired CD83 after treatment with TNF- $\alpha$  (Fig. 5A). While  $85 \pm 5\%$  of TNF- $\alpha$ -treated cells were CD83 $^{+}$  after 48 h (average mean fluorescence intensity (MFI) 4.53), SB203580 pretreatment reduced CD83-expressing cells to  $26 \pm 6\%$  (average MFI 0.6). Interestingly, analysis of CD49d integrin expression revealed a similar inhibitory effect: in the presence of SB203580, the percentage of CD49d $^{+}$  cells and the level of CD49d expression were comparable with those observed on immature MDDC after 48 h (Fig. 5A). As a control, Western blot analysis was performed to verify that SB203580 and PD98059 were actually inhibiting MAPK activation. In agreement with previous results (30), TNF- $\alpha$  induced ERK1/2 and p38 activation after 10 min (Fig. 5B). SB203580 reduced the phosphorylation of p38 in response to TNF- $\alpha$ , while PD98059 completely blocked the activation of ERK1/2 (Fig. 5B), thus demonstrating the activity and specificity of both inhibitors. Therefore, inhibition of p38 MAPK activity prevents the acquisition of the CD83 and CD49d maturation markers on TNF- $\alpha$ -treated MDDC, suggesting that activation of the p38 MAPK pathway is required for MDDC to complete their phenotypic maturation after stimulation by inflammatory agents.

#### Functional activity of CD49d integrins on MDDC

The CD49d/CD29 integrin mediates cell-extracellular matrix and cell-cell interactions via recognition of the CS-1 fragment of FN and the cell surface molecule VCAM-1, respectively (33, 34). To find out whether the increased expression of CD49d conveyed mature MDDC with an increased adhesive capacity, adhesion assays



**FIGURE 4.** CD49d integrin acquisition requires NF- $\kappa$ B-dependent MDDC maturation. CD49d, CD83, and CD54 expression was determined by indirect immunofluorescence on MDDC either untreated (control) or treated with TNF- $\alpha$  for 48 h in the absence (TNF- $\alpha$ ) or in the presence of the NF- $\kappa$ B inhibitor NAC (NAC + TNF- $\alpha$ ). The percentage of positive cells and MFI (between parenthesis) are indicated. A representative experiment, of three, is shown.

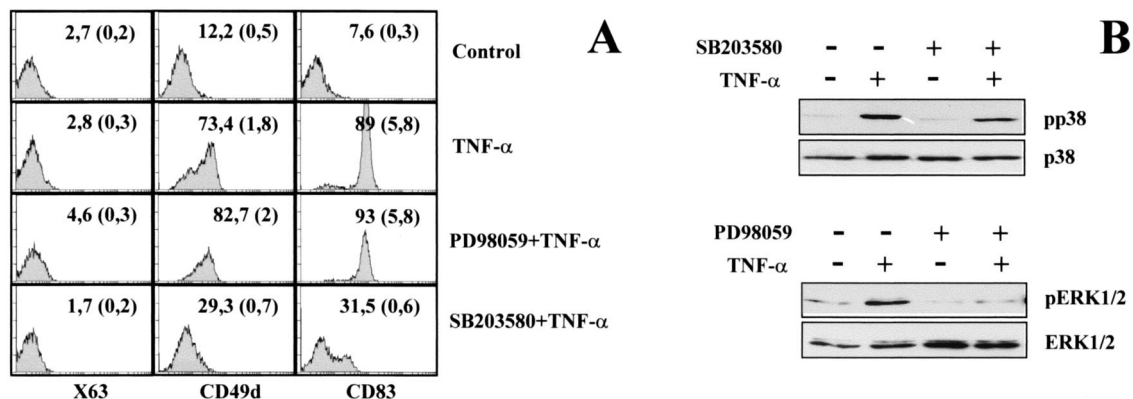
were performed with immature and mature MDDC on the fibronectin FN-H89 fragment, which includes the CS-1 region and does not contain the RGD motif recognized by the CD49e/CD29 integrin (34). Immature MDDC exhibited a very low adhesion to FN-H89, while mature MDDC exhibited adhesion levels that were 10-fold higher than immature MDDC (Fig. 6A). MDDC adhesion to FN-H89 was reduced to 30–40% in the presence of Abs against CD49d or function-blocking anti-CD29 Lia1/2, indicating that the interaction is specifically mediated by CD49d/CD29 and that mature MDDC express functionally active CD49d/CD29 molecules on the cell surface (Fig. 6A). Accordingly, treatment of mature MDDC with the activating anti-CD29 TS2/16 mAb did not increase adhesion significantly (Fig. 6A), further indicating that CD49d integrins are constitutively active on mature MDDC. In accordance with flow cytometry data, additional experiments revealed that an anti-CD49d/ $\beta$ 7 Ab (Act-1) inhibited the adhesion of mature MDDC to FN-H89 (Fig. 6B). The involvement of CD49d/ $\beta$ 7 in MDDC adhesion to FN-H89 might explain the distinct inhibitory effect of CD29- and CD49d-specific Abs (Fig. 6, A and B), and indicates that MDDC attachment to the FN-H89 is mediated by both CD49d-containing integrins. When MDDC were assayed for adhesion to intact FN, it became evident that both

immature and mature MDDC bound to FN in a CD49e- and CD29-dependent manner (Fig. 6, C and D). In fact, comparison of immature and mature MDDC binding to either FN or FN-H89 revealed that immature MDDC exhibited higher binding ability to FN than mature cells (Fig. 6, C and D), while only mature MDDC bound significantly to FN-H89 (Fig. 6C). Therefore, the increase in binding to FN-H89 is not due to an overall augmented adhesiveness of mature MDDC and appears to reflect the augmented expression of CD49d integrins.

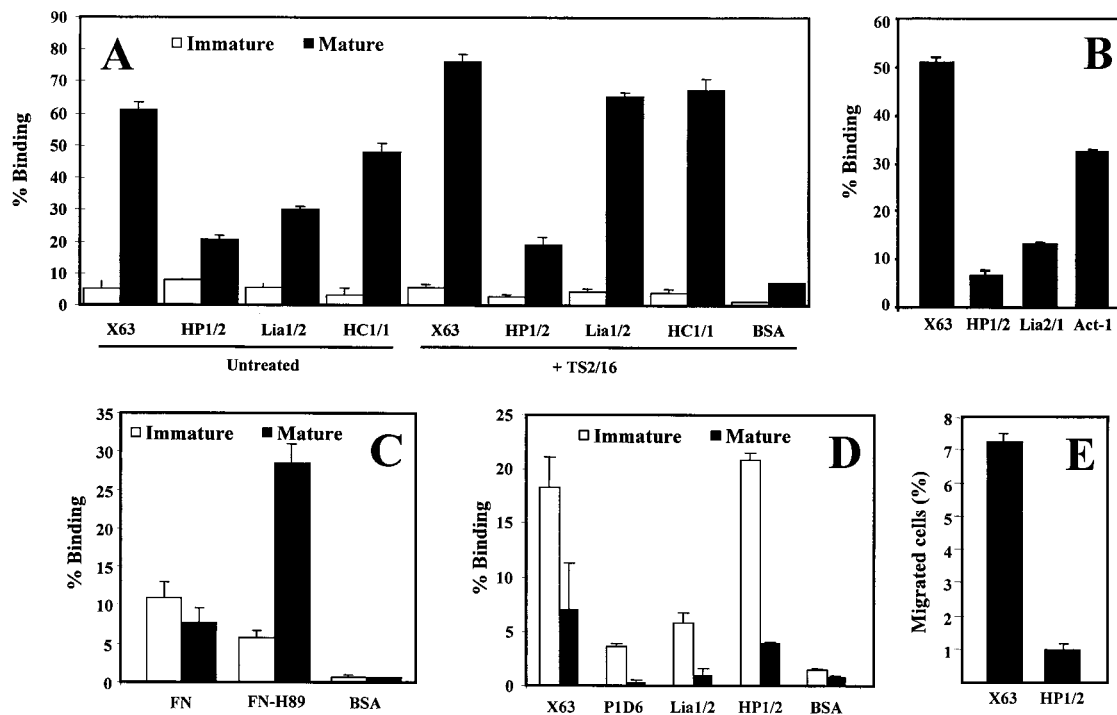
In addition to the adhesive function, the involvement of the newly synthesized CD49d integrins in the MDDC migratory capability was evaluated in a transendothelial migration assay. As shown in Fig. 6E, mature MDDC seeded onto TNF- $\alpha$ -stimulated endothelial cells significantly migrated into the SDF-1 $\alpha$ -containing lower chamber. More importantly, MDDC transendothelial migration was greatly reduced in the presence of blocking anti-CD49d mAbs (Fig. 6E), thus demonstrating that CD49d integrins on mature MDDC mediate both FN attachment and transendothelial migration.

#### Steady-state levels of CD49d and $\beta$ 7 integrins during MDDC maturation

Changes in the level of expression of CD49d integrins have been previously described in differentiating cells of distinct lineages (22, 23). In differentiating myeloid leukemic cells, CD49d/CD29 is dramatically down-regulated as a consequence of a considerable decrease in the steady-state levels of CD49d mRNA (22). In the case of DC, and because immature cells have extraordinarily high levels of endocytic activity (35, 36), changes in integrin expression could be due to molecule recycling and/or internalization. To analyze the molecular basis for the differential expression of CD49d integrins in immature vs mature MDDC, the steady-state levels of CD49d and  $\beta$ 7 mRNA were determined by Northern blot. As shown in Fig. 7A, no hybridization could be detected in RNA from immature cells, while CD49d mRNA species were readily detected in mature MDDC. Similarly,  $\beta$ 7 mRNA was only detected in mature MDDC, while GAPDH mRNA levels were similar in both cell populations (Fig. 7A). As a control, and in agreement with our previous results (23),  $\beta$ 7 mRNA was induced and CD49d mRNA was down-regulated during U937 cell differentiation. To evaluate



**FIGURE 5.** Effect of MAPK inhibitors on the acquisition of CD49d integrins and CD83 along MDDC maturation. *A*, CD49d and CD83 expression was determined by indirect immunofluorescence on MDDC either untreated (control) or treated with TNF- $\alpha$  for 48 h in the absence (TNF- $\alpha$ ) or in the presence of the mitogen-activated protein/ERK kinase 1/2 inhibitor PD98059 (PD98059 + TNF- $\alpha$ ) or the p38 MAPK inhibitor SB203580 (SB203580 + TNF- $\alpha$ ). The percentage of positive cells and the MFI (between parenthesis) are shown in the *right-hand corner*. Six independent experiments were performed and a representative one is shown. *B*, Determination of the levels of phosphorylated (activated) forms of p38 MAPK and ERK1/2 by Western blot. Immature MDDC were incubated in the presence of the indicated inhibitors (SB203580, *upper panel*; PD98059, *lower panel*) for 1 h and either left untreated or treated with TNF- $\alpha$  for 10 min. Levels of activated (pp38, *upper panel*; pERK1/2, *lower panel*) were determined by Western blot using specific polyclonal Abs, and sample loading was controlled using Abs recognizing total p38 (*upper panel*) or ERK1/2 (*lower panel*).



**FIGURE 6.** Functional activity of CD49d integrins on MDDC. *A*, Adhesion of immature (□) and mature MDDC (■) to the FN-H89 fibronectin peptide. Adhesion was performed in the presence or absence of an anti-CD29-activating Ab (TS2/16) and included blocking Abs against CD49d (HP1/2), CD29 (Lia1/2), CD11c (HC1/1), or an irrelevant Ab (X63). *B*, Adhesion of mature MDDC to FN-H89 in the presence of Abs against CD49d (HP1/2), CD29 (Lia1/2), CD49d/ $\beta$ 7 (Act-1), or X63. *C*, Adhesion of immature (□) and mature MDDC (■) to FN, the FN-H89 fibronectin peptide (FN-H89), and BSA. *D*, Adhesion of immature (□) and mature MDDC (■) to FN in the presence of blocking Abs against CD49d (HP1/2), CD29 (Lia1/2), CD49e (P1D6), or control X63. *E*, Migration of mature MDDC through TNF- $\alpha$ -treated endothelial cell monolayers, in the presence of Abs against CD49d (HP1/2) or an irrelevant Ab (X63). Each experiment was performed at least three times, and representative experiments are shown.

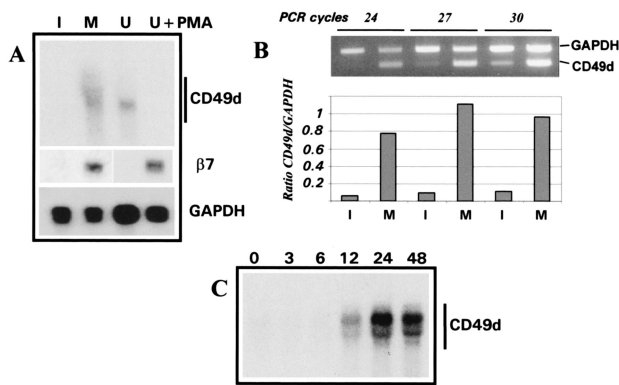
the difference between the CD49d mRNA levels in immature and mature MDDC, relative RT-PCR was performed. Amplification of CD49d mRNA, using oligonucleotides encoded by distinct exons, and control amplification (GAPDH) were accomplished simultaneously on the same sample and analyzed after 24, 27, and 30 cycles. After normalizing according to the GAPDH amplification, mature cells exhibited CD49d mRNA levels, which were at least 10 times higher than those detected in immature MDDC (Fig. 7*B*). The dramatic up-regulation of the CD49d mRNA was further analyzed by determining its steady-state levels along TNF- $\alpha$ -initiated MDDC maturation. As shown in Fig. 7*C*, kinetic analysis indicated that CD49d mRNA increase becomes evident 12 h after TNF- $\alpha$  treatment, reaches a maximum after 24 h, and remains at high levels up to 48 h after cytokine addition. Altogether, these results demonstrate that CD49d integrin membrane expression is strongly up-regulated as a consequence of a dramatic increase in the steady-state levels of CD49d and  $\beta$ 7 mRNA during MDDC maturation.

## Discussion

Once exposed to inflammatory mediators or bacterial products, DC undergo functional maturation and reenter the circulatory system to home to the T cell areas of the draining lymphoid organs. As a consequence, DC maturation and migration take place concomitantly and influence each other. In the present manuscript, we describe the induction of functional CD49d integrins upon MDDC maturation, which reflects an increase in CD49d and  $\beta$ 7 mRNA steady-state levels, and enables mature MDDC to interact with specific domains within the FN molecule. Thus, CD49d integrins can be considered as maturation markers on MDDC. In fact, and like CD83, CD49d induction is dependent on MDDC maturation

and can be prevented with agents inhibiting either NF- $\kappa$ B activation or the p38 MAPK signaling pathway. The relevance of the phenotypic changes that we have noted during MDDC maturation is supported by previous results indicating that CD49d integrins are up-regulated on murine Langerhans cells upon *in vivo* activation (37).

The expression of the CD49d integrin subunit is subjected to a tight control in leukocytes and other cell types. In muscle cells, CD49d/CD29 integrin is induced during myotube formation, and it is exclusively expressed at sites of secondary myogenesis *in vivo* (22). CD49d is down-regulated upon T cell alloantigen or superantigen recognition, after CD43-induced T lymphocyte homotypic aggregation (38), during monocyte activation (26), myeloid cell differentiation (23), and upon cytokine treatment of bone marrow cells (39). In the last two cases, a specific down-regulation of CD49d mRNA was reported, indicating that the loss of CD49d/CD29 cell surface expression reflected the steady-state level of the CD49d mRNA. By contrast, our findings demonstrate that CD49d mRNA is greatly up-regulated during MDDC maturation, with CD49d mRNA levels being at least 10 times higher in mature MDDC than in their immature counterparts. Although leukocyte CD49d expression appears to be mainly regulated at the transcriptional level (40), the possibility that CD49d mRNA levels are also posttranscriptionally regulated during MDDC maturation cannot be ruled out because 1) the 3' untranslated region of the CD49d mRNA contains several AU-rich elements (23, 41) that might be targets of signal-induced mRNA stabilization; and 2) p38 MAPK, whose activity is involved in MDDC maturation and CD49d up-regulation, is known to contribute to mRNA stabilization through an AU-rich element-targeted mechanism (42).



**FIGURE 7.** Changes in CD49d and  $\beta 7$  mRNA steady-state levels along MDDC maturation. **A**, Determination of the steady-state levels of CD49d,  $\beta 7$ , and GAPDH mRNA in immature MDDC (I), mature MDDC (M), untreated U937 cells (U), and PMA-differentiated U937 cells (U + PMA). **B**, Relative levels of CD49d and GAPDH mRNA in immature (I) and mature (M) MDDC by relative PCR. Analysis was performed after the indicated PCR cycles, and the ratio CD49d:GAPDH was determined after densitometric measurement of the PCR results. The experiment was performed on two independent donors with identical results, and one of them is shown. **C**, Immature MDDC were treated with TNF- $\alpha$  for the indicated periods of time, cells were lysed, total RNA was extracted, and CD49d mRNA levels were determined by Northern blot.

In agreement with its induction in terminally differentiated myeloid cell lines (23),  $\beta 7$  integrin mRNA is also induced upon MDDC maturation. Based on the capacity of CD49d/ $\beta 7$  to mediate mature MDDC binding to fibronectin FN-H89 (Fig. 6), CD49d/ $\beta 7$  expression might also potentiate MDDC migratory function by conferring an improved ability to roll on and attach to endothelial cells (15, 16). In addition,  $\beta 7$  integrin expression has recently been found to be up-regulated on apoptotic lymphocytes (43). Because DC undergo apoptosis at the final stages of their maturation, it is therefore possible that  $\beta 7$  expression on mature MDDC might mark DC initiating their apoptotic program.

The phenotypic and functional differentiation/maturation of DC is dependent on the activity of the NF- $\kappa$ B family of transcription factors, and especially RelB (2, 44). In this regard, two well-established NF- $\kappa$ B activators such as TNF- $\alpha$  and LPS (45) trigger MDDC maturation and, subsequently, promote induction/up-regulation of CD49d integrins. The influence of NF- $\kappa$ B activation on CD49d integrin expression on DC can also be inferred from the ability of NAC, which abrogates MDDC maturation, to inhibit CD49d integrin induction. However, CD49d mRNA up-regulation/induction is only observed 12 h after TNF- $\alpha$  treatment, making unlikely a direct effect of NF- $\kappa$ B factors on the regulatory regions of the CD49d gene. Therefore, the inhibitory effect of NAC on the CD49d integrin up-regulation does not imply a direct effect of NF- $\kappa$ B factors on CD49d gene transcription, and most probably reflects the overall NF- $\kappa$ B dependency of the MDDC maturation.

TNF- $\alpha$  stimulation of MDDC activates several MAPK, including ERK2, c-Jun N-terminal kinase, and p38 MAPK (30). Inhibition of different signaling routes during TNF- $\alpha$ -induced maturation has revealed that both NF- $\kappa$ B and p38 MAPK are involved in the induction of the CD83 maturation marker, and in the up-regulation of CD49d integrins and CD54 (Figs. 4 and 5). Like NF- $\kappa$ B, the p38 MAPK signaling pathway appears to be required for MDDC to acquire their complete array of cell surface maturation markers in response to TNF- $\alpha$ , including adhesion (CD49d, CD54) and costimulatory (CD80, CD40) molecules. In this sense, recent reports

have also implicated p38 MAPK in the functional maturation of DC because 1) DC from *Mkk3*<sup>-/-</sup> mice are defective in IL-12 production (46); 2) p38 MAPK appears to be constitutively activated in mature murine DC (47); and 3) CpG-DNA- and CD40-specific activation of DC is strongly blocked by SB203580 (48, 49). Therefore, CD49d integrin up-regulation is a maturation-specific parameter specifically affected by p38 MAPK in DC. Whether p38 MAPK affects CD49d expression by transcriptional or post-transcriptional mechanisms is currently under investigation, as is the search of additional MDDC markers and functional activities that might be regulated by p38 MAPK activation.

In summary, based on the induction of CD49d by TNF- $\alpha$  and LPS, and the inhibitory effect of maturation-interfering agents (NAC, SB203580) on CD49d up-regulated expression, our results support the existence of a link between the phenotypic maturation of MDDC and the acquisition of CD49d integrin expression, thus establishing CD49d integrin expression as a maturation marker for MDDC.

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