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Apoptosis in the immune system: 1. Fas-induced apoptosis in monocytes-derived human dendritic cells

Roxana Nat *, E. Radu, T. Regalia, L. M. Popescu

Division of Cellular and Molecular Medicine
"Carol Davila" University of Medicine and Pharmacy, Bucharest, Romania

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Abstract

Dendritic cells (DC) are cells of the hematopoietic system specialized in capturing antigens and initiating T cell-mediated immune responses. We show here that human DC generated from adherent peripheral blood mononuclear cells (PBMC) after *in vitro* stimulation with granulocyte macrophage colony stimulating factor (GM-CSF) and interleukin-4 (IL-4) express Fas antigen (APO-1, CD95) and can undergo apoptosis upon triggering of Fas by monoclonal antibodies. Immature monocytes-derived dendritic cells (MDDC) upregulate CD86 and HLA-DR expression and develop dendrites and veiled processes. Flow cytometry analysis revealed CD95 expression in approx. 40% of these MDDC and incubation with anti-CD95 mAb (0.5μg/ml) induced apoptosis when compared to untreated controls. The extent of apoptosis induced by the agonist anti-Fas antibody strongly related to the percentage of cells expressing CD 95. Upon tumor necrosis factor α (TNF-α) additional stimulation, MDDC assumed a characteristic mature dendritic cells morphology showing prolonged veils, CD83 expression, and high levels of HLA-DR. These cells have downregulated their Fas receptors (to approx. 20%) and undergo apoptosis to a lesser extent when treated with anti-CD 95, as demonstrated by the hardly noticeable effect of this antibody on the viability of cultured cells as compared to controls. Thus, upon TNF-α induced maturation, MDDC became resistant to Fas-induced apoptosis. The apoptotic episodes surrounding the earlier stage of DC differentiation appeared to be mediated by Fas. In contrast, a Fas independent pathway is probably responsible for the apoptotic events associated with terminally differentiated DC.

Keywords: apoptosis - Fas (CD95/Apo1) - human dendritic cells - monocytes

Introduction

Apoptosis, or programmed cell death, plays a critical role in the immune system, both during the cells development in primary lymphoid organs as well as during immune responses of mature cells [1-3].

Dendritic cells (DC) are the most important antigen presenting cells of the immune system, specialized in capturing antigens and initiating T cell-mediated immune responses [4-6]. In view of the restricted number of recirculating DC or CD34+ stem cells, several studies have focused on the possibility to recruit DC *in vitro* from cord blood stem cells or other precursors in blood [6, 7]. It has been shown that characteristic DC could

* Correspondence to: Roxana NAT, MD
Division of Cellular and Molecular Medicine, "Carol Davila" University of Medicine and Pharmacy, Bucharest, Romania.
Fax: +40-21-312 7721
E-mail: rnat@univermed-cdgm.ro

be induced from human blood monocytes (monocytes derived DC - MDDC) cultured with granulocyte macrophage colony stimulating factor (GM-CSF) and interleukin-4 (IL-4) [8] and maturated with tumor necrosis factor α (TNF-α) [9].

In the present paper, we aimed to study apoptosis in dendritic cells (DC), since the understanding of all aspects of DC immunobiology, including regulation of their death, is crucial for further development of effective DC-based immunotherapies [10-12]. MDDC are readily obtained and represent an appropriate system for apoptosis studies, with both practical and theoretical importance.

Apoptosis induced by cell surface receptors activation or by cytokines/growth factors withdrawal is thought to play a pivotal role in the immune system, and was intensively studied in both T and B cell homeostasis and lymphocyte-mediated cytotoxicity [13, 14].

Certain cytokines of the TNF family and their cognate receptors, including TNF/TNF-R1 and FasL/Fas (CD95/APO1) are classic triggers of the suicide response [15-17].

Fas (APO-1, CD95) is one of the main signaling systems with the specialized function of inducing apoptosis [18-26]. Fas is a cell surface receptor that on activation (cross linkage) by its natural ligand (FasL) or by an agonistic antibody initiates a signaling cascade that leads to apoptosis [27, 35].

Apoptotic events, occurring before or after terminal maturation, play a role also in supporting DC lineage selection and homeostasis. There are recent studies regarding DC apoptosis in various systems, associated with different phases of DC development. Thus, regulated programmed cell death during DC generation from progenitors likely represents a biological homeostasis mechanism, by preventing uncontrolled proliferation and renewal of progenitor/stem [28].

Apoptosis is also associated with elimination of mature, terminally differentiated DC [29]. The spontaneous death of DC in cultures and its regulation by cytokines and growth factors has been evaluated intensively. For instance, it has been shown that the spontaneous decrease of human Langerhans cells (LC) viability during culture is due to apoptosis [30]. GM-CSF, TNFα, and CD40L have been reported to promote DC survival and induce DC differentiation [31-33]. In long-term DC cultures it was demonstrated that growth factor deprivation led to DC growth arrest and cell death [34].

Other *in vitro* experiments suggest that whereas putative immature DC that have not interacted yet with T cells are more prone to apoptosis *via* Fas, DC that have seen the CD40-ligand of T cells become resistant [33].

In our study we investigate the modulator effect of Fas on cultured human monocytes derived dendritic cells. In our system we prove the expression of Fas receptors on immature and mature DC, in different proportions, and the functional role of Fas in inducing apoptosis in immature DC.

Materials and methods

Reagents and antibodies

Endotoxin tested (< 0.1 ng/ml) Dulbecco modified Eagle's medium (DMEM), low endotoxin (< 20 EU/ml) fetal calf serum (FCS), L-glutamine, penicilline-streptomycine, human recombinant granulocyte and macrophage colony stimulating factor (GM-CSF) and Interleukin-4 (IL-4) were obtained from Sigma (St. Louis, MO, USA).

Monoclonal antibodies anti-CD14 PE, anti-HLA-DR PerCP, anti-CD11c PE, anti-CD86 PE, anti-CD83 FITC, anti-CD95 FITC, as well as lin1 FITC (a cocktail of mAbs to CD3, CD14, CD16, CD19, CD20 and CD56) were obtained from BD (San Jose, CA, USA), together with the appropriate control antibodies. Azide-free anti Human Fas (CD95) clone 2R2 antibody was purchased from Boehringer Mannheim (Mannheim, Germany).

Human recombinant TNFα, ApoAlert Caspase-3 Inhibitor, DEVD-fmk (1mM), ApoAlert Caspase-8 Inhibitor, IETD-fmk (1mM) were obtained from BD Clonetech (Palo Alto, CA, USA), Annexin V FITC Apoptosis Detection Kit 1 and APO BRDU Kit (FITC) from BD Pharmingen (San Diego, CA, USA).

Generation of DC from peripheral blood monocytes

Immature DC were generated from human peripheral blood mononuclear cells according to published methods [8]. Briefly, peripheral EDTA-blood samples (20-50ml) obtained from healthy donors (according to the blood transfusion guidelines for human subjects)

were fractionated by gradient density centrifugation (15 min at 1000g) using Sepcel (Babes Institute, Bucharest, Romania). The peripheral blood mononuclear cells (PBMC) fraction was recovered from the interface and repeatedly washed in medium. PBMC were plated on tissue culture plates (Costar, Corning, New York, NY, USA) in DMEM supplemented with 10 % FCS, 1% L-glutamine and 1% antibiotics (penicillin/streptomycin) at 37°C at a density of $2x10^6$/ml for 2 hours. After 5 washes with PBS, the nonadherent cells were removed and adherent cells were cultured in DMEM complete medium containing GM-CSF (50ng/ml) and IL-4 (20ng/ml). The culture medium was changed every other day.

Induction of DC maturation

After 7 days, immature DC were additionally exposed to TNF-α (50pg/ml) to induce maturation, as previously described [9]. Cell cultures were analyzed after a 48 h period of further incubation.

Cell phenotype and morphology

Expression of CD 14, CD 11c, CD 83, CD 86 , CD95 and lineage markers CD 3, CD14, CD 16, CD19, CD20, CD56 was analyzed using specific FITC, PE or PerCP conjugated monoclonal antibodies by flow cytometry, in culture days 0, 3, 5, 7 and 9.

Briefly, aliquots of cultured cells (approx.10^5 cells) were suspended in PBS and stained for 30 min. with specific monoclonal antibodies (10 µl). Cells were then washed and fixed with paraformaldehyde 1%, and analyzed by three-color flow cytometry using a FACSVantage SE cytometer and CellQuest software (BD, San Jose, CA, USA).

Cells were identified on the basis of their relative size (FSC) and granularity (SSC), and cellular markers expression detected using fluorochrome-conjugated monoclonal antibodies. Results were expressed for triplicate assays as percentages of cells expressing a marker out of a gated population. For some markers, mean fluorescence intensity was also calculated.

To study their morphology, cultured cells were observed in phase-contrast under a Nikon Eclipse TE 300 microscope.

Induction of Apoptosis using Anti-human Fas mAbs

Apoptosis in both immature (culture day 7) and mature (culture day 9, after TNF-α treatment) dendritic cells was induced using anti-Fas agonist-like antibodies (0.5µg/ml in culture medium) for 3 hours at 37°C, in presence or absence of caspase-8 inhibitor IETD-fmk (10µl/ml) or caspase-3 inhibitor DEVD-fmk (10µl/ml). Untreated cells were used as controls.

Apoptosis detection

Apoptosis and cell viability were quantified using TUNEL (terminal deoxyinucleotidyl transferase mediated dUTP nick-end labeling) assay for FACS and Annexin V FITC / propidium iodide (PI) staining for fluorescence microscopy and FACS. Apoptotic cells, due to membrane phospholipid phosphatidylserine (PS) translocation to the outer leaflet of the plasma membrane, bind Annexin V, which can identify apoptosis at an earlier stage than assays based on nuclear changes such as DNA fragmentation. Therefore, staining with Annexin V in conjunction PI with using Annexin V FITC apoptosis detection kit I, allows identifying early apoptotic cells (Annexin V-positive, PI-negative) and late apoptotic or necrotic cells (Annexin V-positive, PI-positive).

DNA fragmentation in apoptotic cells has been revealed using APO-BRDU Kit for flow cytometry - a two color staining method for labeling DNA breaks by TdT-mediated BrdU incorporation.

Samples were prepared according to manufacturer instructions.

Statistical analysis

FACS results were analyzed using BD's CellQuest software. By fluorescence microscopy 200 cells were counted for each samples and each category expressed as percentage. Assays were performed in triplicates, and results were computed as mean values ± standard deviation.

Results

Establishing DC cultures from human monocytes

Immature DC were propagated from cultured adherent human monocytes in the presence of GM-CSF and IL-4. 7 days after, DC were induced to maturate using TNFα.

In cultures of adherent human PBMC, most cells formed clusters within 1-2 days of GM-CSF and IL-4 stimulation, which increased in size with time. After 3 to 4 days of stimulation the majority of the cells was pleiomorphic with regard to the cell form and size and displayed hairy, dendritic and veiled surface projections of variable length and number.

After 7 days of stimulation, the number of free and loosely attached cells with dendritic morphology had increased (fig 1a). The size of

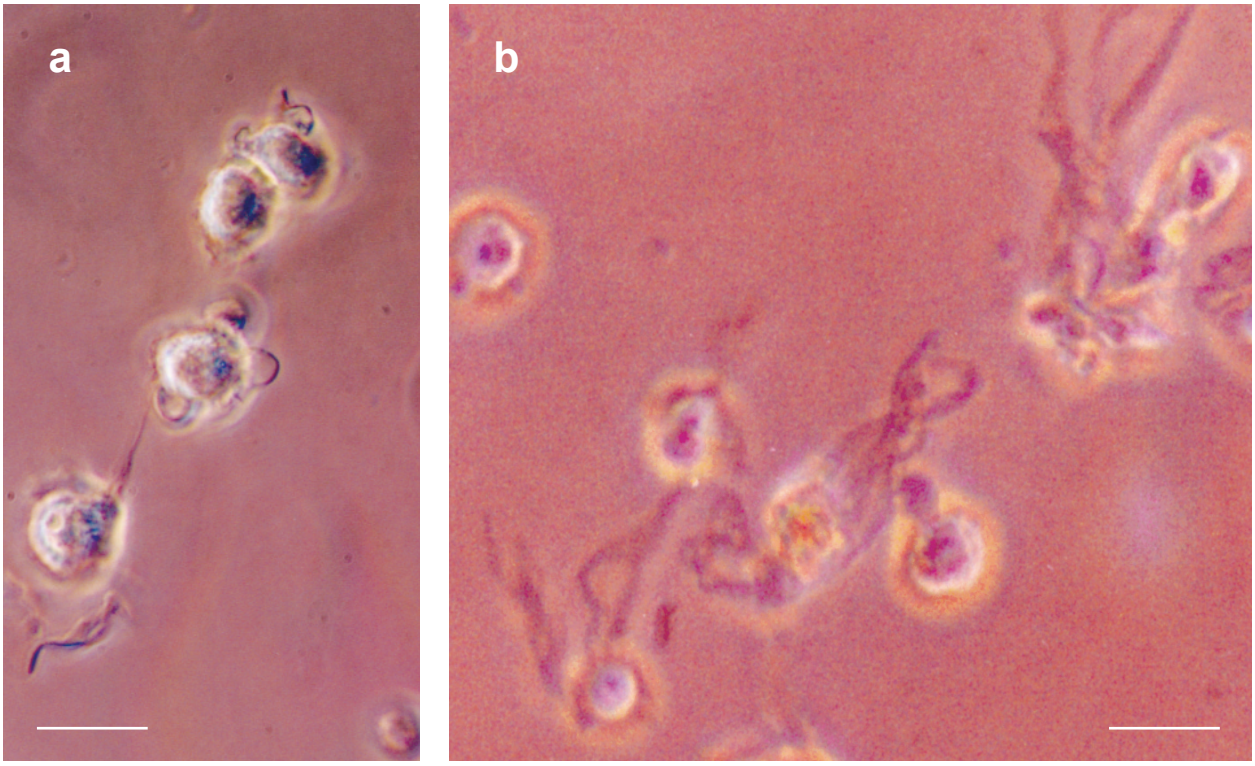


Fig. 1 In vitro human immature and mature monocytes-derived dendritic cells. Immature DC were propagated from adherent human monocytes in the presence of GM-CSF and IL-4. At culture day 7, cells are loosely attached and display dendritic morphology (fig 1a). 48 hours after additional TNF-α stimulation, mature DC appear larger, with very long processes (fig 1b). Phase-contrast microscopy, Nikon Eclipse TE 300. Bars indicate 20 μm.

most cells was also clearly increased at day 7 compared to day 3, in contrast phase microscopy and flow cytometry analysis.

After 48 hours of additional TNFα stimulation, cultured cells appeared larger and with very long processes (fig 1b).

MDDC phenotypes and morphologies

The phenotypes of adherent mononuclear cells (culture day 0), immature DC (culture days 3, 5, 7) and mature DC (culture day 9) were presented in Table 1.

Table 1. Phenotypes of adherent mononuclear cells (culture day 0), immature monocytes derived dendritic cells (MDDC) (culture days 3, 5, 7) and mature MDDC (culture day 9). Flow cytometry analysis results were expressed as percentages out of total cells (for CD14) or lin- cells (for HLA-DR, CD11c, CD 86 and CD 83) and are computed from values obtained in triplicate experiments.

	Monocytes	Immature DC (GM-CSF and IL4 stimulation)			Mature DC (TNFα stimulation)
Days	0	3	5	7	9
CD14	82.4±9.3	42.3±6.1	22.1±2.4	20.3±7.1	10±3
HLA-DR	65.0±8.4	68.4±5.9	72.6±4.5	90.5±9.1	86±10
CD11c	50.3±6.1	60.3±5.4	78.0±4.7	94.4±3.9	76.1±5.2
CD86	10.2±2.0	13.6±5.2	22.4±6.3	77.8±8.3	70.2±5.2
CD83	-	2.1±1.3	4.3±3.6	7.3±2.5	70.3±4.5

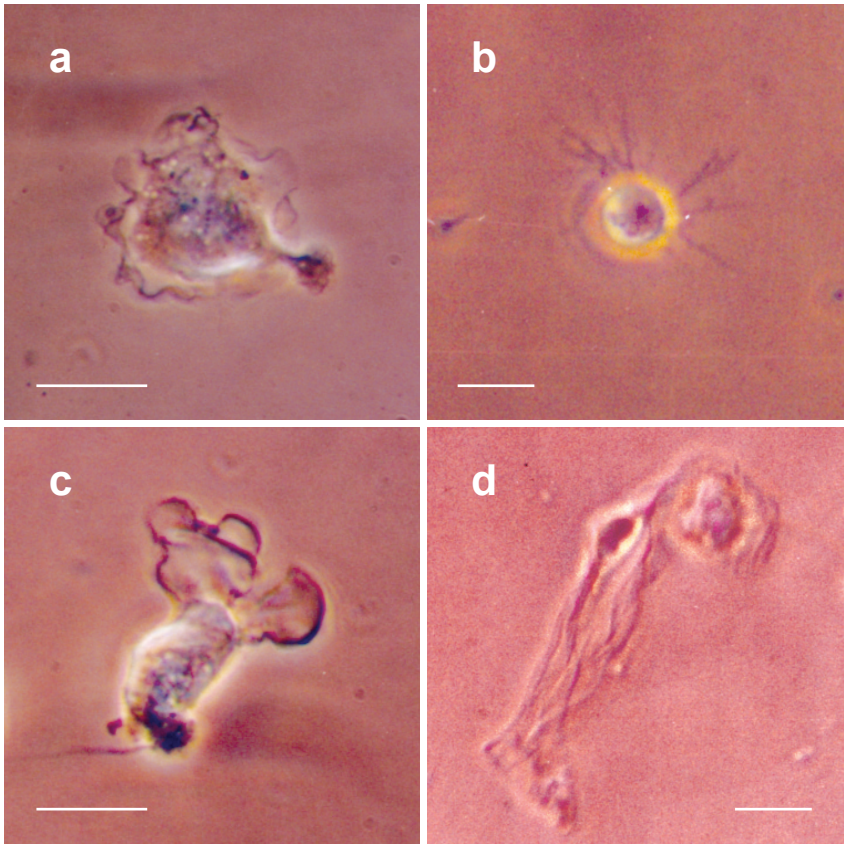


Fig. 2 Various morphologies of *in vitro* generated live DC at culture days 7 (a, b ,c) and 9 (d). Human immature DC display typical veiled appearance (a, b), and dendritic sticking out from the cell body (c). Upon TNFα stimulation, long tails were revealed by phase-contrast microscopy (d). Phase-contrast microscopy, Nikon Eclipse TE 300. Bars indicate 20 μm.

Freshly plated adherent mononuclear cells (culture day 0) expressed in high percentage (more than 85 %) the CD14 antigen and also more than 60% were HLA-DR positive with a mean fluorescence channel (MFC) value of 671±37 on a 1024 channels logarithmic scale. CD86 was only weakly expressed on 10% of the adherent nonstimulated cells at day 0 (mean fluorescence channel 469±30).

Immature, GM-CSF and IL-4 stimulated MDDC grew larger than unstimulated monocytes, and 50% of them continued to express CD14. In stimulated cultures CD86 expression also increased (up to 77%), as compared to cells from culture day 3 (<20%). Almost no CD83 antigen expression could be detected at day 7. HLA-DR expression increased in the population to about 91% and was more intense (MFC 820±52).

The morphology of human immature DC displays typical veiled appearance, with protrusions and dendritic sticking out from the cell body (fig. 2a, b, c).

Upon TNFα stimulation, by culture day 9, an important increase in CD83 expression occurred, while CD14 expression was barely detectable, a phenotype consistent with mature DC. Long tails were revealed by phase-contrast microscopy (fig 2d).

Fig. 3 shows dot-plot representations of relative

Table 2. Fas expression in monocytes (day 0) immature monocyte-derived dendritic cells (MDDC) (days 3-7) and mature MDDC (day 9). Flow cytometry analysis results were expressed as percentages out of lin- cells and are derived from values obtained in triplicate experiments.

Culture day	**0**	**3**	**5**	**7**	**9**
Fas (%)	8.5 ± 2.7	18 ± 5,8	24 ± 7	34.3 ± 9.5	14.6 ± 3.1

Table 3 Percentages of Fas-induced apoptosis in immature and mature MDDC, measured by both annexin V and TUNEL assays are listed in comparison to uninduced apoptosis and to apoptosis partially inhibited by caspases selective inhibitors. Values are expressed as mean ± standard deviation of triplicate experiments.

	Fas-induced apoptosis with anti-Fas mAb		**Caspase 8 inhibitor + anti Fas mAb**		**Caspase 3 Inhibitor + anti Fas mAb**		**Control**	
	Annexin V	TUNEL	Annexin V	TUNEL	Annexin V	TUNEL	Annexin V	TUNEL
Immature MDDC (day7)	38.4±4.0	42.5±3.9	17.9±4.1	18.5±3.1	16.6±1.7	18.1±2.9	12.2±4.4	15.3±2.0
Mature MDDC (day 9, TNFα stimulation)	10.8±4.4	14.3±1.2	8.5±5.2	11.8±7.4	6.8±1.3	7.4±3.2	9.6±4.7	11.5±3.1

size and structural complexity of cells, as well as dendritic lineage markers expression, relevant for MDDC in our experiments.

Fas expression

As shown in Table 2, at the initiation of cultures, mononuclear adherent cells expressed low levels (8.5 ± 2.7%) of CD95; a gradual increase was noticed thereafter, peaking in culture day 7 immature MDDC (34.3 ± 9.5%). Mature MDDC showed significantly decreased Fas expression (14.6 ± 3%).

DC undergo apoptosis upon Fas ligation

The level of Fas-induced apoptosis in immature and mature MDDC, measured by flow cytometry for both annexin V and TUNEL assays is showed in comparison to uninduced apoptosis and to apoptosis partially inhibited by caspase selective inhibitors (Table 3). Significant dot -plots and histograms are showed in fig 4. Similar results were obtain by fluorescence microscopy using Annexin V assay (Table 4 and Fig. 5).

When apoptosis was triggered with Fas agonist-like antibodies, the highest levels of cell death were found as expected within day 7 immature MDDC. Mature MDDC also underwent apoptosis, but at levels comparable with untreated controls.

Caspase-8 inhibitor IETD-fmk markedly antagonized CD95-induced apoptosis in culture day 7 MDDC, while its effect on TNFα matured MDDC was barely noticeable. Inhibition of apoptosis was strongly observed when using caspase-3 inhibitor, DEVD-fmk in both immature and mature cells; caspase 3 inhibitor blocks both Fas induced apoptosis and Fas independent

Table 4 Percentages of Fas-induced apoptosis in immature MDDC, measured by annexin V assay are shown in comparison to uninduced apoptosis and to apoptosis partially inhibited by caspases selective inhibitors. Fluorescence microscopy data are expressed as mean ± standard deviation of triplicate experiments.

	Fas-induced apoptosis with anti-Fas mAb	**Caspase 8 inhibitor + anti Fas mAb**	**Caspase 3 Inhibitor + anti Fas mAb**	**Control**
Immature MDDC (day7)	30±4	15±2	11±2	13±3
Mature MDDC (day 9, TNFα stimulation)	17±2	16±4	9±3	15±5

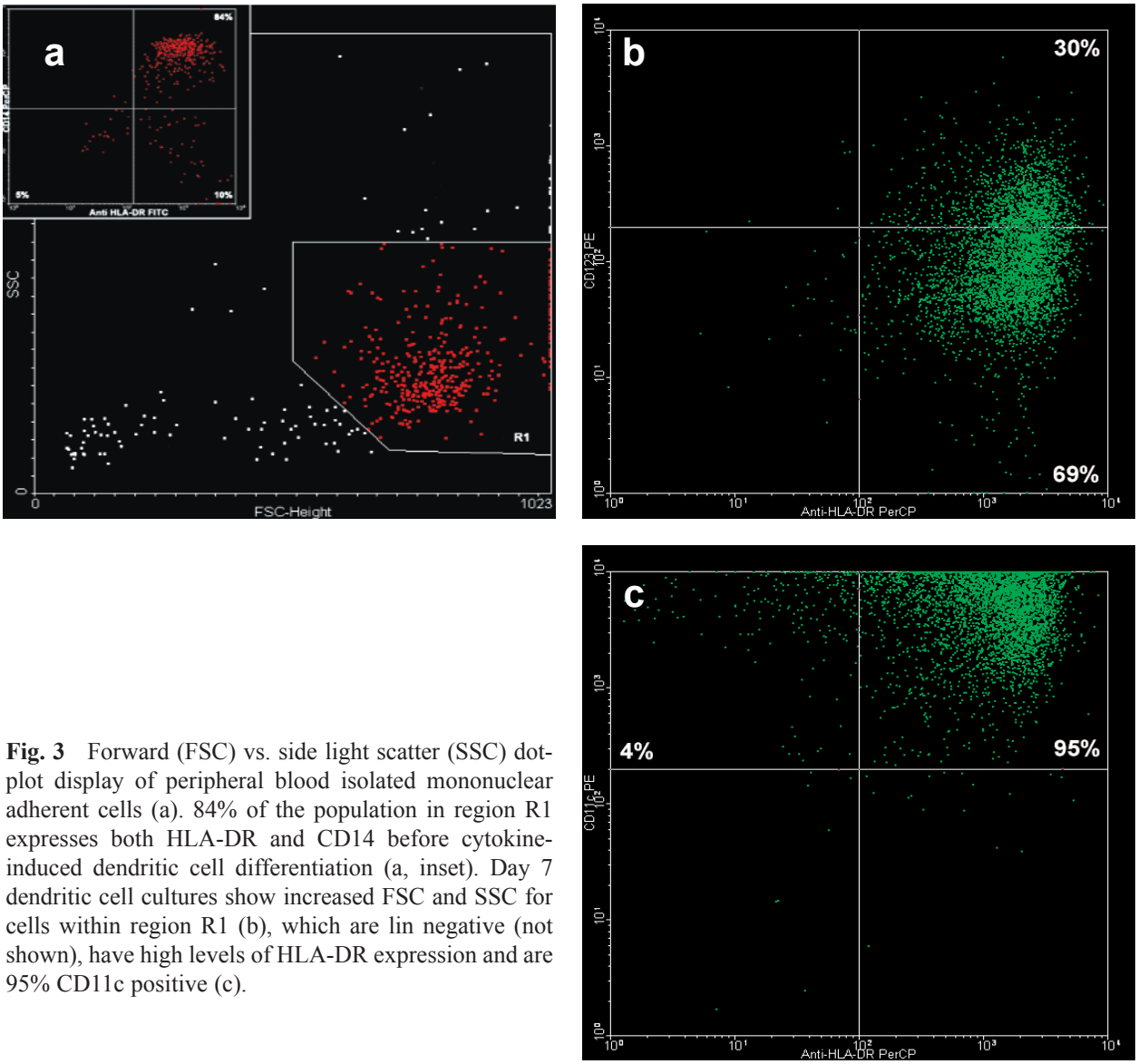


Fig. 3 Forward (FSC) vs. side light scatter (SSC) dot-plot display of peripheral blood isolated mononuclear adherent cells (a). 84% of the population in region R1 expresses both HLA-DR and CD14 before cytokine-induced dendritic cell differentiation (a, inset). Day 7 dendritic cell cultures show increased FSC and SSC for cells within region R1 (b), which are lin negative (not shown), have high levels of HLA-DR expression and are 95% CD11c positive (c).

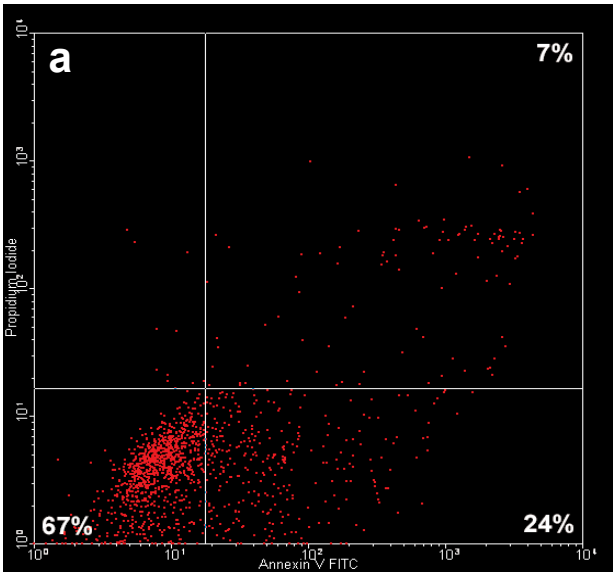
pathways that interact with caspase 3 activation. The apoptosis level in cultures with caspase 3 inhibitor and anti- Fas agonist mAb was lower than in controls.

Discussions

An area of particular interest for the immune system has been the regulation of apoptosis by the receptor- ligand pair: Fas (CD95) and Fas Ligand (FasL).

FasL can induce apoptotic cell death in many cells which express the receptor Fas, providing an important mechanism for cell death mediated by activated cytotoxic T lymphocytes and NK cells. FasL may prevent tissue damage under a number of circumstances, offering immunologically "privileged" sites some protection from inflammatory damage. A more pathological response of this system may be the suppression of cellular immune responses against tumors which may express high levels of FasL and thus evade immune surveillance [13, 14].

FasL signaling is initiated at the cell surface by aggregation of individual Fas receptors *via* binding to the multivalent FasL. Clustering brings into close proximity receptor cytoplasmic domains, a critical requirement for generation of intracellular signaling



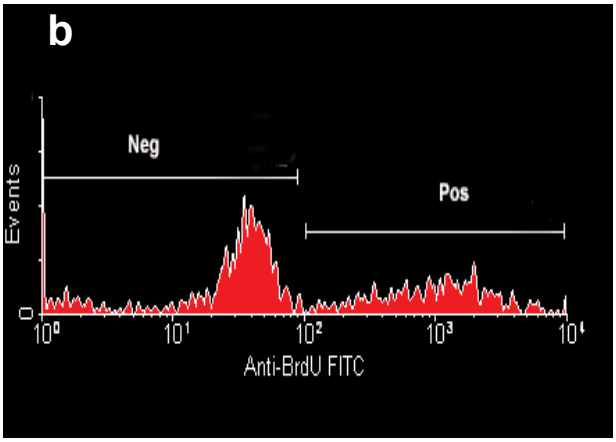


Fig. 4 One representative assay demonstrating culture day 7 immature dendritic cells apoptosis: 24% of the cells bind annexin V-FITC and do not intake propidium iodide (a). A similar proportion of cells show TdT-mediated BrdUTP nick-end labeling, as demonstrated by anti-BrdU FITC antibody binding (b).

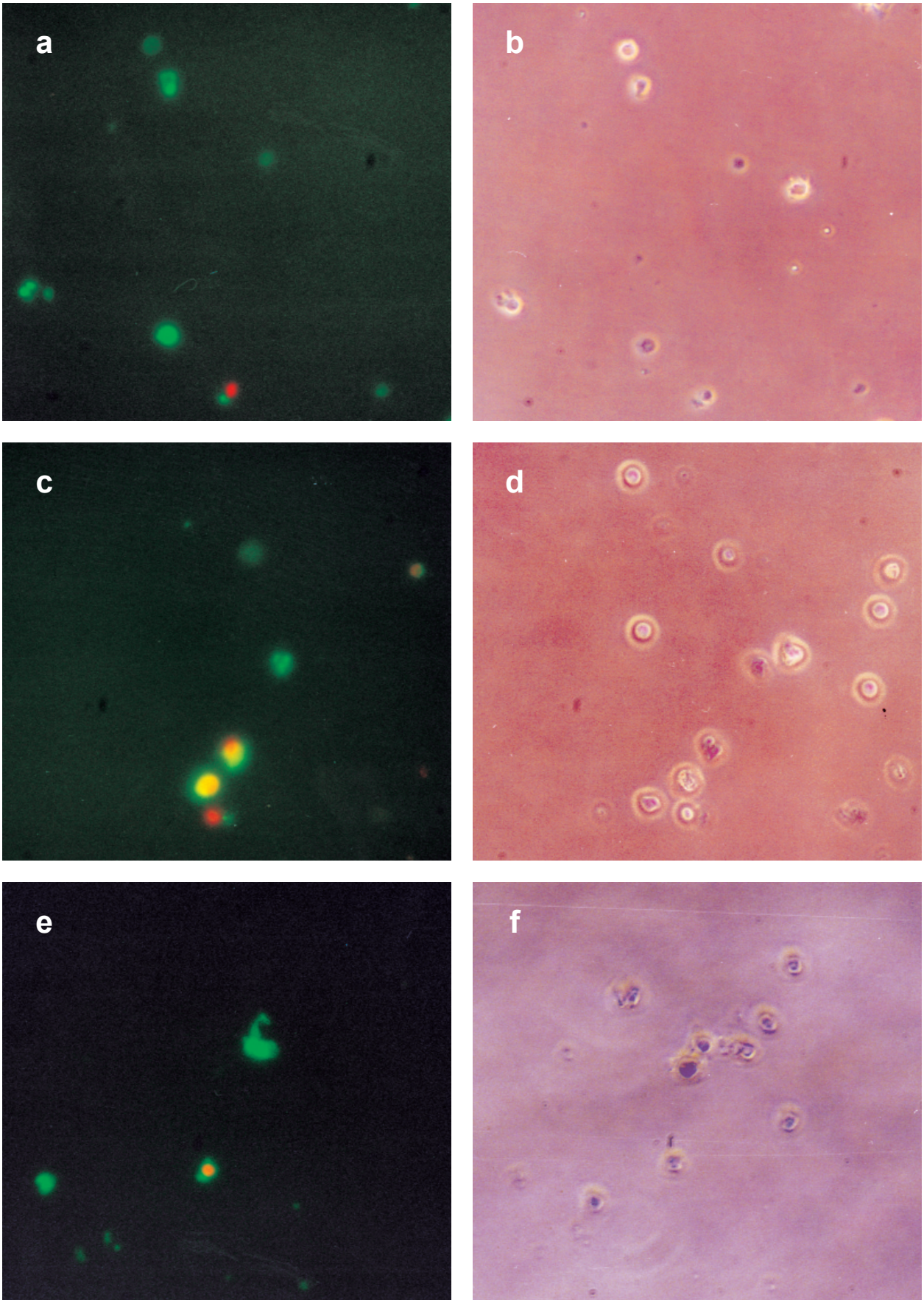
pathways. Antibodies which selectively activate Fas can be used *in vitro* to mimic the apoptotic response associated with FasL. Several cell lines can easily be induced to undergo apoptosis by antibodies directed to human or mouse Fas. It is important to note that there is significant variation between cell lines regarding the level of apoptosis that can be induced *via* Fas receptor. Also, not all cell types which express Fas will necessarily undergo Fas-mediated apoptosis [23, 24].

In the case of Fas-induced apoptosis FADD, an adapter molecule, binds to the receptor and physically engages caspase-8 which represents the most upstream protease involved in the generation of the death signal. Following activation of caspases, including caspase 3, biochemical events occur that lead to DNA degradation and the characteristic morphological changes associated with apoptosis [35].

In spite of the growing evidence for the role of Fas/FasL interaction in the developmental selection and functions of T lymphocytes and NK cells, less attention has been paid to the function of Fas in APC, including DC. Immature murine DC have been shown to express low levels of both Fas and FasL [37]. The role of Fas/FasL interaction in DC apoptosis during antigen-specific interaction with T cells has been demonstrated [38] using a long-term DC line XS52 and a KLH-specific TH1 clone. Thus, ligation of Fas on the DC cell line with FasL expressed on T cells was required and sufficient to trigger cell death. Similar data were also reported earlier, suggesting that activated CD8+ T cells were able to kill APC by both perforin and Fas-mediated mechanisms [39]. Thus, antigen-specific interaction of DC with T cells may induce Fas-mediated apoptosis of DC and might serve as a unique regulatory mechanism to prevent the continual activation of T cells [40, 41].

Interestingly, LC apoptosis in cultures engaged Fas signaling pathways [42]. Expression of Fas (CD 95) was revealed in approximately 40% of isolated LC and addition of anti-CD95 IgM induced and accelerated but not enhanced decrease of LC viability.within the next 72 h. After 2 days of culture without cytokines, CD95 expression was completely downregulated and anti-CD-95 IgM failed to induce apoptosis in these cells. Additionally, spontaneous apoptosis of human DC

Fig. 5 Fluorescence microscopy image of Fas-induced apoptosis in immature MDDC, measured using an annexin V binding assay (a) is shown in comparison to uninduced apoptosis (c) and to apoptosis partially inhibited by caspase-8 selective inhibitor (e). Contrast phase microscopy images of the same fields (b, d, and f). Nikon Eclipse TE 300 microscope, Ob 40x. The percentages of positive cells are presented in table 4.


a
b
c
d
e
f

and LC was efficiently inhibited by CD40 ligation [40]. Using human peripheral blood DC, it was reported that the addition of recombinant soluble CD40L strongly inhibited CD95-mediated apoptosis in these cells [41]. This study indicates that when DC are partially mature they may have a propensity to undergo apoptosis either spontaneously or in response to Fas triggering. In contrast, fully mature DC that have received CD40-mediated stimulation following T cell encounter are apparently resistant to cell death after Fas ligation. The different responses of these two DC populations correlated with differences in the levels of Bcl-2 protein. These findings suggest that the interaction between DC and T cells may have implications for both the activation of naive T lymphocytes and, subsequently, the fate of DC [42]. It is possible that regulation of DC apoptosis by CD95L and CD40L may represent an important mechanism responsible for the feedback control of DC activity by activated T cells.

Our research reveals the presence of Fas and its functional role in DC derived from human monocytes, the easiest and efficient mode to obtain DC analogues.

In accordance to literature data [38-42], about 40% of the immature MDDC in our study expressed CD95 and incubation with anti-CD95 mAb (0.5μg/ml) induced apoptosis when compared to untreated controls. The experimental design did not aim at identifying any subsets within the initial monocyte population, nor within *in vitro* generated DCs, thus excluding the possibility to associate Fas expression to a particular DC subpopulation.

Upon TNFα additional stimulation, MDDC have downregulated their Fas receptors and undergo apoptosis to a lesser extent when treated with anti-CD 95, as demonstrated by the hardly noticeable effect of this antibody on the viability of cultured cells as compared to controls. Thus, upon maturation, MDDC become resistant to Fas-induced apoptosis. Reduced Fas receptor expression seems the most probable cause of this finding, as the extent of apoptosis induced by the agonist anti-Fas antibody strongly related to the proportion of cells expressing CD 95, but other mechanisms cannot be excluded by our experimental data.

Taken together, our findings indicate that the CD 95/CD95L system is implicated in apoptosis seen in immature MDDC cultures and TNFα-matured MDDC are protected against Fas-induced apoptosis.

In conclusion, the apoptotic episodes surrounding the earlier stage of DC differentiation appeared to be mediated by Fas. In contrast, a Fas independent pathway probably mediates apoptotic events associated with terminally differentiated DC. Thus TNFα is considered to play a role in protecting mature DC from Fas-induced apoptosis.

Investigation of factors responsible for DC apoptosis *in vitro* is important for the development of effective strategies designed to protect DC culture from cell death. It is also important to understand the primary signals and intracellular pathways involved in regulation of apoptosis in DC that will bring new means to control both initiation and termination of the immune response. Thus, understanding all aspects of DC immunobiology, including regulation of their death, is crucial for further development of effective DC-based immunotherapies.

Acknowledgements

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