Apoptotic DC-SIGN+ cells in normal human decidua

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ABSTRACT

Background: Normal pregnancy and spontaneous abortion in humans and mice are associated with immune responses. The decidua harbors dendritic cells identifiable in humans by their expression of DC-SIGN. Because dendritic cells are essential for immune response regulation, decidual DC-SIGN+ cells may play a role in normal or pathological pregnancy outcomes. Previous reports suggested that DC interact with NK cells in decidua, although the functional significance of this phenomenon remains unknown. **Objective:** We studied the presence of conjugates of DC-SIGN+ cells with CD56+ NK cells in normal human decidua. **Methods:** Conjugates of DC-SIGN+ cells with CD56+ NK cells were studied in leukocyte suspensions of normal human decidua (6-11 weeks) by flow cytometry and confocal microscopy. The presence of apoptotic cells was determined by the TUNEL assay, incubation with annexin V and confocal microscopy in decidual leukocyte suspensions and by the TUNEL assay in decidual sections. **Results:** We observed conjugates of decidual DC-SIGN+ cells with CD56+ NK cells (40.2 ± 26.1% of all the DC-SIGN+ cells by flow cytometry and 52.3 ± 10.2% by confocal microscopy). We also found that a proportion of DC-SIGN+ cells were in apoptosis, since they were TUNEL+ (40.2 ± 7.2% of all DC-SIGN+ cells in decidual sections) and annexin V+ (34.4 ± 15.2% in leukocyte suspensions). And sorted DC-SIGN+ cells had multilobulated nuclei. **Conclusions:** The conjugates of decidual DC-SIGN+ cells with CD56+ NK cells strongly suggest that these latter cells induce apoptosis in DC-SIGN+ cells during normal pregnancy. We discuss this possibility in the context of maternal-fetal tolerance.

Keywords: Abortion; Apoptosis; Decidua; Dendritic cells; NK cells

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1. Introduction

Human pregnancy is often considered a semiallogeneic allo- graft. Although most abortions occur because of chromosomal abnormalities in the embryo, it has been documented in mice and humans that some sporadic and recurrent spontaneous abortions (and also normal pregnancy) are associated with variations in certain immune responses or in the proportions of peripheral or decidual immune populations [1,2]. Many of the immunological mechanisms that give rise to interactions between the mother and fetus take place in the deciduadthe maternal tissue of the placenta in closest contact with the fetal trophoblast. Dendritic cells (DCs) are immune cells responsible for the balance between immunostimulation and tolerance [3]; therefore DC may be involved in the normal or pathological outcome of pregnancy [4].

Several groups have found immature myeloid DC in normal decidua of humans and mice [5,6]. In human decidua, these cells are identifiable by their expression of DC-SIGN (CD209) [5]. Other authors, however, have suggested that decidual DC-SIGN+ cells are macrophages on the basis of their morphology and co-expression of CD14 [7]. Myeloid DC and macrophages are difficult to distinguish clearly, since they share functions and the expression of many anti- gens; moreover, these two cell types are able to interconvert [8]. This has led some authors to propose that they are the same type of cell in different stages of differentiation [9]. Microarray studies of gene expression by DC and macrophages obtained independently by in vitro cytokine differentiation from monocytes showed that both types of cell expressed DC-SIGN. This antigen, however, was upmo- dulated by DC but downmodulated by macrophages as the two types of cell advanced through their respective differentiation pathways from monocytes [10,11]. In decidua, the differential expression of DC- SIGN, CD14, or both revealed different cell populations that represent steps in the differentiation from monocyte to macrophage or DC [12]. Nevertheless, under the effects of cytokines in vitro, decidual DC-SIGN+ cells differentiated into fully mature myeloid DC [5]. Recent studies showed that decidual DC (dDC) perform different functions that are crucial for pregnancy [13,14], and in spontaneous abortion, a significant decrease in the proportion of these cells has been reported in human decidua [12]. Other reports have demon- strated that human and mice DC are involved in angiogenic responses at the fetalematernal interface [15].

Decidual natural killer (dNK) cells, the most abundant lymphocyte in early human decidua, comprise a distinctive NK cell type different from those of peripheral blood, characterized by their high expression of CD56 and low or absent expression of CD16 [16]. Based on the classical cytotoxic activity of blood NK cells, earlier studies of dNK cells proposed that they exerted cytotoxic control on the trophoblast in order to regulate the physiological invasion of the decidua by these fetal cells. The fact that the trophoblast expresses human leukocyte antigen G (HLA-G), a molecule that binds the inhibitory receptors of dNK cells to block their cytotoxic activity, suggests that this tissue controls this activity via a homeostatic mechanism [17]. Other studies demonstrated that dNK cells were unable to induce spontaneous cytotoxic necrosis in the trophoblast unless they were previously stimulated with IL-2 and IL-15 [18,19]. More recent results suggest that rather than a cytotoxic activity, dNK exert a beneficial control of trophoblast invasion and trophic activity in the decidual vessels [20, 21]. Nevertheless, we and others demonstrated that although human decidual lymphocytes did not induce necrosis, they did induce apoptosis in the trophoblast [22,23]. These findings are consistent with those of Nakashima et al. and Lash et al. [24,25], who showed that granulysin and IFNg, both produced by human dNK cells, induced apoptosis in the trophoblast. Decidual stromal cells and decidual T cells also appear to be susceptible to the induction of apoptosis by dNK cells [26e30].

Kämmerer et al. [5] found clusters of human decidual DC-SIGN+ cells with dNK cells, although the functional significance of this phenomenon remains to be established. In the present study we confirmed the presence of clusters of DC-SIGN+ cells and dNK cells in normal pregnancy decidua and found that a proportion of DC-SIGN+ cells were in apoptosis. We discuss the hypothesis that dNK cells induce apoptosis in DC-SIGN+ cells as a mechanism of maternalefetal tolerance.

2. Materials and methods

2.1. Tissues. Specimens from elective terminations of pregnancy (6-11 weeks) were obtained from the Clínica El Sur in Málaga (Spain) and Ginegranada in Granada. All patients were aged 20-30 years. Women receiving any medication or having recurrent miscarriages, infectious, autoimmune, or other systemic or local diseases were excluded. None of the abortions was pharmacologically induced. Decidual tissue was obtained by suction curettage. None of the samples showed any evidence of necrosis or acute inflammation. All women provided informed consent, and this study was approved by the clinical research ethics committee of the Hospital Universitario de San Cecilio in Granada.

2.2. Immunohistochemistry. For immunostaining, cryostat sections (5 μm) of early human decidua were fixed with acetone and labeled with an indirect immunoperoxidase method. Briefly, samples were rehydrated in PBS and incubated with hydrogen peroxide and AB human serum to block endogenous peroxidase and Fc receptors, respectively. The samples were then incubated for 30 min at room temperature in a humid chamber with an appropriately diluted anti-DC-SIGN monoclonal antibody (mAb) (Sigma-Aldrich, St. Louis, MO, USA). Normal mouse serum or an irrelevant mAb was substituted for the first antibody as a negative control. After three brief washings with PBS, samples were overlaid with peroxidase-conjugated goat antimouse immunoglobulin G (IgG) (Bio-Rad, Richmond, CA, USA) and diluted 1:100 in 1% PBS-BSA, and the reaction was developed with 0.5 mg/mL diaminobenzidine (Sigma-Aldrich) containing 0.01% hydrogen peroxide. The reaction was stopped after 5-10 min by washing in excess PBS. Samples were counterstained with Mayer's hematoxylin (Sigma-Aldrich).

2.3. Extraction of decidual leukocytes. To avoid inducing an allogeneic reaction by leukocytes, samples of decidua from different patients were not mixed. The method of extraction has been described elsewhere. Briefly, samples from the decidua of elective terminations of pregnancy were thoroughly washed in PBS. Decidual fragments were finely minced in a small volume of RPMI 1640 (Sigma-Aldrich) and then pushed through a 53-um sieve (Gallenkamp, Loughborough, UK). The resulting cell suspension was washed with RPMI and layered on an equivalent volume of Lymphoprep (Flow Laboratories, Hertsfordshire, UK) at room temperature, and centrifuged for 20 min at 600 g. The cells, decidual leukocytes, were collected from the interface, suspended in RPMI, washed and suspended in PBS for further analysis. Cell viability was determined microscopically by trypan blue exclusion. Only samples with more than 90% viable leukocytes were used.

2.4. Flow cytometry analysis. Decidual leukocytes were suspended in PBS at 10^6 cells/mL. One hundred µl of the cell suspension was incubated with 10 µL of the appropriate mAb for 30 min at 4°C in the dark. The cells were washed, suspended in 0.5 ml PBS, and immediately analyzed in a flow cytometer (Ortho-Cytoron, Ortho Diagnostic Systems, Raritan, NJ, USA). To identify dead cells we incubated decidual cells with propidium iodide (Sigma-Aldrich). Decidual cells were stained with the appropriate mAb labeled with FITC or phycoerythrin (PE), or with their corresponding control isotype. The percentage of cells that were antibody-positive was calculated with reference to the appropriate isotype control. The mAbs against surface proteins were: CD56 (Caltag, San Francisco, CA, USA), CD83 (BD Biosciences, Denderstraat, Erembodegem, Belgium) and DC-SIGN (R&D Systems, Minneapolis, USA). For flow cytometric analysis we used the electronic gate in which most DC-SIGN+ cells were detected. Decidual leukocytes were treated with a mAb against DC-SIGN labeled with PE (R&D Systems) and sorted in a FACS Vantage cell sorter (Becton-Dickinson, Franklin Lakes, NJ USA). Sorting was done twice and the proportion of DC-SIGN+ cells obtained was higher than 90%.

2.5. Immunofluorescence microscopy. For immunofluorescence microscopy we used the same protocol as for flow cytometry. Cells were stained with mAbs against DC-SIGN labeled with PE (R&D Systems) and against CD56 labeled with FITC (BD Biosciences) or their corresponding control isotype. They were suspended in Vectashield (Vector, Peterborough, UK) and examined with a Leica confocal or conventional immunofluorescence microscope (Leica Microsystems, Wetzlar, Germany).

2.6. Apoptosis detection. To identify apoptotic DC-SIGN+ cells in decidual sections, double staining was used. We first treated the cells with the In Situ Cell Death Detection Kit, POD (TdT-mediated dUTP nick end-labeling (TUNEL) assay with peroxidase according to the manufacturer's instructions (Roche Diagnostic, Mannheim, Germany), and then incubated them with an anti-DC-SIGN mAb (Sigma-Aldrich), followed by the Vector VIP Substrate kit (Vector Laboratories, Burlingame, CA, USA). Positive apoptotic cells were identified by their brown nuclei, whereas DC-SIGN+ cells were purple. As a negative control for DC-SIGN staining, an irrelevant monoclonal antibody was used instead of the anti-DC-SIGN mAb. As a negative control for the TUNEL assay, we followed all the steps in the method described above except for cell permeabilization, which was omitted. To study early apoptosis, decidual leukocytes were suspended in PBS at a concentration of 10^6 cells/mL. One hundred µl of this suspension was incubated with 10 µl annexin-V-Fluos (Roche Diagnostics) and 10 µl DC-SIGN PE (R&D Systems) at 4°C for 30 min in the dark. Finally the cells were washed and suspended in 0.5 mL PBS and immediately analyzed in a flow cytometer. To study late apoptosis, decidual leukocyte suspensions or sorted DC-SIGN+ cells were washed in PBS and then fixed at a concentration 10^7 cells/mL in a solution of 2% paraformaldehyde in PBS, pH 7.4, for 1 h at room temperature on a shaker. The preparation was centrifuged at 400 g for 10 min, and cells were permeabilized by treatment with a solution of 0.1% Triton-X 100 and 0.1% sodium citrate for 2 min on ice. The cells were washed in PBS and analyzed with the TUNEL assay (In Situ Cell Death Detection Kit, Fluorescein, Roche Diagnostic) according to the manufacturer's instructions. Samples were washed again in PBS and suspended in PBS for analysis by flow cytometry, or examined by fluorescence microscopy. To identify apoptotic cells, decidual cells were stained with a PE-labeled mAb against DC-SIGN (R&D Systems) or against CD56 (Caltag) before the TUNEL assay. To observe apoptotic DC-SIGN+ nuclei, sorted DC-SIGN+ cells were permeabilized with 0.5% Tween 20 in citric acid solution before the nuclei were stained with 2 µg/ml 4',6-diamidino-2-phenylindole (DAPI) stain in a solution containing Na2HPO4 for 5 min at 37 °C, and were examined by fluorescence microscopy. All assays were done at least 3 times, and the figures illustrate representative results from a specific assay.

3. Results

3.1. NK-DC-SIGN+ cell conjugates. We and others [5,12] previously reported that decidual DC- SIGN+ cells from elective terminations of pregnancy (normal decidua) exhibited a phenotype compatible with immature myeloid DC. We found that these cells expressed CD11c (a myeloid DC marker), but showed little or no expression of CD123 (a plasmacytoid

DC marker) and HLA-DR was only weakly detected [12]. DC-SIGN+ cells showed little or no expression of CD83 (a mature myeloid DC marker) (Fig. 1A). Like other authors [5], we observed DC-SIGN+ cells predominantly in perivascular localizations (Fig. 1B). Immunohistological assays [5] showed clusters of DC- SIGN+ cells and CD56+ NK cells in human decidua. We confirmed the presence of these clusters by flow cytometry (40.2 26.1% of all the DC-SIGN+ cells, *n* = 15) and confocal microscopy (52.3 10.2% of all the DC-SIGN+ cells, *n* = 3) (Fig. 2). This latter method showed that DC-SIGN and CD56 were expressed on different cells. We also observed that some DC-SIGN+ cells that formed conjugates with CD56+ dNK cells displayed a “boiling” surface, a classical morphological feature of apoptosis (Fig. 2B).

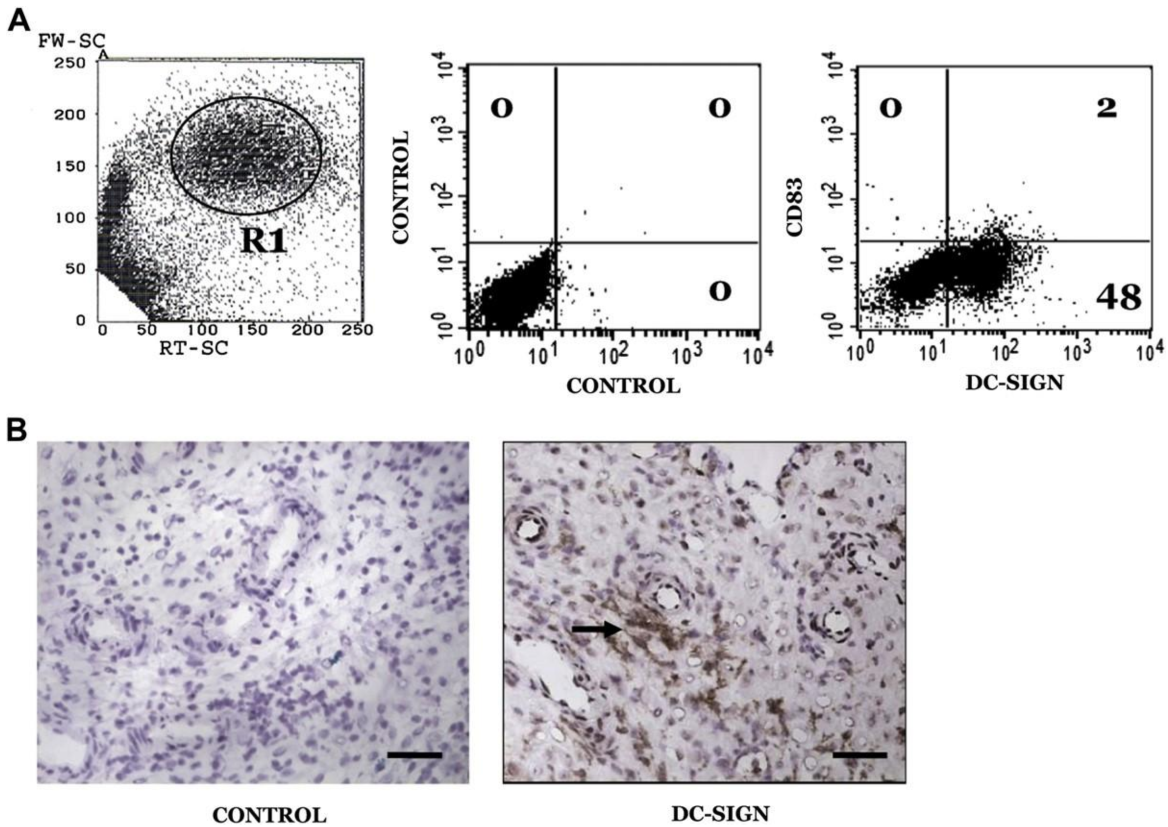

Fig. 1. Decidual DC-SIGN+ cells from elective termination of pregnancy. Decidual DC-SIGN+ cells were analyzed by flow cytometry, confocal microscopy and immunohisto- chemistry. A) Flow cytometric analysis of decidual DC-SIGN+ cells. R1 indentifies the flow cytometry electronic gate in which most DC-SIGN+ cells were detected. DC-SIGN+ cells were studied in R1 in this and the following flow cytometry experiments (left). DC-SIGN+ cells showed little or no expression of CD83 (right). B) Immunoperoxidase staining of DC- SIGN+ cells in a section of early human decidua. The arrow points out perivascular clusters of positive cells. Bar = 50 mm.

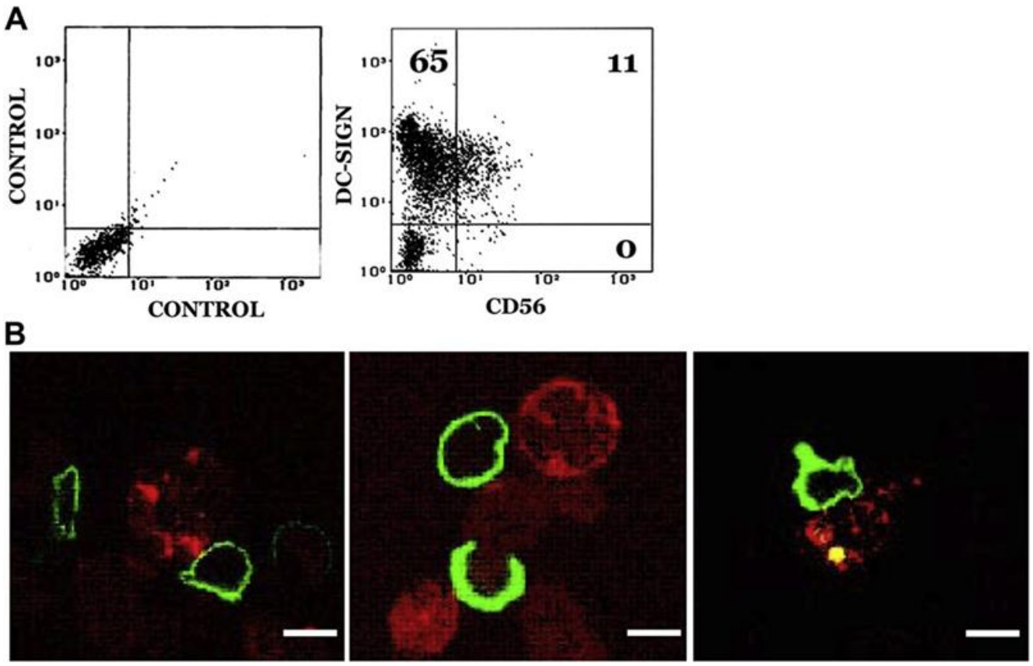

Fig. 2. Conjugates of DC SIGN+ cells with CD56 + NK cells of decidua from elective termination of pregnancy. A) Flow cytometric analysis by double-labeling with anti-CD56 and anti-DC-SIGN mAbs. A proportion of DC-SIGN+ cells were also positive for CD56. B) Confocal microscopic images showing conjugates of DC-SIGN+ cells (PE, red) and CD56 + NK cells (FITC, green). DC-SIGN and CD56 were expressed by different cells; we did not detect double-labeling with these antigens on any cells. The third photo on the right shows a DC- SIGN+ cell with a “boiling” surface morphology typical of apoptotic cells. Bar = 10 mm.

3.2. Apoptotic DC-SIGN+ cells Double-labeling analysis with anti-DC-SIGN and TUNEL assay to identify apoptotic cells by flow cytometry showed that a substantial proportion of DC-SIGN+ cells were also TUNEL+ (Fig. 3A). When annexin V was used instead of the TUNEL assay, a proportion of DC- SIGN+ annexin V+ cells was also detected by flow cytometry (34.4 15.2% of all the DC-SIGN+ cells, n = 10) (Fig. 3A). In decidual sections, we observed by immunocytochemistry that part of the DC-SIGN+ cells were TUNEL+ (Fig. 3B) (40.2 7.2% of all the DC- SIGN+ cells, n = 3), whereas we did not detect TUNEL+ CD56+ NK cells (not shown). When we purified DC-SIGN+ cells by cell sorting, we found by immunofluorescence microscopy that a proportion of these cells were TUNEL+, and apoptotic nuclei in DC-SIGN+ cells were seen with this method as well as with DAPI staining (Fig. 4). These results showed that some of the decidual DC-SIGN+ cells were apoptotic.

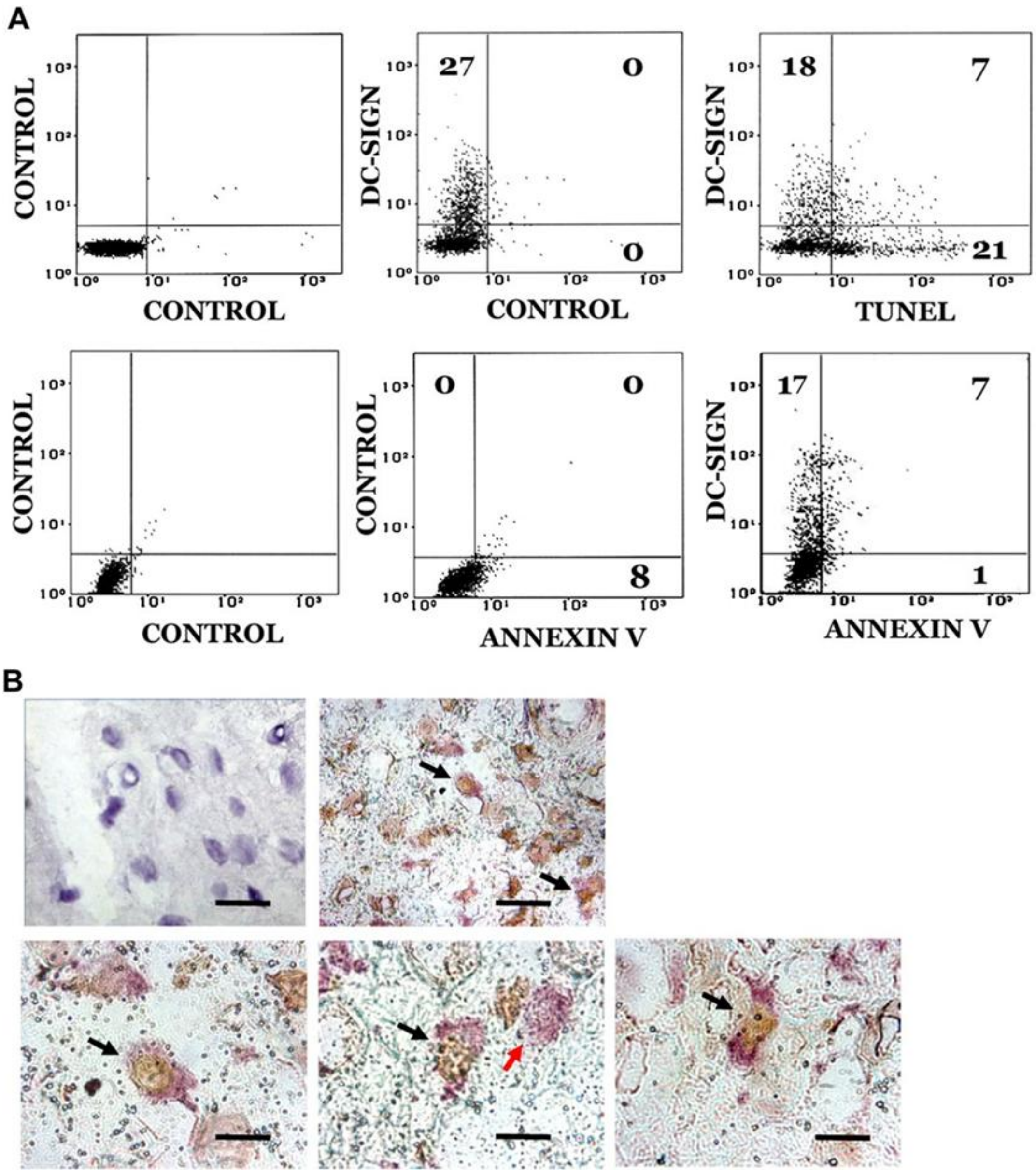

Fig. 3. Analysis of apoptosis in decidual DC-SIGN+ cells from elective termination of pregnancy. A) Flow cytometric analysis by double-labeling with TUNEL or annexin V and anti-DC-SIGN. A proportion of DC-SIGN+ cells were in apoptosis, since they were also TUNEL+ or annexin V+ B) Immunohistochemical analysis by double-labeling with TUNEL (brown) and anti-DC-SIGN (purple) of a section of decidua .The black arrows mark DC-SIGN+ TUNEL+ cells; the red arrow marks a DC-SIGN+ TUNEL— cell. Bar in the two upper panels = 25 mm , and bar in the in the three lower panels = 12 mm.

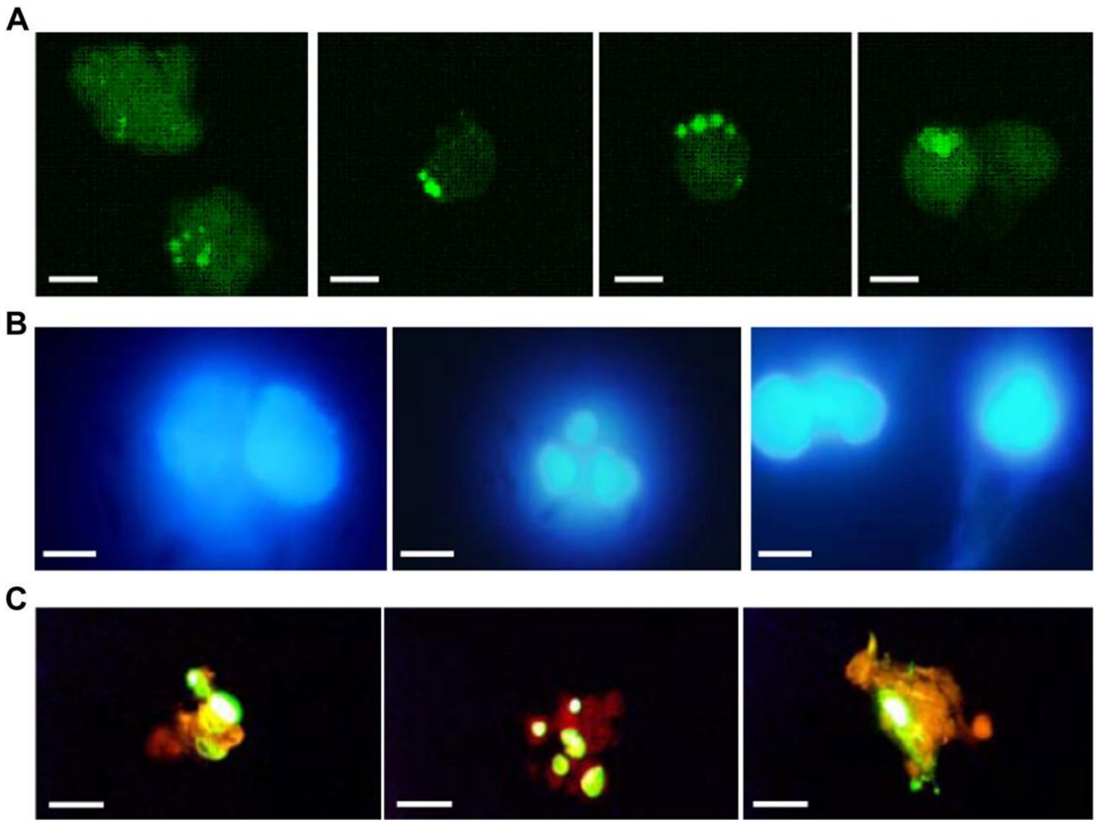

Fig. 4. Fluorescence microscopic images showing sorted DC-SIGN+ cells with multilobulated nuclei as revealed by A) TUNEL staining (FITC), B) DAPI staining, and C) TUNEL staining (FITC, green) with DC-SIGN labeling (PE, red). Bar in A and C = 10 mm, and bar in B = 5 mm.

4. Discussion

Several lines of evidence in mice and humans have confirmed the involvement of DC in normal and pathological pregnancies [6,12,13,15]. Dendritic cells in the human decidua are identified by the expression of DC-SIGN, exhibit an antigen phenotype compat- ible with immature myeloid DC [5,12], and are detected around the vessels (Fig. 1). Kammerer et al.[5] demonstrated by immunohis- tology that approximately half of the decidual DC-SIGN+ cells formed clusters with dNK cells. Conjugates of dDC and dNK cells have also been reported in mice [31]. We confirmed the clusters of human decidual DC-SIGN+ cells and CD56+ NK cells by flow cytometry and confocal microscopy (Fig. 2). Flow cytometry showed that these conjugates were not an artifact of the immu- nohistology (Fig. 2A), and confocal microscopy demonstrated that DC-SIGN and CD56 were expressed on different cells (Fig. 2B). Furthermore, the proportion of clusters of decidual DC-SIGN+ cells-dNK cells detected by flow cytometry was close to the proportion found by immunohistology [5].

Several hypotheses have been proposed to explain the func- tional significance of these conjugates: 1) activation of dNK cells by dDC, 2) activation of dDC by dNK cells, 3) inhibition or killing of dDC by dNK cells, and 4) inhibition or killing of dNK cells by dDC [13,32e35]. Our data neither support nor refute the possibility that dDC activate dNK. However, the proportion of DC-SIGN+ cells that were also TUNEL+ or annexin-V+ (Fig. 3), and that exhibited multilobulated nuclei (Fig. 4), together with the fact that some of the DC-SIGN+ cells that formed conjugates with dNK cells showed a “boiling” morphology (a feature of apoptosis) (Fig. 2B), suggest that dNK cells kill dDC by apoptosis. Dendritic cell killing by NK cells, previously documented for immature myeloid DC, prevents further maturation of DC and thus controls the immunostimulation of Th1 cells [36].

Recent reports are consistent with our hypothesis. Collins and colleagues [37] showed that dDC are entrapped in the decidua in normal murine pregnancy, and these authors suggested that dDC entrapment impaired T cell activation against fetal antigens. The immobilization of dDC may be associated with apoptosis in these cells. Tirado-Gonzalez et al., [12] showed that, unlike normal pregnancy, spontaneous abortion is characterized by a reduction in the proportions of decidual DC-SIGN+ cells, apoptotic DC-SIGN+ cells and conjugates of DC-SIGN+ and dNK cells. This reduction suggested that dDC escape from the decidua to the local lymph nodes, where they activate T cells [12,38,39]. Further differentiation of dDC into fully mature myeloid DC in the local lymph nodes would induce a Th1 response, which would in turn trigger spontaneous abortion [12,23,40e42]. The induction of apoptosis in dDC during normal pregnancy may therefore constitute another mechanism of maternalefetal tolerance that is inhibited in spon- taneous abortion.

The current view, however, is that dNK cells, rather than harming cells of the maternal-fetal interface through their cyto- toxic activity, actually secrete cytokines and growth factors that favor different local cells and activities [20,21,35]. Nevertheless, several lines of experimental evidence showed that dNK cells also induce apoptosis, but not necrosis, in trophoblast, decidual stromal cells and decidual T cells [22e30]. Different dNK subsets may exert distinct or even opposite functions that contribute to local homeostasis [43].

5. Conclusions

Our findings confirm the existence of decidual DC-SIGN+ cell-NK cell conjugates and demonstrate the presence of apoptotic DC-SIGN+ cells in normal human decidua. Functional experiments to study the dynamics of the formation of conjugates and to test the effects of dNK on DC-SIGN+ cells are needed to confirm our hypothesis. These experiments, which should ideally be performed with autologous cells, are challenging because of the low number of DC-SIGN+ cells that can be obtained from a given sample. Our hypothesis should therefore be tested in animal models such as syngeneic mice.

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