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Rosiglitazone promotes the differentiation of Langerhans cells and inhibits that of other dendritic cell types from CD133 positive hematopoietic precursors

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Summary. Dendritic cells and their precursors express PPAR-gamma, whose stimulation has inhibitory effects on the maturation and function of dendritic cells *in vivo*. Dendritic cells can differentiate *in vitro* from CD133+ progenitors; the influence of PPAR-gamma stimulation on this process is unknown. We have addressed the effect of PPAR-gamma agonist rosiglitazone, at a concentration as used in clinics, on the differentiation of dendritic cells from human CD133+ progenitors. Cells were harvested from cord blood by density gradient and immunomagnetic separation, and cultured for 18 days with fetal calf serum, cytokines and 1 μmol/L rosiglitazone. Analyses included flow cytometry, electron microscopy and mixed lymphocyte reaction. As expected, control cells generated without rosiglitazone were dendritic, expressed MHC-II, CD80, CD83 and CD86 and stimulated mixed reaction potently. A minority of cells expressed the Langerhans cell marker CD207/langerin, but none contained Birbeck granules. With rosiglitazone much fewer cells were generated; they were all dendritic, expressed differentiation and maturation-related antigens in higher percentage and were better stimulators of lymphocytes than those generated without the drug. The vast majority of cells expressed CD207/langerin and many contained Birbeck granules, i.e. were full-fledged Langerhans cells. We conclude that stimulation of PPAR-gamma, while negatively affecting the number of generated cells, promotes the maturation of human cord blood CD133 positive precursors into efficient, immunostimulating dendritic cells with a Langerhans cell phenotype

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Introduction

Dendritic cells play a pivotal role in the regulation of the immune response, providing lymphocytes and other cell types with information on invasive pathogens (Banchereau and Steinman, 1998). Immature dendritic cells, located in non-lymphoid tissues, absorb and process antigens (Platt et al., 2010) and then migrate into secondary lymphoid organs. During migration dendritic cells become mature and hence able to present antigens to T and possibly B lymphocytes (Wan et al., 2008), which are stimulated to proliferate. The maturation process implies the rapid down-regulation of endocytic activity, up-regulation of major histocompatibility class (MHC) II antigens and expression of the co-stimulatory molecules CD54, CD80 and CD86 (Pieri et al., 2001).

Among immature dendritic cells, Langerhans cells (LC) are peculiar for location, phenotype, turn over (Romani et al., 2010) and also for function, since they are able to respond to low dose haptens but are damaged by high doses, which on the contrary elicit responses through dermal connective tissue dendritic cells (Bacci et al., 1997). They are identified in squamous stratified

epithelia by the expression of class II molecules, CD1a and CD207/langerin and by peculiar inclusions, Birbeck granules (Birbeck et al., 1961; Romani et al., 2010), which are part of the endosomal recycling network (Mc Dermott et al., 2002). Langerin/CD207 (a C-type lectin receptor) is the main molecular component of Birbeck granules (Valladeau et al., 2000), but langerin can also be found in cells in the absence of Birbeck granules (Uzan-Gafsou et al., 2007; Bonetti et al., 2011) and can reside in endosomal structures independent of those granules (Valladeau et al., 2000; Romani et al., 2003). Given the proposed role of LC in the response to tumours (Gerlini et al., 2005) and virus infections arising in squamous epithelia (Romagnoli, 2001), knowledge of this dendritic cell subtype may be of high interest for basic and applied tissue biology.

Dendritic cells develop from bone marrow hematopoietic stem cells, possibly via both the common myeloid and common lymphoid progenitors (Manz et al., 2001). They can also be generated from peripheral blood monocytes and from hematopoietic CD34 positive progenitors isolated from bone marrow, umbilical cord blood and peripheral blood (Caux et al., 1992; Strunk et al., 1997; Hubert et al., 2005; Forraz and McGuckin, 2011). Hematopoietic stem cells are selected, also for transplantation, on the basis of CD34 expression. A less numerous population expressing CD133/prominin-1 is considered to include non-committed, even earlier hematopoietic progenitors than CD34 positive cells (Miraglia et al., 1997; Mizrak et al., 2008). The majority of CD34 positive cells are also CD133 positive (Gallacher et al., 2000; Bonetti et al., 2011), while virtually all CD133 positive cells in the human cord blood express CD34 (Bonetti et al., 2011). Both human CD34 positive and CD34 negative, CD133 positive hematopoietic cells include cells that behave as stem cells (Gallacher et al., 2000). Since CD133 positive cells have shown a higher clonogenic capacity than CD34 positive, CD133 negative cells (de Wynter et al., 1998; Handgretinger et al., 2003), and CD133 is expressed only on stem and progenitor cells, whereas CD34 is also expressed on some differentiating cells, the CD133 epitope is a promising selection marker for hematopoietic stem cells enrichment (Mizrak et al., 2008; Meregalli et al., 2010).

The information on CD133 positive precursors, their differentiation into dendritic cells and the response to agents possibly interfering with this process is still limited. We have shown that these cells can be driven to generate immunostimulatory dendritic cells (Bonetti et al., 2011). However, in respect to cells deriving from CD34 positive precursors in the same culture conditions (Caux et al., 1996, 1997; Hubert et al., 2005), the cells generated from CD133 positive precursors were relatively few and expressed only some markers of LC, and in a limited percentage of cells, upon culture conditions which draw CD34 positive precursors to generate high numbers of that specific cell subtype.

The function and immunophenotype of dendritic cells may be influenced by numerous genes, cytokines, hormones and lipid mediators. Among nuclear receptors, the regulation of these cells depends - among others - on peroxisome proliferator-activated receptors (PPAR), which are transcription factors playing roles in an ever increasing list of regulatory pathways (Varga and Nagy, 2008; Reka et al., 2011). To date, three isotypes of PPAR called PPAR-alpha, -beta (also designed delta) and -gamma have been identified (Varga and Nagy, 2008).

PPAR-gamma is the most intensively studied PPAR (Luconi et al., 2010); it is expressed by hematopoietic stem cells (Greene et al., 1995) and cells of the immune system such as macrophages and dendritic cells (Nencioni et al., 2002; Asada et al., 2004), including LC (Angeli et al., 2003). During the differentiation *in vitro* of human monocytes and dendritic cells, PPAR-gamma is expressed at a high level in a narrow, early time frame and at lower levels thereafter (Varga and Nagy, 2008).

The agonists of PPAR-gamma exert a complex anti-inflammatory action (Ricote et al., 1998; Hinz et al., 2003), which depends on their ability to antagonize and block the pro-inflammatory transcriptional regulators nuclear factor-kappaB (NF-kappaB), adaptor-related protein complex 1 (AP-1) and signal transducer and activator of transcription (STAT) (Ricote et al., 1998; Chinetti et al., 2000), and to stimulate retinoic acid production (Geissmann et al., 2003). These agonists include glitazones, which have come into use in the clinics for type 2 diabetes. PPAR-gamma ligands are able to alter the maturation process of dendritic cells stimulated by specific immunological receptors, such as Toll like receptors (TLR), by inhibiting extracellular signal-regulated kinase (ERK) and NF-kappaB (Wei-guo et al., 2010); these effects are reportedly concentration dependent (Nencioni et al., 2002; Majai et al., 2010). Dendritic cells differentiated from monocytes in the presence of a PPAR-gamma agonist expressed maturation related antigens less intensely and were impaired in T cell stimulation as compared with controls (Nencioni et al., 2002). The stimulation of PPAR-gamma may also interfere with the migration of dendritic cells out of peripheral tissues and with the expression of relevant signalling molecules during that process both in humans (Nencioni et al., 2002; Li et al., 2011) and the mouse (Angeli et al., 2003; Jung et al., 2011). In a rat model of arterial wall injury the stimulation of PPAR-gamma with rosiglitazone caused a reduction in number of dendritic cells and a lack of interaction between these cells and lymphocytes, although both cell types were present, in parallel with a decreased activation of NF-kappaB, as well as a reduced production of repair tissue (Rinaldi et al., 2009). Local and systemic production of PPAR-gamma activators, for instance arachidonic acid derivatives, may therefore be important in the regulation of immune responses.

We have addressed here the electron microscopic features of CD133 positive hematopoietic precursors, to help in their recognition at microscopy, and assessed the effect of PPAR-gamma stimulation on the differentiation

of those progenitors into dendritic cells, taking advantage of rosiglitazone as a tool to activate those receptors (Hirakata et al., 2004). The lowest effective concentration of the drug was used (Nencioni et al., 2002; Majai et al., 2010), for better comparison with concentrations attained in clinical use (Cox et al., 2000; Chapelski et al., 2003; Pedersen et al., 2005). It was applied since the start of culture, to mimic what would happen in subjects treated with this drug in the clinics.

Material and methods

Isolation of CD133 positive progenitors

Umbilical cord blood samples were obtained from donations unsuitable for banking because of too few nucleated cells (Meyer et al., 2006), according to Italian law and the Institution ethical rules. The CD133 positive cells were obtained from mononuclear cells isolated by density gradient on Ficoll (Lymphoprep, Nicomed Pharma, Oslo, Norway), labelled with colloidal superparamagnetic microbeads conjugated with mouse anti-human CD133 monoclonal antibody (MiniMACS; Miltenyi Biotec, Bergisch Gladbach, Germany) and separated through a magnetic field, following a previously published protocol (Bonetti et al., 2011).

Cell culture

The cells were seeded in RPMI 1640 with 10% heat-inactivated fetal bovine serum, 100 U/mL penicillin and 0.1 ng/mL streptomycin (all from Sigma, Milan, Italy). The cells were expanded until day 7 with GM-CSF (10 ng/mL), TNF-alpha (10 ng/mL), IL-4 (10 ng/mL), TPO (10 ng/mL), Flt-3L (25 ng/mL), SCF (20 ng/mL) and TGF-beta (10 ng/mL; all cytokines from PeproTech, London, UK). The cells were differentiated until day 18 in the same medium without SCF, TPO and Flt-3L; TGF-beta concentration was raised to 20 ng/mL since day 14 (Hubert et al., 2005; Bonetti et al., 2011). Rosiglitazone (1 μmol/L, i.e. 0.36 μg/mL; Glaxo-SmithKline, Verona, Italy) was added since the start of culture; some experiments were conducted without rosiglitazone, as controls. The drug was obtained from the producer as a powder and dissolved in 0.1 mol/L phosphate buffer saline at pH 7.4 without any specific vehicle. The cells cultured in the presence of rosiglitazone were analyzed in detail only at the end of culture (14 days or 18 days, as specified in the results).

Electron microscopy

Cytocentrifugates were fixed in 2% formaldehyde and 2.5% glutaraldehyde in 0.1 mol/L cacodylate buffer, pH 7.4, osmicated and embedded in epoxy resin. Sections 1-2 μm thick were analysed at light microscopy. Sections about 70 nm thick were stained with lead acetate and uranyl acetate and observed in a JEM 1010 electron microscope (Jeol, Tokyo, Japan), at 80 kV.

The morphological features of the freshly isolated cells were evaluated on electron photomicrographs, scanning at least 100 cells for three isolated cord units. The photomicrographs were analysed with ImageJ software (NIH, Bethesda, Maryland) at the single cell level. For each analysed cell, thresholding and - where needed - opto-manual integration were used to delineate the whole cell section and the section of the nucleus, then the surface area and the perimeter of each cell and its nucleus and the surface area of the nucleolus (when included in the cell section) were measured. The results were transformed from pixel and square pixel to μm and μm^2 on the basis of magnification and were used to compute the circumference-equivalent and the area-equivalent diameters of cells and nuclei. The perimeter-equivalent circumference is a circumference as long as the perimeter of the analyzed figure; the area-equivalent circle is a circle with the same surface area as the analyzed figure. For each analyzed figure, the diameter of the perimeter-equivalent circumference, D(eq circumf), was computed from: D(eq circumf) = P/π, where P is the perimeter of the figure; the diameter of the area-equivalent circle, D(eq circle), was computed from D(eq circle) = $2 \bullet (A/\pi)^{1/2}$, where A is the area of the figure. The ratio between the diameter of the perimeter-equivalent circumference and that of the area-equivalent circle, D(eq circumf)/D(eq circle), was assumed as an estimate of the degree of convolution of each examined cell and nucleus. This ratio is 1 in a circle and increases with divergence from circularity: it is 1.13 for a square, 1.29 for an equilateral triangle, 1.43 for a star with a pentagonal core and five equilateral triangular points and so on.

Flow cytometry

The cells were immuno-labelled for 20 min at 4°C. The following monoclonal antibodies were applied: HLA-DR, DP, DQ-fluorescein isothiocyanate (FITC); CD34-phycerythrin (PE) or CD34-FITC; CD45-FITC (BD Pharmingen, San Diego, California); CD80-FITC, CD83-phycerythrin-Cy5 (PC5), CD86-PE (Immunotech, Marseille, France); CD133/2-PE (Miltenyi Biotec); CD11c-PE (BD Pharmingen); F4/80-FITC (Abcam, Cambridge, United Kingdom); purified CD207/langerin (Dendritics, Lyon, France; IgG1, clone DCGM4/122D5) followed by goat anti-mouse IgG F(ab')$_2$ (H+L)-Alexa Fluor 546 (Invitrogen, Carlsbad, California); unspecific IgG1-FITC and IgG1-PE (BD Pharmingen) were used as negative controls. Dead cells were counted by flow cytometry upon labelling with 7-amino-actinomicin D (BD Pharmingen). Flow cytometric acquisition was performed by collecting 10^4 events on a FACScalibur (Becton Dickinson, Sparks, Maryland) or an Epics XL-System II flow cytometer (Beckman Coulter, Fullerton, California) and data were analysed on DOT-PLOT bi-parametrical diagrams using CELL QUEST software (Becton Dickinson) on a Macintosh (Cupertino,

California) personal computer.

Mixed lymphocyte reaction

At culture day 18 the cells were transferred into quadruplicate cultures at $1x10^2$, $1x10^3$ and $1x10^4$ cells per 100 μL per well in 96-well round-bottom plates with $1x10^5$ allogeneic CD4+ T cells (purified from peripheral blood mononuclear cells with MACS CD4 isolation kit II of Miltenyi Biotec). The culture medium was RPMI 1640 with 10% heat inactivated foetal bovine serum (without cytokines, nor rosiglitazone). After 5 days, cells were pulsed overnight with ^{3}H-thymidine (1 μCi/well=0.037 MBq/well). Thymidine incorporation was measured by standard liquid scintillation counting. Results are expressed in counts per minute and shown as mean ± standard deviation (SD) of quadruplicate counts.

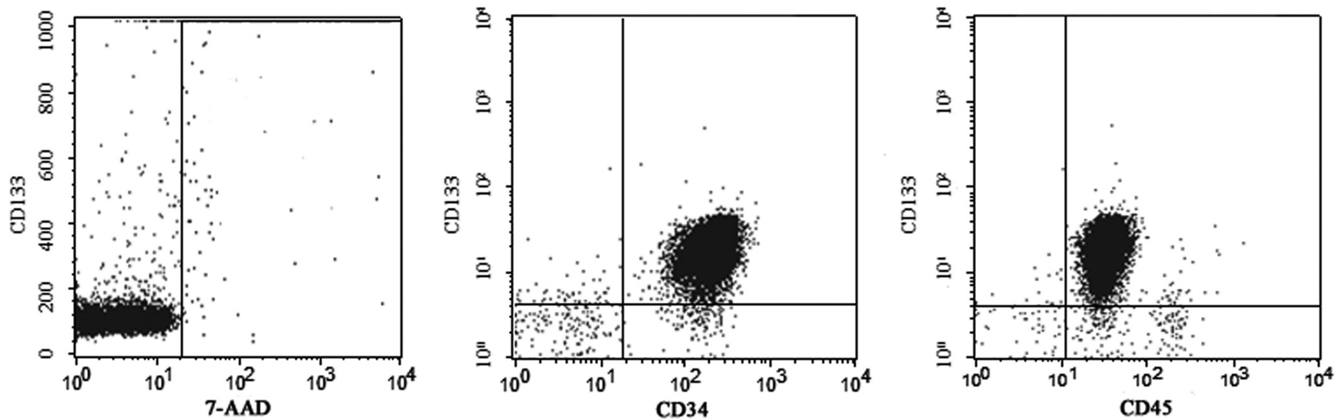
Statistics

The results are presented as mean and SD. Analysis of variance was used with two tails, assuming p<0.01 as significant.

Results

Freshly isolated cells

The average of total nucleated cells present in each sample of cord blood was $1.29x10^9$ cells (range $1.16x10^9$ - $1.35x10^9$) and the average of mononuclear cells obtained from each separation by density gradient



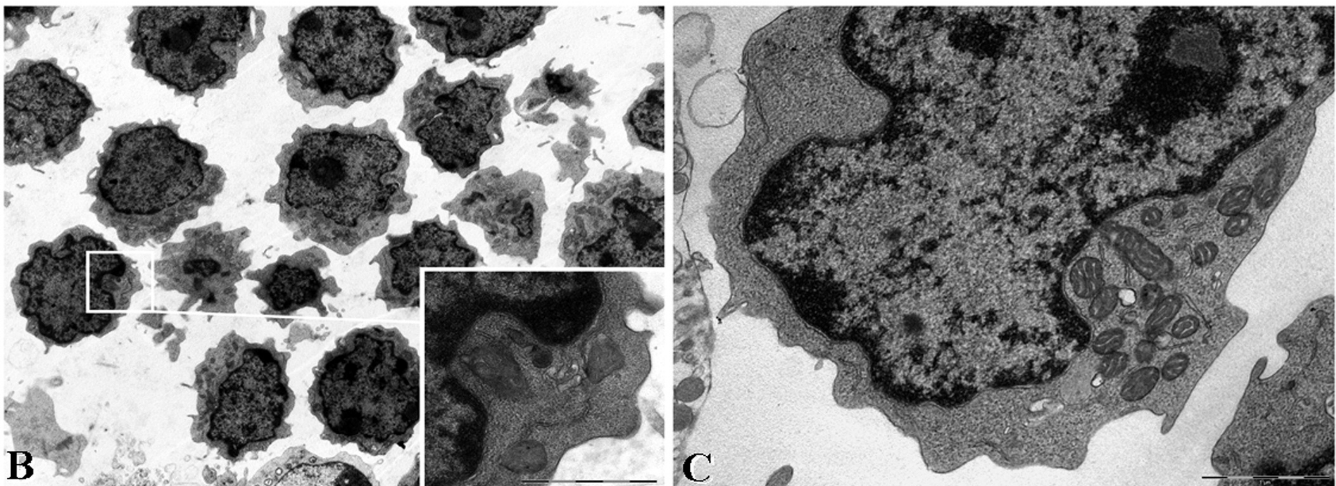


Fig. 1. Flow cytometry at the start of culture and electron microscopy of freshly isolated cells. **A.** Percentages of vital freshly isolated cells and expression of relevant membrane antigens. The simultaneous expression of CD133, CD34 and CD45 is characteristic of hematopoietic stem cells. **B.** CD133 positive cells at the start of culture. **C.** Particular of selected cells showing the slightly wavy surface, the abundance in ribosomes, a few mitochondria with longitudinal cristae and a nucleolus. Images were taken with analySIS 5.0 software through a MegaView III camera applied to a JEM 1010 electron microscope at 80 kV (Jeol, Tokyo, Japan), and were processed for print with Photoshop 6.0 for Macintosh (Adobe Systems Inc., San Jose, California). Scale bar: B, 5 μm; C, inset in B, 1 μm.

on Ficoll was 2.94x10^8 (range 1.70x10^8 - 3.78x10^8). Upon selection for CD133 of Ficoll isolated mononuclear cells, an average of 6.25x10^5 cells (range 4x10^5 - 8x10^5) were obtained from each donation. The CD133 positive cells were 96% (SD ±3) and viable cells were 97% (SD ±2). The CD133 positive cells which also expressed CD34 were around 96% (SD ±3) and those which also expressed CD45 were 99% (SD ±1; Fig. 1A). Seeded cells were a homogeneous population for each donor, characterized by small size, round shape and small, shallow indentures of the profile (Fig. 1B,C). At electron microscopy the cytoplasm was poor in organelles except for ribosomes; the Golgi apparatus was extremely small, with only few, narrow cisternae. The nucleus was round, with shallow indentures; the chromatin was finely granular, with a thin peripheral condensed rim and small, sparse chromocentres; the nucleolus was large and sometimes double (Fig. 1B,C). The nucleus-cytoplasmic ratio was high and similar among subjects. The ratio between the diameter of the perimeter equivalent-circumference and that of an area equivalent circle - an estimate of the degree of indenting - showed a mild deviation from circularity for both the cell as a whole and for the nucleus. The cell size and

Table 1. Morphometric features of freshly isolated CD133 positive cells.

Donor	A[cell] (μm^2)	D(eq circumf)/ D(eq circle) [cell]	A[nucleus]/ A[cytoplasm]	D(eq circumf)/ D(eq circle) [nucleus]	A[nucleoli] (μm^2)
#1	18±7	1.54±0.25	1.38±1.56	1.87±0.99	1.74±1.55
#2	17±6	1.53±0.32	1.30±0.78	2.02±1.45	0.85±0.29
#3	23±10	1.68±0.51	1.58±1.34	1.95±1.80	0.44±0.63
P among donors	<0.01	<0.01	not significant	not significant	<0.01

The number of cells analyzed for the various features, except nucleoli, were 139 for donor 1, 20 for donor 2 and 151 for donor 3; the analyzed nucleoli were 47 for donor 1, 8 for donor 2 and 22 for donor 3. The cell section surface area is representative of cell volume, cell D(eq circumf)/D(eq circle) ratio is representative of the degree of convolution of the cell surface, nucleolar section surface area is representative of nucleolar volume, cell A[nucleus]/A[cytoplasm] is representative of the volume of nucleus relative to cytoplasm and nuclear D(eq circumf)/D(eq circle) ratio is representative of the degree of convolution of the nuclear surface. (mean values ± standard deviation)

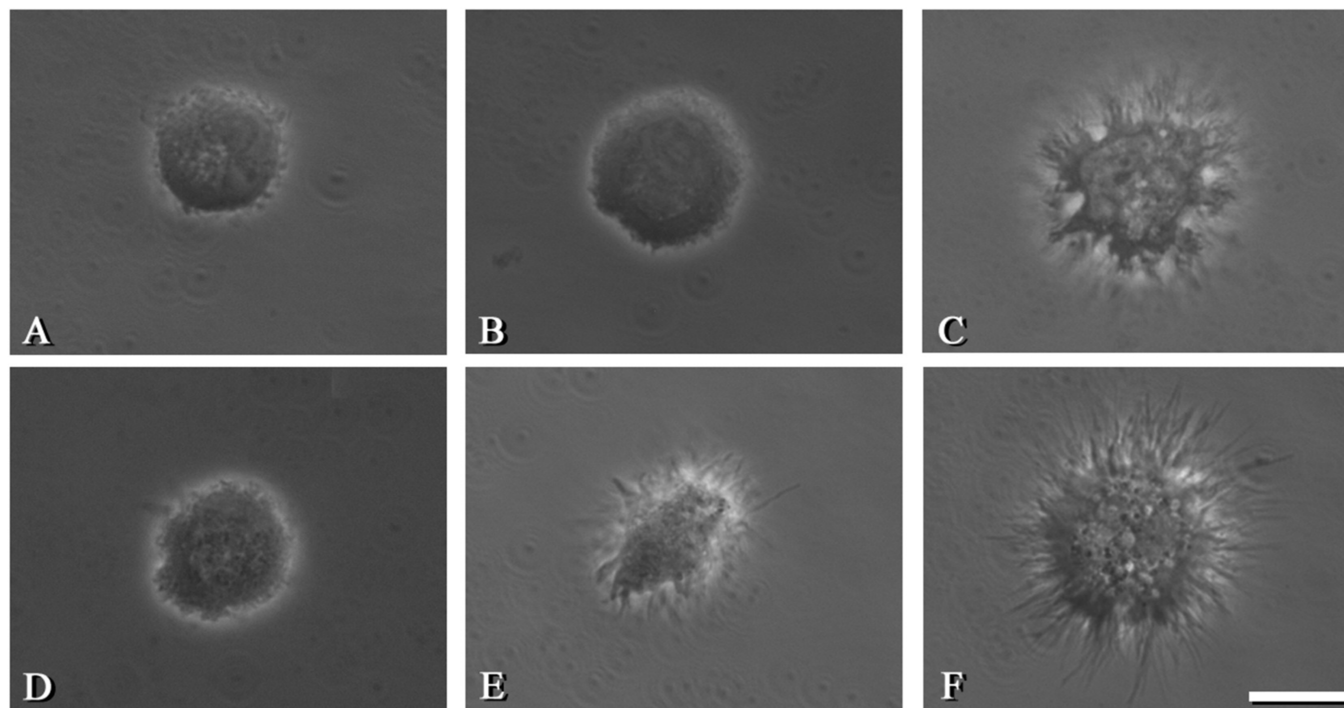


Fig. 2. Phase contrast microscopy. Phase contrast microscopy images of living cells cultured with (**A-C)** or without **(D-F)** rosiglitazone after 7 days culture (**A and D**), 14 days culture (**B and E)** and 18 days culture (**C and F**). Images were taken from slides mounted with 0.1 mol/L phosphate buffer saline at pH 7.4, with Axio Vision 4.1 software through an AxioCam HRm camera applied to an Axioskop microscope with a 40x/0.75 NA air objective (Zeiss, Oberkochen, Germany); they were processed for print with Photoshop 6.0 for Macintosh (Adobe). Scale bar: 20 μm.

degree of convolution were significantly different among subjects ($p<0.01$), while the size and degree of convolution of nuclei were similar among cases (Table 1). The volume of nucleoli relative to the nucleus varied between 1/6 and 1/32 ($p<0.01$ among subjects).

Cell culture

With rosiglitazone the cell growth was heavily impaired: the cells increased 1.01 times (range 0.43-2 times) after 7 days, then decreased to 0.65 times the starting number (range 0.23 - 1.5 times) at day 14 and to 0.37 times the starting number (range 0.13 - 0.83 times) at day 18. By comparison, without rosiglitazone the cells increased 7.25 times (range 6.21 - 8.21 times) after 7 days, then limited to 5.22 times the starting number (range 4.37 - 5.84 times) at day 14 and to 4.48 times the starting number (range 3.96 - 5.01 times) at day 18. Both with and without rosiglitazone the surviving cells adhered to the flask and acquired a dendritic shape from 7 day culture (Fig. 2A,D). Because of the few cells, we analysed only 14 day cultures by electron microscopy, since at this step the cells appeared morphologically mature, and 18 day cultures by flow cytometry and mixed leukocyte reactions.

At electron microscopy on 14 d culture, the cells

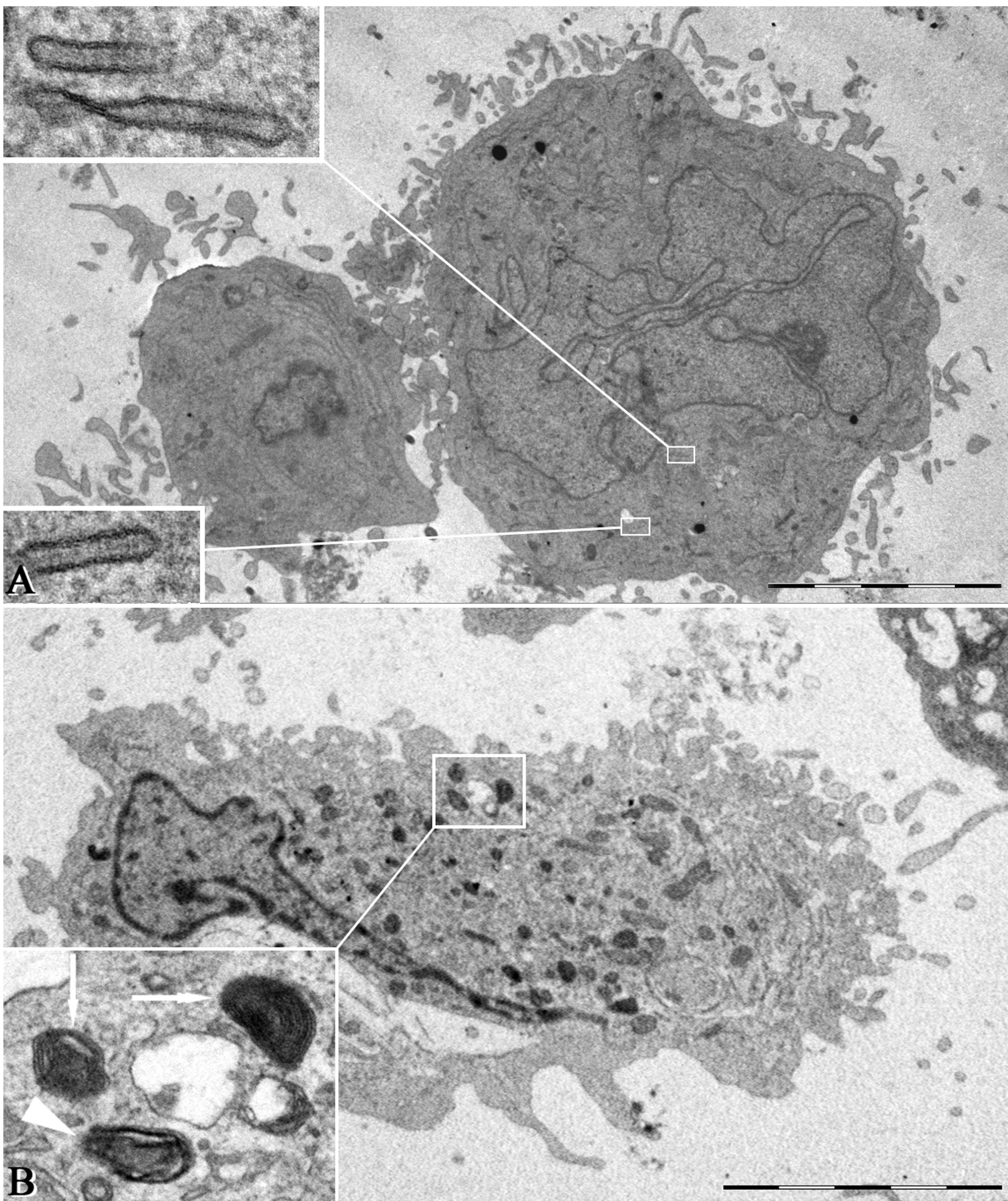


Fig. 3. Electron microscopy at day 14. **A.** Cells cultured 14 days with rosiglitazone. Birbeck granules were present in the cytoplasm (insets). **B.** Cells cultured 14 days without the PPAR-gamma agonist. Variably well developed multilaminar inclusions (arrows in inset) and myelin-like figures (arrowhead in inset). Images were taken with analySIS 5.0 software through a MegaView III camera applied to a JEM 1010 electron microscope at 80 kV (Jeol), and were processed for print with Photoshop 6.0 for Macintosh (Adobe). Scale bar: A, B, 5 μm; insets, 200 nm.

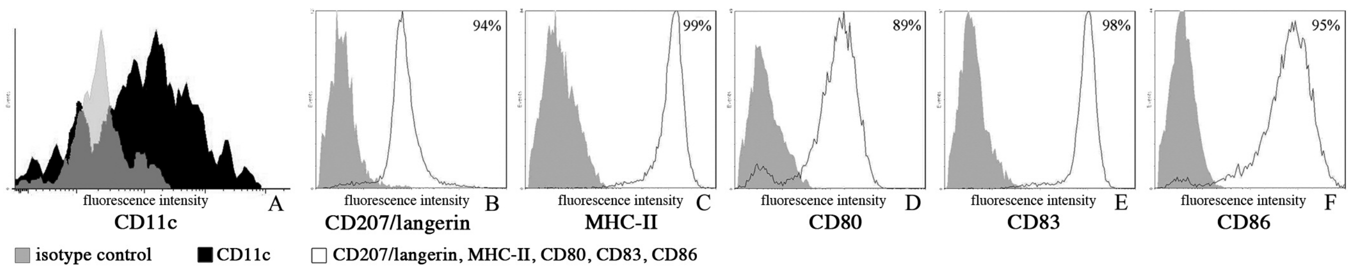


Fig. 4. Flow cytometry at day 18. Results of a representative experiment showing the expression of CD11c **(A)**, CD207/langerin **(B)**, MHC-II **(C)**, CD80 **(D)**, CD83 **(E)** and CD86 **(F)** by dendritic cells after 18 days culture with rosiglitazone.

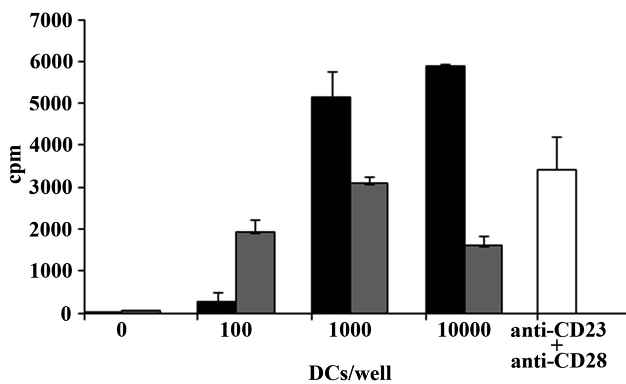


Fig. 5: Mixed leukocyte reaction. Results (mean and standard deviation of four independent measurements) of a representative mixed leukocyte reaction experiment with the indicated stimulatory cell numbers per 10^5 respondent cells. Cells cultured 18 days with (black columns) and without rosiglitazone (gray columns); 0 = no stimulatory cell; anti-CD23+anti-CD28 (white column) = lymphocytes stimulated only with the indicated antibodies. The differences between rosiglitazone treated and untreated cultures were significant for all stimulatory cell numbers.

cultured with rosiglitazone, similar to those cultured without the drug, appeared large with numerous, thin, branched cytoplasmic projections, the nucleus was deeply indented and with loose chromatin. However, with rosiglitazone many cells contained typical Birbeck granules (Fig. 3A) and only rare cells contained multilaminar inclusions, whereas the cells in control cultures contained many, electron dense lysosomes and multilaminar inclusions resembling class II compartments (Fig. 3B) and no Birbeck granules.

At the end of culture (18 d) with rosiglitazone, the surviving cells were very few, although dendritic in shape (Fig. 2C,F). Their dendritic cell nature was confirmed by the expression of CD11c by about 80% cells (Fig. 4A) and by the lack of expression of F4/80 (not shown). About 80% cells expressed CD207/langerin and showed an enhanced expression of stimulatory and co-stimulatory molecules as compared with control cultures: virtually all cells expressed MHC-II, and about 90% of cells expressed CD80, CD83 and CD86 (Fig. 4B-F), whereas in control cultures CD80, CD83 and CD86 were expressed by a lower percentage of cells and only little more than one third cells expressed the Langerhans cell marker CD207/langerin.

In mixed leukocyte reaction, the cells proved to be efficient stimulators of lymphocyte proliferation, more than cells cultured without rosiglitazone (Fig. 5).

Discussion

The present results show that CD133 positive hematopoietic precursors are lymphocyte-like, with mildly scalloped cell surface and nuclear profile, and that they vary in size and cell surface scalloping among isolates from different subjects, while being similar among subjects for nuclear shape, nucleus-cytoplasmic ratio and ultrastructure. More relevant, the results show that the addition since the start of culture of rosiglitazone, at a concentration comparable with that in clinical use, to a culture medium suitable for the generation of dendritic cells from CD133 positive precursors leads to the differentiation of only a small number of cells, which appear as full-blown Langerhans cells (LC) since they both express langerin and contain Birbeck granules, and are endowed with an even higher lymphocyte stimulating activity than those harvested without the PPAR-gamma agonist. To the best of our knowledge this is the first report of a selective generation of LC as a consequence of PPAR-gamma stimulation during culture of highly immature precursors. The effect of PPAR-gamma on cell number appeared to be mainly dependent on increased cell death, which was possibly correlated with the selection of a restricted population of differentiating cells of dendritic lineage, i.e. LC.

The cells selected for seeding were highly homogeneous for antigenic profile. The cell size and the convolution of cell surface were homogenous within each donor but varied among donors, indicating that these parameters are of limited value for cell identification, while the nucleus-cytoplasmic ratio, nuclear scalloping and ultrastructure were similar among different subjects. The extreme reduction of the endoplasmic reticulum and Golgi apparatus suggests a

low level of exocytosis and membrane turnover. The cell features are comparable with those shown for CD133 positive precursors by Pavon et al. (2008), who however did not address any morphometric parameter of these cells. Also, the isolation method used here prevented entirely the mild cell swelling which can be appreciated in the photomicrographs of Pavon et al. (2008). The morphometric data of the cells immediately upon isolation, i.e. of CD133 positive circulating cells, cannot be compared with similar data on immature progenitors, which are lacking in the literature. In a general way, the size and shape of cells shown here correspond to what is known for early progenitors and hematopoietic stem cells, including the irregularity - although mild - of the cell and nuclear profile (Pavon et al., 2008).

The morphologic and antigenic profile varied with culture: the cells showed a progressive loss of stem cell antigens, which was complete after 14 days culture, whereas other antigens came to be expressed indicating a differentiation towards a dendritic cell subtype. This interpretation is further supported by the expression of the Langerhans cell accessory marker CD11c (Chorro et al., 2009) and lack of expression of the macrophage marker F4/80 at the end of culture. We could confirm our previous findings that in the absence of PPAR-gamma stimulation a minority of these cells resembled LC in expressing langerin, but were without Birbeck granules (Bonetti et al., 2011). A similar immunophenotype had been previously shown for cultures of cells selected for CD34, with inconstant outcomes regarding Birbeck granules (see Bonetti et al., 2011, for review). The present findings indicate a dramatic influence of PPAR-gamma stimulation on the final outcome of the process when the starting population is made of CD133 positive cells, with the generation of few cells which are, in high percentages, langerin and Birbeck granule positive LC. The results may be correlated with the previously reported influence of PPAR-gamma stimulation on endocytosis and membrane recycling in immature (Szanto et al., 2004) and mature dendritic cells (Luo et al., 2004), since Birbeck granules are part of the endosomal recycling network (Mc Dermott et al., 2002). The increased expression of langerin and the presence of Birbeck granules in the cells generated with rosiglitazone, together with the increased expression of stimulatory and co-stimulatory molecules, are probably correlated with the enhanced ability of the generated cells to stimulate lymphocytes in mixed reaction.

Rosiglitazone had already been shown to influence the features of dendritic cells generated *in vitro* from circulating monocytes (Nencioni et al., 2002; Szatmari et al., 2007) and to impair the migration to lymph nodes and the development of a local immune response (Angeli et al., 2003). However most studies have focused on the final maturation, migration and function of dendritic cells rather than on the differentiation of dendritic cells from their progenitors. From the morphological standpoint, the finding of Birbeck granules in cells generated with rosiglitazone is at variance with the reported lack of morphological effects of that stimulation (Angeli et al., 2003; Szatmari et al., 2007), possibly because previous studies had not taken advantage of electron microscopy. The effect on differentiation seen here seems to reinforce the stimulation of TGF-beta towards the generation of a specific subtype of dendritic cells, i.e. LC. This suggests that, *in vivo*, endogenous stimulants of PPAR-gamma receptors may contribute to drive dendritic cell differentiation within stratified squamous epithelia, where this type of dendritic cell is found.

A thorough interpretation of the present findings is hampered by the still ongoing uncertainty on what exactly makes LC different from other sub-types of dendritic cells. LC specificity emerges from morphology, immunophenotype and cell cycle (Romani et al., 2010) and is probably correlated with a specific role in the response to low doses of antigens (Bacci et al., 1997; Mc Dermott et al., 2002; Fukunaga et al., 2008). The apparently contradictory effects of PPAR-gamma stimulation shown here between the number of generated cells and their immunostimulatory efficiency suggest that the effects of such a stimulation *in vivo* may differ among anatomical sites and among physiological and pathological conditions as a function of the locally prevailing dendritic cell type, of the degree of maturation of these cells and of concomitant stimuli.

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