A Sofia e Leonardo

UNIVERSITÀ
DEGLI STUDI
FIRENZE

DOTTORATO DI RICERCA IN
Scienze biomediche

CICLO XXIX

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Differentiation of early and late human hematopoietic progenitor cells into dendritic cells in vitro

Settore Scientifico Disciplinare BIO/17

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Anni 2013 / 2017

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Key to abbreviations

BDCA = blood dendritic cell antigen

CCL = chemokine (C-Cmotif) ligand

CCR = chemokine (C-C motif) receptor

CXCR = chemokine (C-X-C) receptor

CD = cluster of differentiation

CLA = cutaneous lymphocyte antigen

CLP = common lymphoid progenitor

CMP = common myeloid progenitor

DC = dendritic cell

DC-SIGN = dendritic cell-specific intercellular adhesion molecule-3-grabbing non integrin

Flt3-L = fms-like tyrosine kinase-3 ligand

GM-CSF = granulocyte-macrophage colony-stimulating factor

HLA = human leukocyte antigen

ICAM = intercellular adhesion molecule

IFN = interferon

IL = interleukin

LC = Langerhans cell

LPS = lipopolysaccaride

MCP = monocyte chemoattractant protein

M-CSF = macrophage colony-stimulating factor

mDC = myeloid dendritic cell

MHC = major histocompatibility complex

MHC-I = major histocompatibility complex class I molecules

MHC-II = major histocompatibility complex class II molecules

MIP = macrophage inflammatory protein

MMP = matrix metalloproteinase

NF-κB = nuclear factor κB

NK = natural killer

NLR = NOD-like receptors

NP = nanoparticle

pDC = plasmacytoid dendritic cell

PPAR = peroxisome proliferator-activated receptor

PPRE = peroxisome proliferator response elements

RANTES = regulated upon activation normal T cell expressed and secreted

RunX3 = runt-related transcription factor 3

RXR = retinoid X receptor

SCF = stem cell factor

SDF = stromal cell-derived factor

SPARC = secreted protein acid and rich in cystein

STAT = signal transducer and activator of transcription

TGF = transforming growth factor

TLR = Toll-like receptor

TNF = tumor necrosis factor

TPO = thrombopoietin

Abstract

Dendritic cells are specialized to capture antigens, process them and present them to T cells to initiate, regulate and fine tune immune responses towards pathogens and tumours. Therefore these cells play crucial roles in infection, cancer, allergy, autoimmunity and graft rejection. Besides immunity, they can cause anergy and trigger active tolerance, depending on the microenvironment conditions. Dendritic cells are heterogeneous for origin, anatomical localization, phenotype and function. Two subsets are recognized, myeloid and plasmacytoid dendritic cells. Myeloid cells include Langerhans cells, the dendritic cells of non-lymphoid connective tissue and those of lymphoid tissue and lymph. Langerhans cells are found in the epidermis and some mucosal epithelia and have an unique antigenic and morphological profile. They express langerin and contain special inclusions evident at the electron microscopy, *i.e.* Birbeck granules. The myeloid dendritic cells of non-lymphoid connective tissue express high levels of DC-SIGN/CD209.

In vivo, myeloid dendritic cells differentiate from haematopoietic stem cells-derived precursors and localize in peripheral tissues, afterwards they migrate from sites of antigen uptake to lymphoid organs and during this process mature to antigen presenting cells capable of interacting with lymphocytes and stimulate both immune response and peripheral tolerance to self. Dendritic cells express peroxisome proliferator-activated receptors (PPAR)-γ. These receptors are involved in many regulatory pathways, such as those regarding lipid, lipoprotein and glucose metabolism, inflammation, endothelial function, cancer and bone morphogenesis. The stimulation of PPAR-γ on dendritic cells *in vitro* has been reported to have controversial effects.

Knowledge of the stages and mechanisms of control of the differentiation of dendritic cells is a necessary premise to exercise control over these cells, useful for medical purposes. In this research the differentiation potential into myeloid dendritic cells *in vitro* and the effect of rosiglitazone, an agonist of PPAR-γ, on that differentiation have been evaluated for three different precursors collected from human peripheral blood, precisely CD14+ monocytes, CD34+ progenitors and CD133+ progenitors. Moreover, the cells differentiated from CD14+ monocytes were evaluated for their interaction with inorganic nanoparticles.

The cells were isolated from buffy coats obtained from healthy donors by magnetic separation, upon labelling with colloidal superparamagnetic microbeads conjugated with mouse anti-human CD14, CD34 or CD133 monoclonal antibodies, and were cultured with different cytokines depending on the cell type, for 9 d (CD14+ monocytes) or 18 d (CD34+ and CD133+ precursors); rosiglitazone (1 µmol/l) was added in some experiments; the differentiation stages and the characteristics of the cells generated at the end of culture were observed. Each experiment (with and without rosiglitazone) was made with cells of a single donor.

The generated cells were analyzed by light microscopy, electron microscopy, immunofluorescence, flow cytometry, mixed lymphocyte reaction and real-time PCR. Cells obtained from CD14+ monocytes after 7 d culture (*i.e.* immature dendritic cells) were incubated for up to 48 h with gold and silica nanoparticles, 10 and 100 µg/ml, and evaluated for their ability to uptake those nanoparticles and for the intercellular compartments of destination.

All precursors generated large dendritic cells that strongly expressed HLA-DR antigens and exhibited lymphocyte-stimulating activity in mixed lymphocyte reaction. Moreover, CD34+ and CD133+ precursors generated also medium size dendritic cells, with fewer organelles and less well developed dendrites than large dendritic cells, and

small, lymphocyte-like cells poor in organelles.

A proportion of large cells, increasing from those generated from CD14+ monocytes to those generated from CD34+ precursors to those generated from CD133+ precursors, expressed CD1a and langerin/CD207; in flow cytometry these cells were all included among those with higher side scatter, while large dendritic cells with low side scatter did not express langerin/CD207. Many cells from any culture expressed DC-SIGN/CD209, even together with langerin/CD207. Rudimentary Birbeck granules were observed only occasionally by electron microscopy in large dendritic cells from any precursor. Variable percentages of large dendritic cells expressed surface molecules related to maturation, the highest numbers were among cells generated from CD133+ precursors.

Rosiglitazone led to more mature features at electron microscopy in all cases (looser chromatin texture, increase in organelles especially lysosomes) and to more numerous cells expressing langerin/CD207 in cells generated from CD34+ and CD133+ precursors, the latter effect was significant for cells generated from CD133+ precursors.

Freshly isolated CD14+ monocytes expressed a low number of copies of mRNA for PPAR-γ; the expression increased during culture, independent of the addition of rosiglitazone. Freshly isolated CD34+ cells showed a much lower expression of PPAR-γ gene than CD14+ monocytes and the expression remained low throughout culture. Freshly isolated CD133+ cells showed a number of copies of mRNA for PPAR-γ higher than CD14+ monocytes; this expression increased during culture although with great variability among experiments; the response to rosiglitazone was also variable among experiments, with a trend towards increase of PPAR-γ mRNA.

Immature dendritic cells absorbed gold and silica nanoparticles by endocytosis and concentrated them in lysosomes; using fluorescent silica nanoparticles it was found that the amount of particles internalized depended on the concentration of particles in

the medium and reached a maximum within 4 h, then remained stable with time. The presence of nanoparticles and their absorption did not influence the structure, immunophenotype and lymphocyte-stimulating capacity of dendritic cells, as evaluated by electron microscopy, flow cytometry and mixed lymphocyte reaction respectively.

These results indicate that the differentiation potential of hematopoietic cells into myeloid dendritic cells with different phenotypes changes with the progression of spontaneous *in vivo* differentiation towards monocytes and that the specific orientation towards Langerhans cells begins very early. The differentiation degree achieved *in vitro* does not match entirely that seen *in vivo*, which indicates that more environmental factors are relevant in this respect than those that can be reproduced *in vitro*. CD133+ cells, which are the most immature progenitors, express PPAR-γ and, if transferred into culture, maintain this expression, that increases during differentiation. Therefore the possibility arises that PPAR-γ agonists or antagonists may be used to modulate this differentiation. Indeed, cells generated from all precursors tested in this research appeared to respond to the PPAR-γ agonist rosiglitazone at the morphological level, with signs of improved differentiation towards more mature dendritic cells. Immature myeloid dendritic cells proved able to incorporate inorganic nanoparticles without apparent damage, at least within the time of culture of the present experiments, which opens the way to explore the possible use of those particles in medicine.

Since dendritic cells play crucial roles in infection, cancer, allergy, autoimmunity and graft rejection, thorough knowledge of their subsets differentiation and behaviour will open the path to tools allowing better control of those cells in clinical conditions.

Introduction

The immune system grants for both innate and adaptive responses to pathogens, which depend on different cell types and functions. The innate immune system involves granulocytes, mast cells, macrophages and natural killer cells. These cells use non-clonal recognition receptors, including lectins, Toll-like receptors (TLRs), NOD-like receptors (NLRs) and helicases, to provide quick but generic responses to pathogens. The adaptive immune system response depends on B and CD4+ and CD8+ T cells which use clonal receptors that specifically recognize antigens or their derived peptides presented by antigen presenting cells and grant for immunological memory (Paluka and Banchereau, 2012).

Dendritic cells (DCs), macrophages and B cells may act as antigen presenting cells; DCs, in particular, are specifically devoted to capture and process antigen since primary response. DCs express several specialized molecules, as the major histocompatibility complex (MHC) and a combination of costimulatory molecules and adhesion molecules for the presentation of antigen to T cells in order to initiate the immune response. They are also known as dendritic leukocytes and are the most efficient cells for antigen presentation, being able to elicit T cell response even in very low numbers and to stimulate primary as well as secondary response, thus explaining their nickname of “professional antigen presenting cells” (Steinman, 2012). The interaction between DCs and lymphocytes during the initiation of an adaptive immune response is one of the most important control points to ensure that the immune response is correct and to direct this response towards reaction or tolerance to pathogenic organisms (van Panhuys, 2017) and tumours. DCs are also responsible for the

peripheral tolerance to self and play crucial roles in allergy, autoimmunity and transplant rejection.

Owing to their properties, DCs are often called “nature adjuvants” and have been proposed for immunotherapy upon antigen loading (Von Bubnoff et al., 2001; Paluka and Banchereau, 2012). However, clinical trials have not yet gone beyond preliminary phases and the limited knowledge on the biology of these cells, both alone and in the context of tissues, may well be a cause of failure. Indeed cancer therapeutic strategies using DC-based immunotherapy alone or in combination with radiation therapy and chemotherapy developed until now have been only partially effective, mainly because there are no techniques yet to monitor the behaviour of DCs *in vivo* (Ahn et al., 2017). The more advanced strategy is the use of DC-based vaccines, consisting in DCs loaded with tumour antigens, that are infused into the patient and generate anti-tumour T-cell responses from CD8+ effector T-cells (Kumar et al., 2017).

Despite their origin from CD34+ bone marrow hematopoietic stem cells, DCs are heterogeneous and many subsets have been identified both in mice and humans (Bancherau and Steinman, 1998; Paluka and Banchereau, 2012; Steinman, 2012). The most important DC subsets are myeloid DCs (mDCs) and plasmacytoid DCs (pDCs) (Dzionek et al., 2000; Shigematsu et al., 2004). The latter are so called because of well developed rough endoplasmic reticulum and less prominent cell projections as compared with mDCs. Knowledge of pDC cell biology is less advanced than that of mDCs.

Among mDCs, Langerhans cells (LCs) are peculiar for localization, molecular phenotype and morphology. They are in an immunologically immature state and localize within epidermis and stratified squamous mucosal epithelia where they can efficiently take up and process a wide variety of antigens, including contact sensitizers, tumor-associated and microbial antigens. LCs express E-cadherin, CD1a and

langerin/CD207 and contain Birbeck granules, a unique organelle detected at electron microscopy (Valladeau et al., 2000; Romani et al., 2003). E-cadherin is used to remain in contact with surrounding epithelial cells (Romani et al., 2010). CD1a is a pattern recognition receptor for lipoarabinomannan, a non-polymorphic bacterial molecule to be presented to T lymphocytes (Adams 2014). Langerin/CD207 is a c-type lectin localized not only at the cell surface, but also in the endosomal recycling compartment, in Birbeck granules and in other tubular and vesicular structures (Valladeau et al., 2000; Romani et al., 2003). Due to their position and the various types of antigens recognized, LCs are critical for the induction of immune responses towards pathogens and tumours and are credited to play a pathogenic role in allergic skin diseases (Paluka and Banchereau, 2012 , Von Bubnoff et al., 2001).

Dendritic cells history

The story of these cells began almost one and a half century ago and the interest for them is ever growing because of their central role in immunobiology.

In 1868 Paul Langerhans described for the first time the dendritic epidermal cells which were thereafter designated with his name. The LC have been indeed the first DC discovered, other DCs have then been found within lymphoid organs, epithelia, connective tissue and lymph (reviewed by Pimpinelli et al., 1994). In the 1950s LCs were considered as melanocytes that had lost the capacity to make melanin pigment (Masson, 1948; Breathnach, 1965). At the beginning of the following decade Birbeck et al. (1961) demonstrated the presence of LCs in vitiligo, showing that they were not melanocytes, and by electron microscopy identified a characteristic organelle, which thence on has been designated Birbeck granule.

Recognition of DCs as a population specific for differentiation and function has come through three distinct lines of research (Hoefsmit et al., 1982; Fossum, 1989; Metlay et al., 1989).

A first line of research, started in the 1970s, was represented by microscopic studies on lymphoid organs and tumours. These studies were aimed at defining the proliferative diseases of the lymphoid organs and included a detailed description of the cell types present in the lymphoid organs. Cells with dendritic shape were identified among the stromal elements of the T-dependent areas of secondary lymphoid organs (Veldman, 1970; Kaiserling and Lennert, 1974). These cells have cytoplasmic extensions that make contact with other similar cells and lymphocytes, for this feature have been called interdigitating cells or interdigitating reticulum cells. Veerman (1974) proposed that the interdigitating cells in the thymus-dependent area of the rat spleen form a microenvironment allowing T cells to differentiate and proliferate. Bone marrow precursors give rise to these cells, that migrate into lymphoid organs via peripheral tissues and lymphatics, becoming interdigitating reticulum cells (Kelly et al., 1978; Hoefsmit et al., 1979). A role has been proposed for these cells also in directing the structural organization of lymphoid tissue in T-cell lymphomas (Ribuffo et al., 1983), including those of the skin (Romagnoli et al., 1986; Pimpinelli et al., 1994).

A second line of research was the histopathological analysis of dermatological immune-mediated diseases, where LCs were found in contact with lymphocytes in the inflamed skin (Silberberg-Sinakin, 1973; Silberberg et al., 1976). It was concluded that LCs are implicated in allergic reactions and in other immunologic reactions, so that they were regarded as cells of immune system (Rowden et al., 1977; Stingl et al., 1977); they were also recognized to derive from progenitors present in the bone marrow (Katz et al., 1979; Perreault et al., 1984; Goordyal and Isaacson, 1985). Studies in the mouse and humans demonstrated the antigen-presenting function of LCs (Stingl et al., 1978;

Braathen and Thorsby 1980; Green et al., 1980; Bjercke et al., 1984). LCs were eventually recognized as members of the DC family of the immune system (see below) and their life cycle was analyzed in the mouse (Schuler et al., 1985).

Steinman's group (Steinman and Cohn, 1973, 1974) started a third line of research, isolating cells with a peculiar dendritic shape from the mouse spleen, hence their designation as dendritic cells. It was then demonstrated that DCs present exogenous antigen to T cells in an MHC-restricted fashion and can induce specific cytolytic T cell responses by cross-priming lymphocytes (Bevan, 1976). These cells have a low phagocytic activity and stimulate T-dependent immune responses very efficiently, in particular the proliferation and maturation of helper T cells to effectors elements. Although they are not the only cells in the body with this property, DCs seem to be mandatory for the stimulation of primary immune responses (reviewed by Metlay et al., 1989). High expression of major histocompatibility complex class II molecules (MHC-II), of which the principal representative in humans is HLA-DR, is instrumental to that function (Steinman e Witmer, 1978 Steinman et al., 1979) even if it is not enough to explain their differential role in respect to other MHC-II expressing cells.

Human DCs in peripheral blood were characterized in detail by Van Voorhis et al. (1982). It was also reported that the T cell areas in lymphoid organs are the main location where DCs are found and activate T cells which, once activated, may leave the lymph node and migrate to sites of inflammation (Witmer et al., 1984). Indeed, DCs were discovered to be responsible for the activation of naïve T cells, that subsequently interact with B cells and stimulate them to generate plasma cells and produce antibodies (Inaba and Steinman, 1985). Witmer-Pack et al. (1987) documented the requirement of granulocyte-macrophage colony stimulating factor (GM-CSF) for DC maturation. Moreover, it was shown that T cells fail to proliferate upon antigen presentation in the

absence of co-stimulatory signals and instead become unresponsive to further activation (Jenkins and Schwartz, 1987).

Later on, cells with abundant rough endoplasmic reticulum (like plasma cells) but with irregular cell surface were shown to behave as antigen presenting cells and to be able to secrete huge amounts of type I interferons (IFN; Fitzergerald-Bocarsly, 1988; Facchetti et al., 1989). They were first designated plasmacytoid T cells or plasmacytoid monocytes and later on have been recognized as a peculiar type of DCs, named pDCs (Cella et al., 2000; Colonna et al., 2002; see McKenna et al., 2005, for review), which are - among others - associated with protection from opportunistic infections (Siegal et al., 1999). The other DCs, including LCs, have been designated mDCs (Banchereau and Steinman, 1998). pDCs have been associated with protection from opportunistic infections (Siegal et al., 1999).

In the last decade of the XX century it was observed that DCs are also able to effectively stimulate the activation of CD8+ cytotoxic T lymphocytes (Young and Steinman, 1990; Shen et al., 1997), the differentiation of plasma cells from B lymphocytes (Fayette et al., 1998) and the survival of plasmablasts (De Vinuesa et al., 1999). The B7-1/CD80 and B7-2/CD86 ligands and their receptors on T cells, respectively CD28 and CTLA-4 (cytotoxic T lymphocyte antigen-4), were identified as crucial regulators of T cell responses and additional co-stimulatory molecules were identified on DCs (Springer, 1990; Caux et al., 1994a; Caux et al., 1994b). A method was devised to obtain DCs from monocytes (Sallusto and Lanzavecchia, 1994), allowing to study the basic functions of DCs in humans. Also, studies in apparently different model organisms led to the identification of TLR, first in the fruit fly and then in the mouse (Lemaitre et al., 1996; Poltorak et al., 1998), and DCs were shown to express these receptors, possibly implied in antigen recognition and capture (Medzhitov et al., 1997). It was eventually shown that DCs are involved also in tolerance and

immune regulation (Banchereau and Steinman, 1998), although the details on when these functions, instead of immune activation, occur and how they are regulated remain still unclear.

In the new millennium, the calcium dependent, C-type lectin receptor (CLR) DC-SIGN (dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin) was found to be expressed by mDCs and to mediate transient adhesion with T cells, necessary for their activation (Geijtenbeek et al., 2000a, 2000b). C-type lectin receptors (CLRs) are an important family of pattern recognition receptors: they interact with pathogens through the recognition of mannose, fucose and glucan carbohydrate structures and are involved in the induction of specific gene expression profiles in response to specific pathogens, either by modulating TLR signalling or by directly inducing gene expression (Geijtenbeek, 2009).

Plasmacytoid DCs were shown to regulate events during the course of viral infections, atopy, autoimmune disease, and metastatic cancer (Liu, 2005; McKenna et al., 2005).

Given their potential to stimulate both innate and adaptive antitumor response, during the past few decades DCs have been the subject of numerous studies seeking new immunotherapeutic strategies against cancer (Kumar et al.2017). The basic concept behind the use of DCs in immunotherapy relies on the intrinsic antigen-presenting properties of DCs to elicit a potent tumour antigen-specific T-cell-driven immune response (Figdor et al., 2004; Tacken et al., 2007; Palucka and Banchereau, 2012; Klechevsky and Banchereau, 2013; Tel et al., 2013). DCs have also been used in nanoparticle (NP)-based immunotherapeutic strategies trying to take advantage of such particles - most frequently organic - to deliver immunogenic molecules to antigen presenting cells. Little information is available regarding inorganic-NP-mediated DCs stimulation (Shen et al. 2016).

Maturation and function of dendritic cells

Migration of dendritic cell precursors and dendritic cells through body compartments

Myeloid DCs differentiate to an immature, antigen absorbing phenotype and then proceed to a mature, lymphocyte stimulating phenotype. The precursors of mDCs enter tissues and quickly differentiate to immature mDCs. Immature DCs are characterized by a high capacity for antigen capture and processing and low T cell stimulatory ability, because they express low levels of co-stimulatory molecules and have a limited capacity to secrete cytokines (Engering et al., 1997; Palucka and Banchereau, 2012). Circulating DC precursors may also directly encounter pathogens, which induce secretion of cytokines that in turn can activate eosinophils, macrophages and NK cells (Palucka and Banchereau, 2012). After antigen capture, DCs migrate to lymphoid organs MHC-II molecules on the cell surface together with co-stimulatory molecules, leading to the selection of circulating antigen-specific lymphocytes. During this process DCs lose the ability to absorb antigens and come to express the co-stimulatory molecules CD54, CD80 and CD86 together with increased amounts of MHC-II, as well as a peculiar chemokine ()C-C motif) receptor (CCR), precisely CCR7, that allows them to localize to lymphoid tissues. Maturation requires the ligation of CD40 and is associated with the expression of the maturation marker CD83 and the acquisition of the ability to secrete cytokines (Cella et al., 1997; Steiman, 2012). Once arrived to lymphoid tissue, DCs localize in the para-follicular zone and differentiate into interdigitating cells to activate naive T-cells. DCs are able to polarize the immune response to either Th1 o Th2 types and to improve T cell memory (Steinman et al., 2003) by the production of different cytokines that determine the classes of immune

response (Bancherau et al., 2000). Furthermore a few DCs move to the germinal centers where they collaborate with T lymphocytes to initiate B cell mediated immune responses. DCs can activate naïve and memory B cells (Bancherau et al., 2000) and induce the differentiation of activated-naïve B cells to plasma cells by the secretion of interleukin (IL)-12 and IL-6 (Dubois et al., 1998).

In turn, activated T cells help DCs to achieve terminal maturation, further promoting the expansion and differentiation of lymphocytes themselves. It is believed that, after interaction with lymphocytes, DCs die by apoptosis (Banchereau et al., 2000).

Interaction between DCs and T cells during primary responses occurs within lymphoid tissue, which is reached by naïve T cells from the circulation. During secondary T cell-mediated responses, DCs can interact with lymphocytes also in peripheral and inflamed tissues (Fujita et al., 2011; Eyerich and Novak, 2013; Trucci et al., 2013), also on account of the fact that memory T lymphocytes circulate also through peripheral tissues (Shin et al., 2013).

The maturation of pDCs occurs before entering the circulation; the steps and regulation of this process are still under investigation. Swiecki and Colonna (2015) have emphasized that pDCs express MHC-II and the co-stimulatory molecules CD40, CD80 and CD86 and can present antigens to CD4+ T cells, albeit not as efficiently as myeloid DCs (Reizis et al., 2011); when pDCs receive activation signals through TLRs or other pattern recognition receptors, they act as immunogenic cells.

Antigen capture

Antigen capture may occur in several ways. Immature DCs can internalize large amounts of material through macropinocytosis, through fluid phase and receptor-mediated endocytosis (Engering et al., 1997; Mommas et al., 1999), and through

phagocytosis of apoptotic and necrotic cell fragments (Albert et al., 1998), viruses, bacteria and parasites (Inaba et al., 1993; Rescigno et al., 1999; Banchereau et al., 2000).

Receptors that have been identified on DCs include lectins such as the mannose receptor langerin/CD207, DC-SIGN/CD209 and DEC-205/CD205, Fc receptors such as FcεRI, FcγR type I (CD64) and type II (CD32) (Engering et al., 1997; Banchereau et al., 2000), and TLRs (Erbacher et al., 2009). The mannose receptor, langerin/CD207 (Romani et al., 2003) and DC-SIGN/CD209 (Geijtenbeek et al., 2000) contain multiple carbohydrate-binding domains; mannose receptor in particular is involved in the internalization of sugar-containing proteins and mediates phagocytosis of various microorganisms which expose mannosylated glycoproteins (Engering et al., 1997). DEC-205/CD205 has in its extracellular portion a cysteine-rich domain, a fibronectin type II domain and multiple C-type lectin-like domains and in the cytoplasmic portion an amino acid motif which efficiently targets the endocytosed molecule to late endosomes/class II compartments (Mahnke et al., 2000; Erbacher et al., 2009). TLRs are able to recognize a broad spectrum of different pathogen-associated molecular patterns, including conserved components of protozoa, bacteria, fungi, and viruses (Takeda and Akira, 2005). The recognition of microbial components by DC TLRs results in the expression of inflammatory cytokines and co-stimulatory molecules followed by antigen presentation, linking innate to acquired immunity. FcγRI/CD64 and FcγRII/CD32 are members of a family of cell surface molecules which includes also FcγRIII/CD16 and bind the Fc portion of Ig G (van de Winkel et al., 1993). Binding to FcγRs leads to endocytose immune complexes or opsonized particles (Fanger et al., 1996; Amigorena et al., 1998; Döbel et al., 2013). CD1 proteins are known as non-classical antigen-presenting molecules and bind microbial lipid and glycolipid antigens to present them to T cells (Peiser et al., 2003; Porcelli and Modlin, 1999). Four CD1

proteins (CD1a-d) are expressed by mDCs; LCs express almost exclusively CD1a and CD1c (Elder et al., 1993; Banchereau et al., 2000); humans pDC do not express CD1d (Montoya et al., 2006). The phagocytosis of apoptotic cells is restricted to the immature stage of DC development, and this process is accompanied by the expression of some receptors as CD36, that recognizes apoptotic cells (Savil, 1997; Albert et al., 1998). Upon maturation, these receptors and the phagocytic capacity of DCs are downmodulated (Albert et al., 1998).

The phagocytosis of uninfected, apoptotic cells may lead to prevent immune responses against self antigens within or on the surface of dying cells (Fadeel et al., 2010); the responsible cells (macrophages, but possibly also DCs) would acquire a "pro-resolution" phenotype and protect against firing inflammation. Alternatively, the phagocytosis of apoptotic cells which hosted infectious agents is critical for the induction of a subset of T cells involved in host defense against infections, which may also become involved in autoimmunity and tissue inflammation (Torchinsky et al., 2009).

Antigen processing and presentation

Antigens captured by immature DCs are degraded in endosomes. The generated polypeptides are transported into the MHC class II-rich compartment (which is part of the endosomal compartment) and are loaded on HLA-DR II molecules, while DCs mature. Mature DCs reduce their endocytic capacity in favour of high surface levels of MHC and co-stimulatory molecules, which make them capable of activating even naïve T lymphocytes. The peptide-MHC-II complex is exported to the cell surface, where it remains stable for days and is available for recognition by $CD4^+$ T cells (Chow and Mellman, 2005; Cresswell, 2005). DCs can also load nascent MHC-I molecules with antigenic peptides which derive not only from newly synthesized molecules but also

from controlled proteolysis of exogenous ones in the proteasome followed by transfer into the endoplasmic reticulum through TAP (transporter associated with antigen processing: Reits et al., 2000). The peptide-MHC-I complex is transferred to the cell surface and is recognized by CD8+ T cells (Banchereau et al., 2000), leading to cross-priming (Ackerman and Cresswell, 2004; Whiteside et al., 2004). The pathway from endosome to cytosol, where the proteasome is located, is still obscure. Recognition of MHC-peptide complexes on DCs by antigen-specific T cell receptor (TCR) constitutes the primary signal in DC-T cell interaction, necessary to stimulate a T-lymphocyte response (Hart, 1997). The activation of T cells is facilitated by the interaction of membrane molecules expressed by these cells, LFA-1 (a CD11b CD18 heterodimer) and CD2, with co-receptors expressed by antigen presenting cells, respectively LFA-3/CD58 and ICAM-1/CD54 (Wang et al., 2003). These bonds are in synergy with TCR-mediated signals and promote efficient intercellular adhesion, T cell proliferation and differentiation (Zumwalde et al., 2013). Although presentation of antigen to TCR is necessary for the initiation of T cell activation, further co-stimulatory signals delivered by antigen presenting cells (and also by neighbour cells) are needed for full-blown immune response (Caux et al., 1994a). Interactions between co-stimulatory molecules expressed by DCs and their ligands expressed by T cells are among those signals (Banchereau et al., 2000). In turn, ligation of CD40 on DCs by CD40-ligand (CD40L/CD154) expressed by lymphocytes stimulates the maturation of DCs, with the consequent expression of high levels of MHC-II, CD80 (B7-1) and CD86 (B7-2), the latter two molecules are ligands for CD28 on lymphocytes and stimulate secretion of cytokines by the latter cells (Caux et al., 1994a). Contemporaneous block of the interactions mediated by CD54 and CD80 or CD86 leads to anergy (Caux et al., 1994b; Wang et al., 2003; Palucka and Banchereau, 2012).

Dendritic cells and regulation of tolerance

Besides being immunostimulatory, DCs also induce tolerance in the thymus (central tolerance) and in the periphery (peripheral tolerance). In the thymus this depends on deletion of autoreactive T cells and differentiation of CD4+ T lymphocytes to CD4+ CD25+ Treg cells. DCs present self-antigens to thymocytes in the thymic medulla: if T cells have too high affinity for self-antigens, such as occurs with autoreactive T cells, they are deleted by negative selection (Bancherau and Steinman, 1998; Rathmel and Thompson, 2002).

In peripheral lymphoid organs anergy or active tolerance may be induced (Bancherau and Steinman, 1998; Steinman et al., 2003). If DCs come to present an antigen while remaining immature, *e.g.* because of antigen load in the absence of inflammatory signals, antigen-specific CD4+ and CD8+ T cells undergo transient activation and proliferation followed by deletion which establishes antigen-specific T cell tolerance (Probst et al., 2014). In these immature, or semi-mature conditions DCs express the Th1-inducing cytokine IL-12, which is part of a tolerogenic phenotype of these cells (Steinman et al., 2003). T cells may go into apoptosis induced via Fas/Fas-ligand pathway (Süss and Shortman, 1996) or through activation of the pro-apoptotic mitochondrial protein Bim (Chen et al., 2007). Another pathway leading to tolerance is activation of CD4+ CD25+ Treg cells, which provide active suppression of response to self and to not-self antigens (Steinman et al., 2003; Fehérvari and Sakaguchi, 2004); also these cells are stimulated by DCs (Probst et al., 2014).

Immune suppression is elicited by Treg cells through secretion of soluble factors, such as the immunosuppressive cytokines IL-10 and transforming growth factor (TGF)-β, and through intimate cell contact (Fehérvari and Sakaguchi, 2004). Among

other effects, IL-10 and TGF-β inhibit the maturation of DCs. Treg cells express on their cell surface molecules, such as CTLA-4/CD152 and lymphocyte activation gene 3 protein (LAG3/CD223), that suppress DC activation. CTLA4/CD152 mediates the down regulation of its ligands, the co-stimulatory molecules CD80 and CD86 on DCs - that are capable of interacting both with CTLA4/CD152 and CD28 (Carreno et al., 2002) - or even the trans-endocytosis of their extracellular domain by lymphocytes, and triggers the induction of the enzyme indoleamine 2,3-dioxygenase (IDO) (Fallarino et al. 2003; Probst et al., 2014) that catalyzes the conversion of tryptophan to kynurenine and other metabolites which have potent, short-range immunosuppressive effects (Fehérvari and Sakaguchi, 2004). LAG3/CD223 is a CD4-related transmembrane protein that binds MHC-II on DCs and suppresses their maturation and immunostimulatory capacity (Liang et al., 2008). Finally, Treg stimulate an increase in the cytoplasmic levels of cyclic adenosine monophosphate (cAMP) in DCs and thus suppress their activation. This may occur by transfer of cAMP from Treg cells to DCs via gap junctions (Probst et al., 2014) and through the generation of pericellular adenosine from extracellular nucleotides by the ectoenzymes CD39 and CD73 (Probst et al., 2014). The effects of extracellular adenosine are mediated by the four members of the P1 purinergic G-protein coupled receptor (GPCR) family (A1, A2A, A2B and A3), which are expressed on both mDCs and pDCs (Hasko et al., 2008). Binding to A2A and A2B causes stimulation of adenylate cyclase and subsequent increase in intra-cellular cAMP, whereas A1 and A3 have an opposing effect (Challier et al., 2013). A2A receptor activation on mature DCs shifts their cytokine profile from a pro-inflammatory to an anti-inflammatory one, with increased production of IL-10 (Hasko et al., 2008).

An example of physiological immune tolerance occurs in the gut, where Treg cells maintain immune homeostasis and prevent effector cells from causing immunopathology in response to commensal bacteria. Here macrophages secrete IL-1β

upon sensing microbial products in the gut; this cytokine triggers a subset of innate lymphoid cells (ILC), precisely ILC3, to produce GM-CSF; and GM-CSF exposed DCs release retinoic acid, which promotes the generation of Treg cells (Aychek and Jung, 2014). ILCs are found in the intestine, lung, skin and liver and are divided into three groups depending on the cytokines secreted; ILC3 secrete mainly IL-17 and IL-22 (Hazenberg at al., 2014).

The mechanisms of tolerance are activated also during pregnancy and allow conception, embryo implantation and foetus development (Hsu and Nanan, 2014; Schumacher and Zenclussen, 2014). The foetus is semiallogeneic since it expresses antigens of both maternal and paternal origin and the immune system of pregnant women must defend both the mother and foetus from foreign pathogens while tolerating the semiallogeneic foetus. It has been shown that maternal immune T cells specifically recognize paternal alloantigens but pregnancy induces a state of transient T cell tolerance specific for those antigens (Tafuri et al., 1995; La Rocca et al., 2014). The exposure of the female reproductive tract to seminal fluid can promote paternal antigen-specific hyporesponsiveness, inducing a state of transient active immune tolerance in the mother (La Rocca et al., 2014). The seminal fluid contains potent immune-regulatory molecules such as TGF-beta and PGE2-related prostaglandins, which promote the generation of Treg from CD4+ CD25- T cells (Robertson et al., 2009) and the conversion of DCs to a tolerogenic phenotype (Schumacher and Zenclussen, 2014). The continuous release of conceptus-derived paternal antigens into the maternal circulation allows Treg cells to continue emerging and expanding throughout pregnancy, migrate to the uterus and contribute to the generation of a friendly environment for the embryo. In peripheral blood, Treg cells are likely involved in the suppression of maternal effector T cells responsive to foetal antigens (Schumacher and Zenclussen, 2014).

Some tissues are endowed with an immune privilege, so that foreign antigens do not induce a conventional immune response; specifically this happens in the eye and brain (Medawar, 1961). DCs and macrophages come to the eye through blood and are localized in some ocular tissues, predominantly the uvea (iris, ciliary body and choroid); few cells enter the peripheral retina and cornea. When ocular tissues are perturbed, bone marrow-derived cells carrying antigen from the eye are found in the lymph nodes and the antigens are detected even in the spleen after several hours (Forrester and Xu, 2012). Nonetheless, the local response may be low or absent, in order to preserve the transparency of the eye. Several mechanisms have been proposed to explain ocular immune privilege, including induction of Treg cells, the expression of molecules such as Fas or CTLA4/CD152 and the secretion of cytokines that generate an immunosuppressive microenvironment (Stein-Streilein, 2008; Denniston et al., 2011; Wang et al., 2011).

Subsets of dendritic cells

Dendritic cells derive from hematopoietic stem cells (see reviews by Steiman, 1991, and Cella et al., 1997). DC progenitors in the bone marrow give rise to circulating precursors that home to tissues, where they reside as immature cells (Banchereau et al., 2000). DCs are a heterogeneous cell population whose members may acquire diverse maturation states and functions (Schmidt et al., 2012). As anticipated, in both humans and mice two distinct types of DCs have been identified, mDCs and pDCs. The origin of the former is from common myeloid progenitor cells, while that of the latter has yet to be conclusively defined (Bancherau and Steinman, 1998; Dzionek et al., 2000; Shigematsu et al., 2004).

During differentiation DCs may go through an immature and a mature phase. The transition of mDCs to maturity is marked by modifications of the immunophenotype and the acquisition of strong immunostimulatory power (Villadangos and Heath 2005; Johnson and Ohashi, 2013). LCs are part of the immature DCs (Shortman and Caux, 1997; Hubert et al., 2005). pDCs appear in blood as already mature (McKenna et al., 2005).

Myeloid dendritic cells

Myeloid CD34+ progenitors differentiate into monocytes (CD11c+ CD14+ DC precursors) that yield immature mDCs in response to GM-CSF and IL-4 (Sallusto and Lanzavecchia, 1994), and macrophages in response to macrophage colony stimulating factor (M-CSF).

Myeloid DCs typically express CD11c, MHC-II (in our species, human leukocyte antigen-D Related, *i.e.* HLA-DR), CD33 and CD40, moreover upon maturation they express CD83 and the co-stimulatory molecules CD54, CD80 and CD86 (Freeman et al., 1995; Banchereau et al., 2000). They are specialized for antigen presentation through MHC-II to CD4+ T-cells (Banchereau et al., 2000; Lewis and Reizis, 2012), but can also present antigens to CD8+ T-cells and interact with natural killer (NK) cells (Albert et al., 1998; Moretta et al., 2003).

Immature mDCs may be found in any connective tissue proper; a subset, *i.e.* LCs are found also within epithelia (see below). Mature mDCs reside in T areas of the lymphoid tissue including the spleen white pulp (Banchereau et al., 2000).

Peripheral blood contains mDC precursors (Banchereau and Steinman, 1998), immature (Bancherau et al., 2000) and mature mDCs (Franks et al., 2013), pDCs (Liu, 2005) and so called inflammatory DCs (see below; Schäkel et al., 2002). The subsets of

DCs in peripheral blood may be distinguished by the differential expression of three cell-surface molecules: CD1c, also know as BDCA-1, CD303 or BDCA-2, CD141 or BDCA-3 and CD304 or BDCA-4. BDCA-1/CD1c is expressed by mDCs (Palucka and Banchereau, 2012). The expression of BDCA-2/CD303 and BDCA-4/CD304 is confined to pDCs, as described below (Bancherau et al., 2000; Dzionek et al., 2000; Palucka and Banchereau, 2012; Franks et al., 2013). BDCA-3/CD141 is expressed by mDCs with high capacity to capture exogenous antigens for presentation on MHC-I class molecules, such as epidermal Langherans cells, also described later.

In stratified squamous epithelia of the skin and mucosae mDCs are represented by LCs, which express E-cadherin, CD1a and langerin/CD207, and in connective tissue there are interstitial DCs, which express CD1c, DC-SIGN/CD209, DEC205 and, sometimes, also CD1a. There are phenotypic and functional differences between these two cell types, as detailed below (Nestle et al., 1993; Palucka and Banchereau, 2012; Klechevsky and Banchereau, 2013). mDCs are also observed in lymph vessels as veiled cells and in lymphoid tissue as interdigitating cells, as detailed below.

Langerhans cells

Langerhans cells are located in stratified squamous epithelia of the epidermis and mucosae. They have an oval body and some long branches. The nucleus is indented, with pale chromatin except for a thin peripheral rim. The cytoplasm contains cytoskeletal intermediate filaments not clustered into fibrils, mitochondria, few cisternae of rough endoplasmic reticulum, many smooth vesicles and tubules, a large Golgi apparatus and Birbeck granules (Montagna and Parrakkal, 1974).

The Birbeck granules are disc shaped with a central paracrystalline array of particles and a dilated rim along part of their margin, so in transverse section they

appear as rods, with parallel membranes on either side enclosing a central striated lamella, and are sometimes dilated at an end, resembling a tennis racket (Birbeck et al., 1961 and Wolff, 1967; Rodriguez and Carosi, 1978).

Birbeck granules are found exclusively in LCs of human and other mammals, but not in other DCs. They are part of an endocitic pathway and are implicated in antigen processing. Antilangerin antibodies are internalized upon binding to the cell surface, and delivered to Birbeck granules (Valladeau et al., 2000). Some authors believe that these inclusions also traffic between the endosomal compartment and the plasma membrane along the recycling route (McDermott, 2002). Birbeck granules are closely associated with the expression of langerin/CD207; transfection of langerin cDNA leads to Birbeck granule formation in fibroblasts (Valladeau et al., 2000), however not all langerin positive cells exhibit Birbeck granules when observed at electron microscopy (see below).

Langerin/CD207 is a mannose-specific C-type lectin (Romani et al., 2003); an intracellular epitope of this molecule is Lag antigen (Valladeau et al., 2000). Langerin/CD207 recognizes mannose, n-acetylglucosamine, fucose and sulfated sugars; it binds microbial fragments and pathogens such as HIV and Candida albicans (Mizumoto and Takashima 2004; Idoyagaet al., 2008). Remarkably, langerin/CD207 can be found in cells also 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).

Another characteristic marker of LCs is CD1a, that has been identified before langerin/CD207; it recognizes lipid antigens and can participate in the uptake and presentation of non-polymorphic molecules of bacterial cell wall (Mizumoto and Takashima 2004). CD1a is transported from the cell surface to early recycling vescicles and Birbeck granules; glycolipid antigens captured by langerin/CD207 are also

internalized to these granules, where CD1a is coupled with lipid moieties. This pathway enables LCs to uptake specific lipid and glycolipid antigens and to present them via CD1a to T cells (Hunger et al., 2004).

This indicates that LCs have a role in the presentation of non-peptide antigens to T cells, mediated by the LC-specific pattern-recognition receptor langerin/CD207 as well as the antigen-presenting molecule CD1a. The location of LCs in the epidermis allows the innate immune system to respond rapidly to microbial invaders in the skin (Hunger et al., 2004). Targeting of non-peptide antigens to LCs may therefore become a vaccine strategy that could be used for the generation of immunity to cutaneous pathogens.

Langerhans cells express other molecules such as the myeloid markers CD13 and CD33 (Valladeau et al., 2005), DEC205/CD205, DCIR (dendritic cell immunoreceptor)/CLEC4A (C-type lectin domain family 1 member-4A)/CD367, TLRs including TLR 1, 2, 3, 6 and 10 (respectively CD281, CD282, CD283, CD286, CD290), the high affinity receptor for the crystallizable fragment of immunoglobulins G (FcγRI/CD64a), that for the third component of the complement system (C3R/CD11b CD18), interleukin-2 receptor (IL2R/CD25 CD122 CD132), MHC class I (HLA-A, B and C in humans) and class II (HLA-D, DR, DP in humans) molecules, the lymphocyte common antigen CD45, ICAM-1/CD54, the integrin molecules of the CD11/CD18 family - *i.e.* CD11a, CD11b, CD11c and CD18 - (see for review: Romagnoli et al., 1991; Klechevsky and Banchereau, 2013) and E-cadherin/CD324, which mediates the binding of LCs to keratinocytes in epidermis (Jakob et al., 1999), plays a role in cell migration and is expressed even by some DCs in the blood (Blauvelt et al., 1995). The presence of E-cadherin/CD324 may be important also for adherence to carcinoma epithelial cells. LCs also express the cutaneous lymphocyte antigen (CLA/CD162)

which is a ligand for E-selectin/CD62E (Koszik et al., 1994), that in basal conditions is specifically expressed by skin endothelial cells (Ley, 2003).

As already stated, myeloid CD34+ progenitors differentiate into monocytes (CD11c+ CD14+ DC precursors) that yield immature DCs in response to GM-CSF and IL-4 (Sallusto and Lanzavecchia, 1994), and macrophages in response to M-CSF. Myeloid progenitors also differentiate into CD11c+ CD14- precursors, which yield LCs in response to GM-CSF, IL-4 and TGF-β, and macrophages in response to M-CSF (Bancherau et al., 2000). Myeloid progenitors can differentiate into LCs also through CD1a+ CD14- precursors, generated upon five days culture of CD34+ cells with GM-CSF and tumour necrosis factor (TNF)-α, by continuing culture with the same factors for seven more days (Caux et al., 1996; Cella et al., 1997). A research of Geissmann et al. (1998) has shown that also CD14+ monocytes cultured with GM-CSF, IL-4 and TGF-β can give rise to LCs containing Birbeck granules, however this result has not been confirmed by other studies. In the literature the differentiation of LCs from monocytes has given inconstant results, and most often the generated cells did not show a strong polarization of differentiation into LCs. Good results have been obtained in the differentiation of LCs starting from CD34+ or CD133+ stem cells, isolated from cord blood (Hubert et al., 2005; Bonetti et al., 2014). The differentiation of LCs is dependent on TGF-beta, as indicated by the fact that TGF-beta 1 knockout mice totally lack epidermal LCs while bone marrow cells from these mice can give rise to LCs after transfer into wild type recipients. Thus, the lack of LCs in TGF-beta 1-deficient mice is not a result of a defect in bone marrow precursors but of the production of TGF-beta 1 within the microenvironment where LC differentiate (Borkowski et al., 1996).

Therefore it is actually conceived that DCs may arise from dedicated circulating precursors in steady state ("classical" or "conventional" DCs) and from monocytes in inflammation (Kushwah and Hu, 2011).

The bone marrow precursors of the LCs pass into the circulation, migrate into tissues through the wall of blood vessels and enter epithelia crossing the basement membrane (reviewed by Romagnoli et al., 1991). The influence of the epithelial microenvironment induces the maturation of precursor cells into LCs; it cannot be excluded that CD1 antigen may be acquired by these cells even before they enter the epithelium, while Birbeck granules appear after this entry (Bani et al., 1988). In situ, some cells express low levels of CD40, CD83 and CD86, but not CD80. Isolated LCs express high levels of all these antigens, which reflect enhanced expression during cell isolation (McLellan et al., 1998). LCs behave as immature DCs, able to capture antigens but expressing low levels of co-stimulatory molecules and with a limited capacity to secrete cytokines (Paluka and Banchereau, 2012). These cells acquire co-stimulatory molecules and secrete abundant cytokines upon maturation, a process that occurs during migration of these cells from the epidermis to lymph nodes and is accompanied by down-regulation of E-cadherin/CD324 and langerin/CD207 and loss of Birbeck granules (Tang et al., 1993; Romani et al., 2001; Van den Bossche et al., 2012), not always complete (Schuler, 1991).

Langerhans cells are also capable of transferring endocytosed antigens into the cytoplasm, process them and eventually present them on the cell membrane within MHC-I class molecules, to stimulate CD8+ T lymphocytes (Palucka and Banchereau, 2012). LCs stimulate CD8+ T responses also through secretion of IL-15, that is critical for promoting differentiation of naive CD8+ T cells into granzyme B+ cytotoxic lymphocytes (Klechevsky and Banchereau, 2013). The turn over in the epidermis is not high: LCs in the epidermis are long-living. This may in part be attributed to the longevity of individual LCs within the epidermal environment, in part to a low level of cell division in the periphery (Romani et al., 2003). Blood-borne precursors are recruited to the epidermis in large numbers only in inflammatory states of the skin: two

cytokines are critical for immigration of LC precursors into the inflamed skin: TGF-β and macrophage inflammatory protein (MIP)-3α/chemokine (C-C motif) ligand (CCL)20 (Borkowski et al., 1996; Merad et al., 2002; Schmuth et al., 2002). MIP-3α is produced by keratinocytes and LC precursors express the appropriate receptor, CCR6/CD196 (Dieu et al., 1998).

Dendritic cells of non-lymphoid connective tissue

The normal human dermis hosts highly dendritic cells (Headington, 1986). Some of them express factor XIIIa in normal and diseased skin (Cerio et al., 1989; Nestle et al., 1993; Muszbek et al., 2011). The normal skin contains three separate populations of dermal DCs, which have distinctive phenotypic markers. Nestle et colleagues (1993) showed that factor XIIIa positive dermal DCs may be negative for CD1a and CD14, positive for CD1a only or positive for CD14 only and that the expression of CD1a is anyway weaker than that of LCs. CD14+ dermal DCs express C-type lectins, including DC-SIGN/CD209, LOX-1 (lectin-like oxidized LDL receptor-1), CLEC-6 (C-type lectin domain family 1 member-6)/CLEC4D/CD368, CLEC7A/dectin-1/CD369, DCIR/CLEC4A/CD367; they also express TLRs, such as TLR2, 4, 5, 6, 8, 10/CD282, CD284, CD285, CD286, CD288, CD290 (Klechevsky et al., 2009). The DC-SIGN/CD209 expression is not restricted to DCs in normal dermis but is expressed also by macrophages together with other monocyte/macrophage markers, as CD14, CD68, CD163, and CD206 (Ochoa et al., 2008; Töröcsik et al., 2014). The cytotoxic lymphocyte response induced by CD14+ dermal DCs is less potent than that induced by LCs. In addition, CD4+ T cells primed by CD14+ dermal DCs are unique in their ability to promote antibody response, either directly or by priming T follicular helper (T_{FH})-like CD4+ T cells (Klechevsky and Banchereau, 2013). CD14+ dermal DCs secrete IL-10,

that limits cytotoxic lymphocyte response and induces regulatory T cells (Treg or Tr), and express Ig-like transcript (ILT)-2 and ILT-4, inhibitors of CD8 binding to MHC-I because of sterical hindrance. Conversely, CD1a+ dermal DCs, like LCs, secrete IL-15 in immunological synapses, inducing a cytotoxic response (Klechevsky and Banchereau, 2013). Moreover, even if both LCs and dermal DCs are effective in stimulating immune responses, LCs seem to be particularly effective in stimulating responses to low concentrations of antigens, while dermal DCs are primarily responsible for the response to high dose haptens (Bacci et al., 1997). Low concentrations of antigens may be found in the early stages of viral infection and carcinogenesis (Romagnoli, 2001).

Dendritic cells in lymph and lymphoid organs

During maturation DCs come to express CCR7/CD197 that allows their localization to lymphoid tissue, upon migration through lymph vessels and lymphoid tissue in order to efficiently launch immune responses (Paluka and Banchereau, 2012). While in transit through lymph vessels, DCs acquire a peculiar morphology with wide lamellipodia, for which they have been called veiled cells (Hoefsmit et al., 1982; Bancherau et al., 2000). During maturation DCs process the captured antigen into small fragments which are then conjugated with MHC-II and MHC-I class molecules for the presentation to CD4+ and respectively CD8+ T cells in order to initiate the immune response (Paluka and Banchereau, 2012; Bancherau and Steinman, 1998).

In the spleen there are marginal DCs and in the T-cell rich areas of lymph nodes there are interdigitating reticulum cells, which present antigens to T-lymphocytes (Bancherau et al., 2000; Palucka and Banchereau, 2012). The name evokes the cell shape, indeed they have long branches running among lymphocytes. The nucleus is large, convoluted and lined by a thin band of chromatin (Veldman and Kaiserling, 1980;

Steinman et al., 1997). LCs, peripheral connective tissue DCs, veiled cells and interdigitating reticulum cells are all closely related. The latter two types may contain a few Birbeck granules when they come from the epidermis and mucosal squamous stratified epithelia (Schuler et al., 1991).

Dendritic cells in the thymus

Dendritic cells are present also in the thymus, where they are key players during thymocyte development because they present self antigens and induce negative selection of potential auto-reactive T-cell clones (Vandenabeel et al., 2001). Those cells are localized almost exclusively in the medulla (Brocker, 1999; Steinman et al., 2003), which seems to be a major site of deletion of thymocytes upon positive selection (Hengartner et al., 1988; Sprent and Webb, 1995). Thymic DCs are presumably comparable to those of other tissues for antigen capture and processing. This would lead to the production of MHC-peptide complexes, needed to delete self-reactive T cells; indeed, the cells that express a T-cell receptor (TCR) with specificity for self antigens are removed (Spits, 2001; Steinman et al., 2003). The human thymus contains two populations of mature DCs, expressing different lineage markers and displaying different capacities for IL-12 secretion; the latter is necessary for the negative selection of thymocytes (Lúdvíksson et al., 1999). The major thymic DC population is distinguished by being CD11b- and expresses markers of fully mature DCs. The minor thymic DC population is CD11b+. Both populations are CD4+ (Vandenabeel et al., 2001). After CD40 ligation only CD11b- thymic DCs release substantial amounts of IL-12, while CD11b+ DCs release scarce amounts of this cytokine (Lúdvíksson et al., 1999; Vandenabeel et al., 2001).

Inflammatory dendritic cells

Inflammatory DCs can be found in peripheral blood and in tissues upon inflammation (Hespel and Moser, 2012). They have been designated as 6-sulfo acetyl-N-lactosamine $(LacNAc)^+$ DCs ('slanDCs') based on the expression of that residue on the P-selectin glycoprotein ligand 1 membrane molecule, also known as M-DC8 (Schäkel et al., 2002)/CD162. They express transcription factors involved in DC differentiation, including DC specific ZBTB46 (Satpathy et al., 2012), and their differentiation does not depend on GM-CSF, at variance with conventional DCs (Greter et al., 2012). Inflammatory DCs are able to induce the differentiation of Th17 lymphocytes (Segura et al., 2013) and take part in adaptive and innate immunity (Hespel and Moser, 2012; Segura and Amigorena, 2013).

Plasmacytoid dendritic cells

The CD11c- CD14- DCs, or pDCs, spontaneously express high levels of DC-SIGN/DEC-205 and do not express CD1d; on mDCs these molecules are upregulated upon in vitro culture or LPS treatment (Vremec and Shortman, 1997). Moreover, pDCs regularly express CD11b, CD32 (FcRγ), CD123 (IL-3R) and - in mice - DC specific 33D1 antigen (Nussenzweig et al., 1982; Dudziak et al., 2007; Benitez-Ribas et al., 2008). Initially pDCs were considered to derive from lymphoid CD34+ progenitor cells (Bancherau et al., 2000; Shigematsu et al., 2004), but it has been shown that they are produced in the bone marrow and, in steady-state conditions, circulate in the blood and migrate to lymphoid tissues. At variance with mDCs, they exit the bone marrow already fully differentiated (Sawai et al., 2013). They represent 0.2%-0.8% of peripheral blood mononuclear cells (Liu, 2005) and can migrate to and accumulate in inflammatory sites where they may contribute to the inflammatory response by releasing cytokines and

chemokines and activating lymphocytes (Zhang and Wang, 2005). In physiological conditions pDCs are localized in the spleen white pulp and in lymph nodes but not in peripheral tissues (Liu et al., 2005). Circulating pDCs express BDCA-2/CD303 and BDCA-4/CD304, which are expressed also on pDCs in the bone marrow. BDCA-2/CD303 is a novel type II C-type lectin that can internalize antigen for presentation to T cells (Dzionek et al., 2001), BDCA-4/CD304 is identical to neuropilin-1, a neuronal receptor recognizing both the axon guidance factors belonging to the class-3 semaphorin subfamily and vascular endothelial growth factor (VEGF) and expressed also on endothelial and tumour cells (Dzionek et al., 2002). Its role in pDCs is still obscure. Moreover pDCs express TLR7, 8 and 9/CD287, CD288, CD289 (Zhang and Wang, 2005). These cells represent a front line of anti-viral immunity owing to their ability to secrete large amounts of IFNα in response to viruses (Siegal et al., 1999). At an early stage of viral infection, pDCs produce large amount of interferon (IFN)-α and -β (type I IFN; Dzionek et al., 2000) and can then differentiate into antigen presenting cells (Siegal et al., 1999; Zhang and Wang, 2005). The pDC-derived IFNα may also promote the activation of NK cells and the maturation of other subsets of DCs, thus helping to activate novel T and B cell clones (Zhang and Wang, 2005; Palucka and Banchereau, 2012). IFN-α also has an autocrine effect (McKenna et al., 2005). pDCs appear highly efficient at cross-presenting antigens to CD8+ T lymphocytes, using pre-synthesized stores of MHC-I molecules to stimulate a rapid cytotoxic response to viral infections; moreover they upregulate the expression of MHC-II molecules by mDCs and induce the maturation of these cells to antigen presenting cells thanks to the secretion of IFN-α and TNF-α. pDCs can also be tolerogenic by stimulating CD4+ CD25+ Treg cells (Banchereau and Steinman, 1998; Liu, 2005; Zhang and Wang, 2005; Dudziak et al., 2007; Palucka and Banchereau, 2012), which may be particularly important in case of intracellular infections and tumours (McKenna et al., 2005). Some studies have reported

that pDCs recruited to the tumour microenvironment often display a non-activated state and are associated with tumour progression and poor outcome, especially in cases of breast, cancer, lung and skin cancer (Treilleux et al., 2004; Jensen et al., 2012; Labidi-Galy et al., 2012; Rega et al., 2013).

Dendritic cells and disease

Langerhans cells in pathology

Neoplasia

Substantial alterations in the frequency, distribution, and phenotype of LCs occur in pathological conditions.

Langerhans cell histiocytosis results from the clonal proliferation of immunophenotypically and functionally immature, round LCs. This conditions is characterised by inflammatory lesions containing abundant CD1a positive and CD207-positive cells (Haroche et al., 2017), that show high expression of costimulatory molecules (Laman et al., 2003) and contain Birbeck granules which are pathognomonic of the disease (Harrist et al., 1983).

Oral squamous cell carcinoma is the most frequent malignant tumour of the oral cavity and the sixth most common cancer in the world. It may develop from oral epithelial dysplasia. LCs from the oral epithelium can present tumour antigens to T cells, activating an anti-tumour response. The LC counts in normal, dysplastic and cancerous oral epithelia have given controversial results (Wang et al., 2017). A gradual decline in LC number has been reported at first during the progression of oral carcinogenesis (Upadhyay et al., 2012), while further studies have described a gradual

increase in LC number from normal oral mucosa to dysplasia to squamous cell carcinoma (Costa et al., 2016). Wang et colleagues (2017) found a significantly lower LC number in oral dysplastic lesions with malignant transformation than in those without malignant transformation. A high number of LCs in the latter lesions indicates that the patient has the ability to elicit an immune response against the dysplastic cells. Thus, a low LC number in the lesions progressing to cancer suggests at least a partial loss of immunosurveillance against dysplastic cells; this in turn favours the malignant transformation of a dysplastic lesion into cancer. The authors interpreted the findings to indicate that the patient has the ability to elicit an immune response against the dysplastic cells and that a drop in that response is related to at least partial loss of immunosurveillance against dysplastic cells which may favour the malignant transformation. The issue is still open to debate, because the above reported results of Costa et al. (2016) remain contradictory to the latter hypothesis.

Malignant melanoma can lead to changes in LC population thus altering the host-against-tumour response. Already thirty years ago it was proved that melanoma-associated LCs decline in number as melanoma progress (Stene et al., 1988). Melanoma is one of the most aggressive forms of human cancer and is considered one of the most immunogenic tumours: it has been suggested that the number and the maturation state of LCs could correlate positively with an overall better prognosis of the patient (Neagu et al., 2013; Dyduch et al., 2017). Immature or incompletely matured DCs, in turn, may mediate tolerance instead of immune activation (Toriyama et al., 1993).

A study *in vitro* on LCs generated from CD34+ hematopoietic stem cells has shown that melanoma-derived factors may play a role at an early LC differentiation stage, preventing the generation of LCs from their precursors. Thus, CD34+ progenitors leaving the blood circulation and entering the dermis at the tumour site would be unable to generate LCs and repopulate the epidermis because of the inhibitory factors secreted

by melanoma cells (Berthier-Vergnes et al., 2001). The observations that dermal LCs are not found around deeply invasive melanomas (Stene et al, 1988) while tumour infiltrating CD1 cells are detected in the dermis deep to melanoma (Toriyama et al, 1993) reinforce this hypothesis. The naturally occurring LC turnover thus appears severely impaired inducing a decline in the number of epidermal LCs (Berthier-Vergnes et al., 2001). The regression of primary cutaneous melanoma has been associated with the presence of LCs infiltrating the tumour (Ma et al., 2013).

Another type of analysis on cells taken from sentinel lymph nodes of melanoma patients has shown that LCs cells in those nodes have feature of immature rather than mature LCs (Gerlini et al., 2012a).

Viral diseases

Dendritic cells have a major role in immune defence against viral infection by generating and regulating innate and adaptive immune responses (Randolph et al., 2005; Woodham et al. 2016). LCs have been investigated in relation to viral diseases because of their proximity to pathogen entry portals of the epidermis and mucosae (Merad et al., 2008), including sexually transmitted ones (Botting et al., 2017).

Human immunodeficiency virus-1 (HIV-1) can infect DCs and can transmit the virus to lymphocytes which in turn can exfoliate the virus in the environment. This virus therefore manipulates the natural function of DCs to interact with CD4+ T cells, which are the main target of HIV and in which rapid replication occurs (Ahmed et al., 2015; Botting et al., 2017). The receptors used by HIV to enter DCs and LCs are CD4, that is a primary receptor for glycoprotein envelope gp120, and CCR5/CD195 or CXCR4/CD184 that can act as coreceptors. Entry through these pathways can yield productive infections. Alternatively, HIV has been shown to interact with DC-SIGN/CD209 and langerin/CD207 at the surface of DCs and LCs respectively.

Association with DC-SIGN/CD209 promotes HIV infection, binding gp120 and cointernalizing the virus (Woodham et al. 2016); this can result in viral transmission to T cells via trans-infection, through immune synapse (Woodham et al. 2016). HIV has been detected by immunoelectron microscopy in Birbeck granules, presumably upon adhesion to langerin/CD207 (de Witte et al., 2007). In patients with HIV infection the onset of opportunistic cutaneous and oral infections is more likely, as facial and perioral molluscum contagiosum or oral candidiasis; the latter opportunistic infection is presumably favoured by alterations in number and differentiation state of lymphocytes and LCs that have been found in these patients (Ficarra et al., 1994; Romagnoli et al., 1997).

Molluscum contagiosum and other viral infection, such as warts and condyloma acuminatum, may depend on interactions between virus and LCs for the pathogenesis (Bhawan et al., 1986). Molluscum contagiosum is caused by a poxvirus (Epstein, 1992; Gold and Moiin, 2007; Leung at al., 2017) and the lesions are usually small, discrete, pearly and dome-shaped with central umbilication, but atypical and rare varieties could be found in some patients, in whom the lesions may be large, confluent or non-umbilicated (Yin and Li, 2017). A total absence of Langerhans and indeterminate cells was observed in molluscum contagiosum, while their number appeared to be normal or increased in the perilesional normal skin (Bhawan et al., 1986).

Warts are caused by human papilloma viruses (a double-stranded DNA virus) and quite common (Gold and Moiin, 2007; Thappa and Chiramel, 2016). Warts are clinically identified as papular or nodular structures that have a horny layer on their surface; they can range from 1-2 mm to several centimetres in size and may become confluent leading to the appearance of even larger lesions; viral replication takes place in differentiated keratinocytes in or above the stratum granulosum (Gold and Moiin, 2007). In verruca vulgaris and plantar warts few LCs and indeterminate cells were seen

in the suprabasal location, while normal numbers were noted in the perilesional skin (Bhawan et al., 1986). Jackson et al. (1994) studied epidermal LC number in the warts, in 75% of cases their number was reduced in comparison with normal skin. A decrease in LCs and a lack of adhesion molecule expression in the epidermis of warts may lead to a reduced influx of T cells into the lesion itself, although T cells are present in the dermis underlying the infected site (Jackson et al., 1994). Thus the reduction of LCs may lead to a poor immune response (in particular, T cell-mediated immunity) to the virus in the epidermis (Bhawan et al., 1986). It is also possible that alterations of keratinocytes due to viral infection affect the migration of LCs to the epidermis.

Condyloma acuminatum is induced by human papillomavirus and is characterized by the proliferation of stratified squamous epithelial tissue; it affects the anogenital and oral mucosa (Morelli et al., 1994; Curi et al., 2017). In a study of 20 condylomata of the vulva the intraepithelial LCs showed abnormal morphology and a significantly lower number density than controls. CD1a positive LCs were observed in the underlying dermis, suggesting an abnormal epithelial traffic of DCs. T lymphocytes were the main cellular infiltrate in vulvar papillomavirus infection (Morelli et al., 1994). A study of Giomi et al. (2011) confirmed the suitability of the treatment with aminolevulinic acid and photo-dynamic therapy for the treatment of condylomata and showed that LCs and CD4+ and CD8+ lymphocytes infiltrate heavily the underlying dermis in the responding patients. This seems to temper or even eliminate viral infection in more than half of the treated patients, generally within one month from the beginning of treatment. Stimulation of DCs with imidazolquinoline may be helpful in the treatment of condylomata in HIV infected patients (Cyru et al., 2017).

Dendritic cells in therapy

Given the role of DCs in immunity, the manipulation of skin-derived DCs has a potential to generate defensive immunity or for inducing tolerance in a number of disease conditions (Banchereau and Paluka, 2005).

Immunotherapy can be defined as the treatment of disease by inducing, enhancing or suppressing the immune response (Galluzzi et al., 2014). Immunotherapy can be active or passive (Lesterhuis et al., 2011). In passive immunotherapy, preformed antigen-targeting monoclonal antibodies or T cells endowed with intrinsic anti-target cell activity are transferred to the patient. In active immunotherapy, vaccines and checkpoint inhibitors exert effects mediated by the action of the host immune cells. DC-based vaccines are a form of active immunotherapy (Galluzzi et al., 2014; Coosemans et al., 2015).

In recent years, there has been a growing interest towards cancer immunotherapy, taking advantage of the host immune system to fight cancer by regulating the induction of protective immunity (Steinman and Mellman, 2004; Banchereau and Paluka, 2005). In search of the best vaccination strategies, attention has been drawn also on DCs (Kantoff et al., 2010; van Dinther et al., 2017).

Several forms of DC-based immunotherapy have been developed, most of which involve the isolation of patient- or donor-derived circulating CD14+ monocytes or CD34+ progenitors and their amplification/differentiation ex vivo followed by maturation; the latter step is particularly important because immature DCs exert immunosuppressive, rather than immunostimulatory functions (Palucka and Banchereau 2012). These cells can be re-infused into cancer patients upon exposure to tumour associated antigens (TAAs); exposure may be directly to TAA derived peptides (Mayordomo et al., 1995; Hirayama and Nishimura, 2016), bulk cancer cell lysates (of autologous or heterologous derivation) (Fields et al., 1998; Fucikova et al., 2011), bulk

cancer cell-derived mRNA (Boczkowski et al. 2000), vectors coding for one or more specific TAAs (Irvine et al., 2000), or mRNAs coding for one or more specific TAAs (Zeis et al., 2003).

The use of DCs in immunotherapy against cancer is still a difficult task, since the conditions for their activation as immune stimulating cells are still largely unclear and cells not properly activated may be totally ineffective to elicit a protective response or even counter-productive (Figdor et al., 2004).

Considerable clinical research has focused on improving systemic treatments for melanoma, that is immunogenic and, on the contrary, is generally resistant to standard chemotherapy after surgical excision which remains the primary treatment option. Complete spontaneous regression of melanoma has been observed in some patients, a phenomenon thought to be mediated by the immune system (Faries and Morton, 2005). For this reason, thirty years ago studies have started on vaccine efficacy in patients with metastatic melanoma, exploiting melanoma-associated antigens (Morton et al., 1989; Morton et al., 1992), but until now limited success has been observed in the clinics despite encouraging biological results (Fay et al., 2006).

An *ex-vivo* DC-based vaccine for metastatic melanoma consisting of DCs pulsed with autologous, *ex vivo* expanded tumour cells has given encouraging results (Dillman et al., 2004, 2009). Two recent phase I studies have also reported encouraging clinical results with personalized vaccines made from molecules generated by molecular biology and including peptides potentially attaching to patient's MHC-I and MHC-II molecules (Ott et al., 2017) or corresponding mRNAs (Sahin et al., 2017); mRNA can be uptaken by peripheral DCs and elicit an immune response in lymph nodes (Kreiter et al., 2010). A few patients who experienced recurrence underwent regression with anti-PD1 therapy, which interferes with T-cell suppression by tumour cells (Hamid et al., 2013; Ott et al., 2017; Sahin et al., 2017). This type of treatment does not directly

interfere with DCs, rather potentiates T lymphocytes by inhibiting the activation of PD-1 by its ligand PD-L1, which would lead to death of CD8+ T cells.

Nanoparticles as possible adjuvants in immunotherapy

Nanoparticle (NP)-based vaccines are also being studied for cancer immunotherapy, taking advantage of materials with at least one dimension under the micrometer (Park et al., 2013). Nanotechnology has drawn attention of scientists from many fields in the last years, prompting to technological request for high performance. Since nanomaterials have very different properties compared with bulk ones they are appealing for biomedical applications, which has led to the new branch of nanomedicine (Lynch and Dawson, 2008). Nanoparticles can be employed as carriers for other chemical species, such as fluorescent or radio-opaque or magnetic molecules for imaging (McDonald et al., 2017) and drugs for therapy (Park et al., 2013; Valentini et al., 2013; Bobo et al., 2016). They offer the possibility of a localized delivery of these substances, as they can be functionalized with specific receptors which allow the selective binding to a target tissue or organ (Ghosh et al., 2008).

Dendritic cells are the first cells to act in the sequence of events leading to immune response toward antigens, it is therefore important to understand how these cells interact with nanomaterials candidate to be used as regulators of the immune response. However, it must always be considered that the interactions of NPs with their biological environment depends by a complex interplay between the controllable properties of the particles, such as size, shape and surface chemistry, and the largely uncontrollable properties of the surrounding medium (Bobo et al., 2016). Particle size plays a key role in the clearance of these materials from the body, with small particles

(<10 nm) being cleared via the kidneys and larger particles (>10 nm) through the liver and the mononuclear-phagocyte system (Choi et al., 2007; Sadauskas et al., 2007). For biomedical applications, organic (Hu et al., 2004; Foerster et al., 2016) and inorganic NPs are under evaluation. Silica and gold NPs are among the most investigated inorganic ones (Fogli et al., 2017). Both are characterized by well-established and easy-to-follow synthetic routes, high biocompatibility and tunable physicochemical properties (Walkey et al., 2012). Nanoparticles can be modified to carry an antigenic cargo, which can in turn be internalized together with the NP and processed for presentation to lymphocytes (Prasad et al., 2011; García-Vallejo et al., 2012).

Peroxisome proliferator activated receptors (PPAR) and dendritic cells

PPAR

A very large number of genes, cytokines, various hormones and lipids may influence the function and immunophenotype of DCs, contributing to lineage, subtype, and functional specification. Among nuclear receptors, the regulation of these cells depends on those for glucocorticoids, vitamin D, retinoic acid and peroxisome proliferator activated receptors (PPARs). The latter belong to a large group of related transcription factors (48 members identified in humans) referred to as the nuclear receptor superfamily, which can bind small lypophilic ligands that induce their transcriptional activities (Varga et al., 2011). PPARs play roles in an ever increasing list of regulatory pathways, such as those regarding lipid, lipoprotein and glucose metabolism, inflammation, endothelial function, cancer, atherosclerosis and bone morphogenesis (Reka et al., 2011; Varga et al., 2011).

Three isotypes of PPAR have been identified: PPAR-α, -β (also known as δ) and -γ (Issemann and Green, 1990; Fajas et al., 1997). Each of these three subtypes display differential tissue distribution and mediate specific functions such as early development, cell proliferation, differentiation, apoptosis and metabolic homeostasis (Misra et al., 2002). The three types of PPARs exhibit also different ligand affinity but share a common feature: upon ligand binding they form a hetrodimer with the retinoid X receptor (RXR) and bind to canonical peroxisome proliferator response elements (PPREs) that are usually located upstream of acutely regulated PPAR target genes (Nagasawa et al., 2009; Luconi et al., 2010).

PPAR-α is mainly expressed in cells with high catabolic rates of fatty acids, such as liver, muscle, heart and skin (Issemann and Green, 1990; Braissant et al., 1996).

PPAR-β/δ is ubiquitously expressed with a higher level in gut, epidermis, placenta, skeletal muscles and adipose tissue (Michalik et al., 2003) and plays a role in embryonic development and adipocyte physiology (Braissant et al., 1996).

PPAR-γ is highly expressed in adipose tissue and in multiple other tissues, such as breast, colon, lung, ovary, prostate, thyroid (Lambe and Tugwood, 1996), in the vascular wall (Marx et al., 1999), in cells of the immune system such as monocytes, macrophages and DCs - which suggests its involvement in immunity (Nencioni et al., 2002; Asada et al., 2004) - and in hematopoietic stem cells (Greene et al., 1995). PPAR-γ has gained medical relevance through the discovery of drugs, that are members of the thiazolidinedione class as pioglitazone, rosiglitazone and troglitazone (Hirakata et al., 2004), which can be used in the clinics to improve insulin sensitivity in type 2 diabetic patients and are high-affinity ligands for PPAR-γ (Kliewer et al., 1995). Due to its importance in the treatment of type 2 diabetes and its complications, PPAR-γ is the most intensively studied PPAR (Varga and Nagy, 2008).

For certain nuclear receptors, including PPARs, the identity of their endogenous

ligands remains largely elusive; preliminary evidence suggests that several lipids, including fatty acids and their derivatives, eicosanoids and phospholipids activate target gene expression either directly as PPAR ligands, or indirectly as precursors for other lipid that ligate PPARs (Forman et al., 1997; Kliewer et al., 1997; Chakravarthy et al., 2009; Haemmerle et al., 2011). Upon ligand binding, the nuclear receptors can activate or repress the transcription of a broad spectrum of genes (Mangelsdorf et al., 1995).

It seems that PPAR-γ binds lipophylic ligands, including unsaturated fatty acids, arachidonic acid metabolites 15-deoxy-Δ12,14-prostaglandin J2 (15d-PGJ2) and 15-HETE (hydroxyeicosatetraenoic acid), and fatty acid-derived components of oxidized low-density lipoproteins (oxLDL), 9- and 13-HODE (hydroxyoctadecadienoic acid); also, PPAR-γ covalently binds a subsets of fatty acids and can even bind two ligand molecules at a same time. This latter behaviour could explain why the ligand binding pocket of PPAR-γ is unusually large compared to that of related nuclear receptors. Simultaneous binding of two ligands would also support the hypothesis that PPAR-γ is not a specific target for one particular fatty acid but samples the intracellular mixture of various fatty acid molecules (Varga and Nagy 2008; Itoh et al., 2008). As a transcription factor, PPAR-γ directly regulates the expression of several genes participating in pre-adipocyte differentiation, fatty acid uptake and lipid storage (Lehrke and Lazar, 2005; Varga and Nagy 2008) and regulates glucose homeostasis (Tontonoz et al., 1994; Spiegelman, 1997, 1998); moreover, it exerts anti-inflammatory actions (Cunard et al., 2002; Nencioni et al., 2002). PPAR-γ agonists were shown to abrogate the expression of pro-inflammatory genes such as nitric oxide synthase (iNOS), matrix metalloproteinase (MMP)-9 and scavenger receptor A in murine macrophages (Ricote et al., 1998), and TNF-α, IL-1β and IL-6 in human monocytes (Hinz et al., 2003). The anti-inflammatory activity of PPAR-γ is dependent on its ability to antagonize and block the pro-inflammatory transcription factors nuclear factor-κB (NF-κB), adaptor-related protein

complex 1 (AP-1) and signal transducer and activator of transcription (STAT) (Ricote et al., 1998; Chinetti et al., 2000; Welch et al., 2003; Celinski et al., 2011). A consequence is the block of the production of inflammatory cytokines by several cell types of the immune system (Gosset et al., 2001; Nencioni et al., 2002)

Among cells of the immune system, PPAR-γ is expressed in humans by monocytes, macrophages, mDCs and a subset of S100-positive antigen-presenting cells of the tonsils (Szatmari et al., 2004), and in the mouse by macrophages and mDCs, including LCs (Hammad et al., 2004). Both mouse and human DCs express an undetectable or a very low amount of PPAR-α (Gosset et al., 2001; Nencioni et al., 2002; Szatmari et al., 2004). In contrast, the mRNA of PPAR-β has been detected in monocyte-derived human DCs and murine mDCs (Hammad et al., 2004; Szatmari et al., 2004).

PPAR-γ in dendritic cells

Dendritic cells are used as a model to investigate how the trascriptional activities of PPAR-γ can lead to the modulation of immune functions and some information has been obtained for DCs derived from peripheral blood monocytes, using PPAR-γ agonists (Szatmari et al., 2006; Varga and Nagy 2008). PPAR-γ agonists may be used as tools to address the role of these receptors in the differentiation, maturation, migration, cytokine production and antigen uptake and presentation by the DCs (Ahmadian et al., 2013). It has been seen that rosiglitazone, a PPAR-γ agonist, favours the full differentiation of LCs from CD133+ haematopoietic precursors of the cord blood (Bonetti et .al, 2014).

The expression of PPAR-γ is induced immediately at the start of culture leading to differentiation of these cells and is down-regulated soon afterwards, but not

abolished, so that PPAR-γ is expressed at high level only in a narrow timeframe and at lower levels thereafter; the highest level of expression and ligand responsiveness occurs in the first 24 hours of culture, but after 5 days culture with a PPAR-γ agonist a change occurs in gene expression and consequently in phenotypic features (Varga and Nagy 2008), as shown below.

Upon ligand binding PPAR-γ binds in turn to canonical PPREs upstream of acutely regulated PPAR-γ target genes. PPAR-γ belongs to the group of nuclear receptors that bind to their response elements when they form heterodimers with their obligate partner retinoid X receptor (RXR), a promiscuous nuclear receptor. PPAR-γ/RXR heterodimer recruits a protein complex that consists of co-activators or co-repressors to direct transcriptional activation or repression, respectively. The binding of PPAR-γ/RXR heterodimer to PPREs has been shown to result in the stimulation of transcription; in the absence of ligands, PPAR-γ/RXR heterodimers bind to PPREs in a conformation that favours the binding of co-repressor molecules leading to the inhibition of transcription. PPAR-γ activation by ligand binding can also lead to the repression of gene expression, possibly through a trans-repression mechanism (Varga and Nagy, 2008). Upon ligand binding, PPARγ can also interfere with the activity of distinct transcription factors, such as NF-κB, through protein–protein interactions (Pascual and Glass, 2006).

PPAR-γ ligands are able to alter the maturation and immunogenicity of DCs stimulated by specific receptors, such as TLR, by inhibiting extracellular signal-regulated kinase (ERK), NF-κB and mitosis-activated protein (MAP)-kinase pathways (Appel et al., 2005; Wei-guo et al., 2010), and to impair the expression of co-timulatory molecules (CD83, CD80, CD40) (Szatmari et al., 2006).

Differentiation of DCs in the presence of a PPAR-γ agonist leads to reduced expression of CD1a and CD80 compared to culture without that agonist; the treatment

apparently does not affect the light microscopical aspect of the differentiated cells (Nencioni et al., 2002; Szatmari et al., 2004, 2007). PPAR-γ agonists treatment of differentiating DCs leads also to lower expression of CCR7, hindered migration and lower secretion of several cytokines, namely IL-6, IL-10, IL-12, IL-15, IFN-γ-inducible protein (IP)-10 also known as chemokine (C-X-C motif) ligand (CXCL)10, MCP-2, CCL5 also known as regulated upon activation, normal T-cell expressed and secreted (RANTES), EBI1 ligand chemokine (ELC) and TNF-α. These modifications indicate a general negative effect of PPAR-γ on the production of inflammatory cytokines by DCs and favours the differentiation of T cells into T-helper 2 rather than T-helper 1 lymphocytes (Gosset et al., 2001; Nencioni et al., 2002; Szatmari et al., 2006). On the contrary, PPAR-γ agonist-treated monocyte-derived DCs undergo up-regulation of CD86 and MHC-II and express more CD1d than untreated cells. CD1d expression in monocytes is high and sustained throughout the differentiation process of DCs. This marker recognizes and presents lipid antigens to invariant NK cells, that are activated, proliferate and release inflammatory cytokines, as INF-γ, TNF-α, IL-4, IL-5 and MIP1-α (Szatmari et al., 2006; Varga and Nagy 2008). DCs differentiated in the presence of PPAR-γ agonists have shown up-regulation also of the receptor for apoptotic cells, *i.e.* CD36 (Tontonoz et al., 1998, Gosset et al., 2001; Nencioni et al., 2002; Szatmari et al., 2007), enhanced phagocytic activity and induced expression of a multidrug trasporter molecule, that confers protection against xenobiotics (Agostini et al., 2006),

Upon stimulation with LPS, DCs differentiated in the presence of a PPAR-γ agonist do not express CD1a and express CD80 and CD83 at lower levels than cells generated without that agonist (Nencioni et al., 2002). The effects of PPAR-γ agonists on differentiating DCs have been reported to be concentration dependent (Nencioni et al., 2002; Majai et al., 2007).

It is not yet clear whether these phenotypic changes are due to direct

transcriptional effects on inflammatory gene expression or to secondary changes induced in lipid metabolism (Szatmari et al., 2007). Asada et al. (2004) suggested that PPAR-γ modulates the gene expression of CD36 through direct interaction with the proximal promoter of that gene via a specific response element. The PPAR-γ agonists glitazones also stimulate endocytosis in immature (Szanto et al., 2004; Szatmari et al., 2006) and mature DCs (Luo et al., 2004) and phagocytosis by immature DCs, but the pro-inflammatory response and the capacity to trigger T cell activation are not enhanced, suggesting that the functions of DCs undergo very complex regulation upon stimulation of PPAR-γ (Majai et al., 2010). The addition of PPAR-γ agonists during the generation of DCs in vitro has controversial effects on the generated cells. Nencioni et al. (2002) and Appel et al. (2005) reported that DCs generated in the presence of PPAR-γ agonists had impaired T cell stimulating activity. In a mouse model of atopic dermatitis, PPAR-γ agonists led to decreased severity of the disease, while they inhibited the maturation in vitro of DCs (Jung et al., 2011). Szatmari et al. (2004) reported, upon PPAR-γ stimulation, a normal T cell-activating capacity of DCs and an increased stimulatory activity on invariant NK-T cells in the presence of galactosyl-ceramide; the latter effect was considered dependent on increased expression of CD1d and glycolipid presentation through this molecule. In a later review, Szatmari et al. (2006) considered that PPAR-γ agonist impaired the allostimulatory capacity of DCs. The stimulation of PPAR-γ may also interfere with the migration of DCs out of peripheral tissues, indeed Nencioni et al. (2002) reported that the activation of PPAR-γ in DCs inhibited the expression of CCR7, a receptor involved in the localization to lymphoid tissue and maturation of DCs (Li et al., 2011). Using a murine model of LC migration induced by TNF-α, Angeli et al. (2003) found that the PPAR-γ agonist rosiglitazone specifically impairs the departure of those cells from the epidermis. Local production of PPAR-γ activators, for instance arachidonic acid derivatives, in the

peripheral sites during inflammatory reactions may therefore be important for the regulation of immune responses. In human DCs the activation of PPAR-γ also leads to the generation of retinoic acid, a molecule that regulates DCs phenotype and may concur to fine tuning the function of these cells (Szatmari et al., 2006). In particular, in the absence of inflammatory signals retinoids induce apoptosis of DCs, while in the presence of inflammatory cytokines they potentiate DC activity by increasing NF-κB binding to DNA, triggering the expression of MHC-II and costimulatory molecules at the cell surface, promoting the transition to mature DCs and leading to enhanced antigen-specific T cell response (Geissman et al., 2003; Szatmari et al., 2006).

Aim

Given the ability of dendritic cells to regulate the immune system and their essential role in inflammatory processes in response to tissue injury and in generating antitumor immunity, these cells are considered possible clinical targets. Also, these cells - depending on intrinsic properties and the microenvironment - may cause anergy or stimulate active tolerance; this prompts to understand how these different functions are regulated in order to control dendritic cell function for clinical purposes.

In order to better understand the steps and control mechanisms of the differentiation of dendritic cells, this research addresses the potentials for such differentiation of different precursors present in adult blood and the possible role of PPAR-γ stimulation on that process, given the possibility that dendritic cells, including Langerhans cells, express those receptor.

Therefore the following steps were undertaken:

1) characterize CD14, CD34 and CD133 positive peripheral blood progenitors and verify the possibility to use them as starting population to differentiate dendritic cells and Langerhans cells;

2) assess the steps of this differentiation and the phenotype of generated cells;

3) using cells generated from CD14+ monocyte precursors, assess the interactions of dendritic cells with inorganic nanoparticles and the effects of this interaction on the morphological, immunophenotypical and functional properties of the cells;

4) establish the effect of PPAR-γ stimulation on these processes, taking advantage of the antidiabetic drug rosiglitazone as a tool to activate those receptors;

5) evaluate the expression of PPAR-γ with and without the stimulation of rosiglitazone, at different times of cell culture.

Materials and Methods

Isolation and culture of CD14+ cells from peripheral blood

Human CD14+ cells were isolated from buffy coats obtained from healthy donors and stored at +4°C for 24 h before use, by density gradient centrifugation on Ficoll (Lymphoprep, Euroclone, Pero, Italy) for 15 min at 800 x *g*. The buffy coat was suspended in 2,5% dextran (Amersham Pharmacia Biotech, Sweden) in 0.9% NaCl and stabilized at 37°C for 30 min. Upon dilution with phosphate buffered saline pH 7.4 mol/litre (PBS) the suspension was stratified over Ficoll and centrifuged as indicated. Upon washing in PBS, the CD14+ cells were selected by a magnetic field upon labelling with colloidal superparamagnetic microbeads conjugated with mouse anti-human CD14 monoclonal antibody (Miltenyi Biotec, Bergisch Gladbach, Germany), following a previously published protocol (Miltenyi et al., 1990; Paccosi et al., 2014); beads were added at the cell suspension (20 μl beads per 10^7 total cells) and were let incubate at 4°C for 15 min. After wash with PBS the cell suspension was injected into the column for separation. After elution of unlabelled cells, the column was extracted from the magnet and CD14+ cells were recovered with PBS.

The cells were seeded at mean concentration di 1 x 10^6 cells/ml in RPMI 1640 with 10% heat-inactivated foetal bovine serum (FBS), 100 U/ml penicillin and 0.1 ng/ml streptomycin (all from Sigma-Aldrich, St Louis, Mo, USA), GM-CSF (10 ng/ml), IL-4 (10 ng/ml), TNF-α (10 ng/ml) and TGF-β (10 ng/ml) for 7 days, thus obtaining immature DCs; final maturation was induced with the same cytokines plus IL-1β (10 ng/ml), IL-6 (1000 U/ml) and TGF-β (20 ng/ml) for additional 24 h. All

cytokines were purchased from PeproTech. For TGF-β the isoform 3 was used: the three isoforms of TGF-β signal through the same receptor and elicit similar biological responses (Sporn and Roberts, 1992).

The isolated cells from each donor were split and seeded with and without rosiglitazone (Selleckchem, TX). The same was done also for the culture of the other precursors indicated below. For all precursors, each donor was used alone for one experiment.

Rosiglitazone (1 μmol/l, *i.e.* 0.36 μg/ml) was added since the start of culture; culture without rosiglitazone was used as control. The drug was obtained from the producer as a powder and was dissolved in dimethyl sulfoxide at a concentration of 100 mmol/l for storage. It was then diluted in 0.1% bovine serum albumin (BSA) down to 100 μmol/l before adding to the culture medium at the indicated final concentration.

Isolation and culture of CD34+ cells

Human CD34+ cells from healthy donors were isolated from freshly recovered buffy coats. The mononuclear cells were isolated by Ficoll density gradient centrifugation and were washed in phosphate buffered saline, pH 7.4 (PBS) with 1% FBS. Up to 10^8 cells were resuspended in a final volume of 300 μl PBS and labelled with superparamagnetic microbeads conjugated to mouse anti-human CD34 monoclonal antibodies for 30 min at 4° C, according to the producer instructions (Microbeads UltraPure, human, Miltenyi Biotec). The cell suspension was loaded onto a MACS Column (Miltenyi) which was placed in the magnetic field MACS Separator (Miltenyi). The magnetically labelled CD34+ cells are retained within the column while the unlabeled cells run through. After removing the column from the magnetic field, the CD34+ cells were eluted and counted.

Purified CD34+ cells (500,000 cells/ml) were seeded in RPMI 1640 with 10% FBS, 100 U/ml penicillin and 0.1 ng/ml streptomycin and cultivated for 7 days with SCF (20 ng/ml), TPO (10 ng/ml), Flt3-L (25 ng/ml), GM-CSF (10 ng/ml), IL-4 (10 ng/ml), TGF-β (10 ng/ml) (all from Peprotech). The culture was further continued for 7 more days with GM-CSF, IL-4, TNF-α and TGF-β, at the same concentrations indicated above for CD14+ cells. For last additional four days, *i.e.* from day 14 to day 18 of culture, TGF-β concentration was raised to 20 ng/ml and IL-1β (10 ng/ml) and IL-6 (1000 U/ml) were added, while GM-CSF, IL-4 and TNF-α, were maintained at the same concentration indicated above (Tab. 1).

Table 1 - Cytokines used for culture of CD34+ and of CD133+ precursors. Concentrations are ng/ml.

	0 -7 d	**7 - 14 d**	**14 - 18 d**
TPO	10		
FLT-3	25		
SCF	20		
GM-CSF	10	10	10
IL-4	10	10	10
TNF-alpha	10	10	10
TGF-beta	10	10	20
IL-1 beta			10
IL-6			15

Rosiglitazone (1 μmol/l, i.e. 0.36 μg/ml) was added since the start of culture in some experiments, as specified above.

Isolation and culture of CD133+ cells from peripheral blood

Human CD133+ cells were isolated from fresh buffy coats. The mononuclear cells were isolated by Ficoll density gradient centrifugation, the cells were washed in PBS, and resuspended in a final volume of 200 µl PBS for up to 5 x 10^7 cells, following the indications of the producer (Diamond CD133 isolation kit, human, Miltenyi Biotec), The cells were incubated with a cocktail of biotin-conjugated mouse monoclonal antibodies against human CD2, CD3, CD11b, CD14, CD15, CD16, CD19, CD56, CD61 and CD253a, for 10 min at 4° C, washed in PBS and resuspended in 400 µl PBS. Anti-biotin microbeads were added for 15 min. at 4° C, then the cell suspension was loaded onto a MACS Column placed in the magnetic field MACS Separator (all from Miltenyi) so that the magnetically tagged cells were retained within the column while the unlabelled ones passed through. The unlabelled, effluent cells were washed and resuspended in 200 µl PBS (up to 5 x 10^7 per sample), adding 50 µl of micro beads conjugated with monoclonal CD133 antibodies for 30 min at 4° C. Upon subsequent magnetic separation the CD133+ cells and subsequent elution of the retained cells, the latter were placed in culture with the same cytokines for the same times indicated for CD34+ cell culture.

Isolation of lymphocytes for mixed lymphocyte reaction

Allogeneic lymphocytes were recovered from buffy coats - obtained from healthy donors and stored at +4°C for 24 h before use - by density gradient centrifugation on Ficoll, as above. The cells were washed in PBS and seeded in RPMI 10% FBS for 45 min. Non-adherent cells were collected and were centrifuged at 160 x g, for 10 min at 20°C, counted and used for mixed lymphocyte reaction (MLR).

Analysis of in vitro generated dendritic cells

At the end of culture the morphology, the immunophenotype and lymphocyte-stimulating activity of the generated cells were analysed by immunofluorescence, flow cytometry, electron microscopy and mixed lymphocyte reaction.

The same methods were used to evaluate the interaction of DCs (obtained from CD14+ cells) with NPs.

Also, the expression of the PPAR-γ by the cells grown with and without rosiglitazone (not treated with NPs) was evaluated at different times from the start of culture (0, 7, 14 and 18 days) by quantitative real-time polymerase chain reaction (qRT-PCR).

Immunofluorescence

Double immunofluorescence analyses were performed on cytospins of mature DCs, that were fixed with cold acetone for 3-5 min at room temperature. After blocking non-specific binding sites with 10 ng/mL BSA (Sigma-Aldrich) in PBS with the addition of 0.5% triton X-100 (Sigma, Milan, Italy) for 30 min at room temperature, primary antibodies (anti-human) were applied over night at 4°C at the indicated dilutions. The following antigens were tagged first: ICAM-1/ CD54 (Merck Millipore, Darmstadt, Germany; monoclonal mouse IgG1, 1:50), langerin/CD207 (Dendritics, Lyon, France; mouse monoclonal IgG1, 1:50), DC-SIGN/CD209 (Sigma-Aldrich; rabbit polyclonal, 1:50). Secondary goat anti-mouse and anti-rabbit polyclonal antibodies conjugated with Alexa Fluor AF594 (red fluorescence), all from Life Technology (Thermo Fisher Scientific, Waltham, MA), were applied for 2 hours at room temperature. Afterwards, fluorescein isothiocyanate (FITC) conjugated anti-human HLA-DR sntibody (Miltenyi Biotec, mouse monoclonal IgG2aκ, 1:20) was added over night at 4°C. The signal was amplified with anti-FITC goat polyclonal

antibody conjugated with AF488 (green fluorescence; Thermo Fisher Scientific; 1:100). Nuclei were labelled with Hoechst 33342 (20 µg/mL; Sigma). Omission of primary antibodies or substitution with irrelevant ones were used as negative controls.

The slides were mounted with Gel/Mount (Fluoromount, Diagnostic BioSystems, Pleasanton, CA), observed in an Axioskop microscope equipped for epifluorescence (Zeiss, Oberkochen, Germany) and captured with an Axio Vision 4 system, consisting of a digital multichannel fluorescence module and dedicated software (Zeiss), or observed in a DMR HC microscope equipped for epifluorescence (Leica Microsystems GmbH, Wetzlar, Germany).

Flow Cytometry

For some samples fixation and permeabilization were performed to evaluate intracytoplasmic langerin/CD207 expression, using the cell permeabilization kit of Nordic-Mubio (Susteren, Netherlands) according to the directions of the manufacturer. The indicated amounts of the following monoclonal antibody solutions were added to 100 µl of cell suspension containing 10,000-60,000 cells, as suggested by the producer: 5 µl HLA-DR-Horizon violet (HV450) or 7.5 µl HLA-DR-FITC (BD Biosciences, Franklin Lakes, NJ), 6.5 µl CD1a-phycoerythrin (PE) (BD Biosciences), 6.5 µl CD11c--allophycocyanin (APC) H7 or CD11c-PE (BD Bioscience), 5 µl CD14-APC (BD Biosciences), 5 µl CD33-phycoerythrin with cyanine dye Cy7 (PeCy7), 5 µl CD34-peridinin chlorophyll protein with the cyanine dye Cy5.5 (PerCP-Cy 5.5) (BD Biosciences), 7.5 µl CD45-FITC or 5 µl CD45-HV450 (BD Bioscience), 7.5 µl CD80-FITC (Miltenyi Biotec), 6.5 µl CD83-PE, 6.5 µl CD86-PE or 5 µl CD86-APC (BD Bioscience), 10 µl langerin/CD207-APC (Miltenyi Biotec). The cells were immuno-labelled for 15 min at room temperature, protected from the light. Dead cells were

counted by flow cytometry upon labelling with 7-amino-actinomicin D (BD Biosciences). 7-aminoactinomycin D (AAD) was used to recognize dead cells and exclude them from analysis. Isotype-matched antibodies were used as negative controls.

Flow cytometry was performed by collecting more than 10,000 events on a FACSCanto II (Becton Dickinson, Sparks, MD) and data were analysed with Infinicyt 1.7 (Citognos, Salamanca, Spain).

Electron microscopy

Cell pellets 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 were stained with gadolinium acetate (Electron Microscopy Sciences, Hatfield, PA; Nakakoshi et al., 2011) and either lead citrate or bismuth subnitrate (Riva, 1974), and observed in a Jeol JEM 1010 electron microscope (Tokyo, Japan) at 80 kV. Photomicrographs were taken with a digital camera MegaView III (Soft Imaging System, Muenster, Germany) connected with a personal computer (Dell, Round Rock, TX) with dedicated software (AnalySIS, Soft Imaging Software, Muenster, Germany).

Mixed lymphocyte reaction

Lymphocytes were stained with the fluorescent dye carboxyfluorescein succinimidyl ester (CFSE) following manufacturer instructions. $2x10^5$ lymphocytes were cultured 5 days in complete medium with $4x10^4$ DCs. Mature DCs generated at the end of incubation period from different precursors and immature, monocyte derived DCs pre-incubated with NPs were subjected to this analysis. Lymphocytes stimulated with 5 µg/ml phytohaemoagglutinin (PHA, Biochrom, MA) were used as positive controls. After 5 d lymphocytes were recovered and stained with fluorescent mouse

monoclonal antibodies following manufacturer's instructions: anti-CD3-PerCP-Cy5.5, anti-CD4-PE and anti-CD8-APC (BD Bioscience).

Gene expression

The expression of PPAR-γ was evaluated by quantitative qRT-PCR using Cells-to-CT 1-Step TaqMan Kit (Thermo Fisher Scientific), that allows to measure relative gene expression by qRT-PCR analysis directly from cultured cells, without preliminary RNA purification and amplification. The lysis technology is designed for 10–100,000 cultured cells per sample, thus for each assay ~80,000 cells were washed in PBS, counted and lysed for 5 min at room temperature; genomic DNA was simultaneously removed with DNase. Lysis was terminated at room temperature by 2 min incubation with Stop Solution. The lysate was mixed with TaqMan® 1-Step qRT-PCR Mix (Thermo Fisher Scientific) and with TaqMan® Gene Expression Assays for PPAR-γ and GAPDH housekeeping gene (Applied Biosystems for Thermo Fisher Scientific), at the volumes indicated by the producer. The results were read in a Rotor-Gene Q (Qiagen, Germany) with the following settings: 1 cycle of reverse trascription at 50°C for 5 min, 1 cycle of reverse trascription inactivation/initial denaturation at 95°C for 20 seconds, 40 cycles of amplification at 95°C for 15 seconds and at 60°C for 1 minute. For the acquisition we have used Rotor-Gene Q series software. A lysate of human pre-adipocytes was used as positive control for PPAR-γ

Synthesis of NPs

Gold and silica NPs (AuNPs and SiO_2NPs respectively) were synthesized from a collaborating research group at the Department of Chemistry "Ugo Schiff" of the University of Florence, directed by Prof. Debora Berti. Briefly, according to established

protocols (Turkevich et al., 1951; Stöber et al., 1968; Frens, 1973;) Rhodamine B isothiocyanate was added to produce fluorescent SiO_2NPs.

Dendritic cell culture with nanoparticles

Nanoparticles were added to cultures of immature DCs, obtained from CD14+ peripheral blood mononuclear cells as described above. NPs were added for 48 h at concentrations of 10 and 100 µg/mL, while the concentration of FBS was reduced to 1% to minimize a possible influence of serum factors on DC maturation. In some experiments maturation-inducing cytokines, i.e. IL-1β, IL-6, TNF-α, at the concentrations indicated above, were added together with NPs. Cells were checked by phase contrast microscopy throughout culture.

The DCs cultivated with NPs were analysed by fluorescence microscopy, flow cytometry and electron microscopy. In particular, to follow the incorporation of fluorescent silica NPs, for each experiment and experimental condition a drop of culture medium containing about 100 unfixed cells was transferred to a microscopic slide, covered with a coverslip and observed and photographed as detailed above. The number of unlabelled and that of labelled cells were counted and the percentage of labelled cells per slide was computed. Among labelled cells, the intensity of fluorescence was measured with ImageJ for Windows (NIH, Bethesda, MD): each labelled cell was outlined by hand and the software was used to measure the surface area (in square pixel, pixel size 0.0256 µm2) and the mean labeling intensity (in arbitrary units, maximum intensity = 255) of the cell. The two measures were multiplied for each other to obtain the total labeling per labelled cell.

The immunophenotype of DCs exposed to NPs was evaluated by flow cytometry for CD80, CD83 and CD86 (BD Biosciences) as indicated above. The results were read

in a FACSCanto II (BD Biosciences) and analysed by FACs Diva software (BD Biosciences).

The capacity of DCs challenged with NPs to stimulate lymphocytes was evaluated by MLR as indicated above.

Statistics

Quantitative data were expressed as mean ± standard error (SE) and analyzed as appropriate by ANOVA and Student t-test for paired data. Values of $p<0.05$, $p<0.01$ and $p<0.001$ were recorded separately and assumed as significant.

Results

Cell culture

Most cells obtained from CD14+ precursors showed a dendritic morphology after 7 days, with or without rosiglitazone (fig. 1). Some DCs obtained from CD34+ and CD133+ precursors acquired a dendritic morphology between 7 and 14 days, whether with or without rosiglitazone (figs. 2, 3). Non-dendritic cells had a round shape and were smaller than dendritic ones. Vitality was not affected by the addition of rosiglitazone to the culture medium.

The cell number at the start of culture was variable, it was possible to isolate more than 10^7 CD14+ cells, while one could isolate about $6x10^5$-$1.5x10^6$ CD34+ or CD133+ cells.

The cell mortality at the end of culture was different, depending on the cell type of origin. The mortality of the cells obtained from CD14+ precursors was ~25%, that of the cells obtained from CD34+ and from CD133+ precursors was ~40%.

Immunofluorescence

At immunofluorescence, DCs generated from CD14+ monocytes were positive for HLA-DR and DC-SIGN; a faint positivity for langerin/CD207 was observed in a few cells (fig. 4).

At immunofluorescence almost all mature DCs from CD34+ and CD133+ precursors expressed HLA-DR with intensity from moderate to bright, both with and without rosiglitazone (figs. 5 and 6, left panels). Part of the cells obtained at the end of

DCs obtained from CD14+ cells after 6 days culture

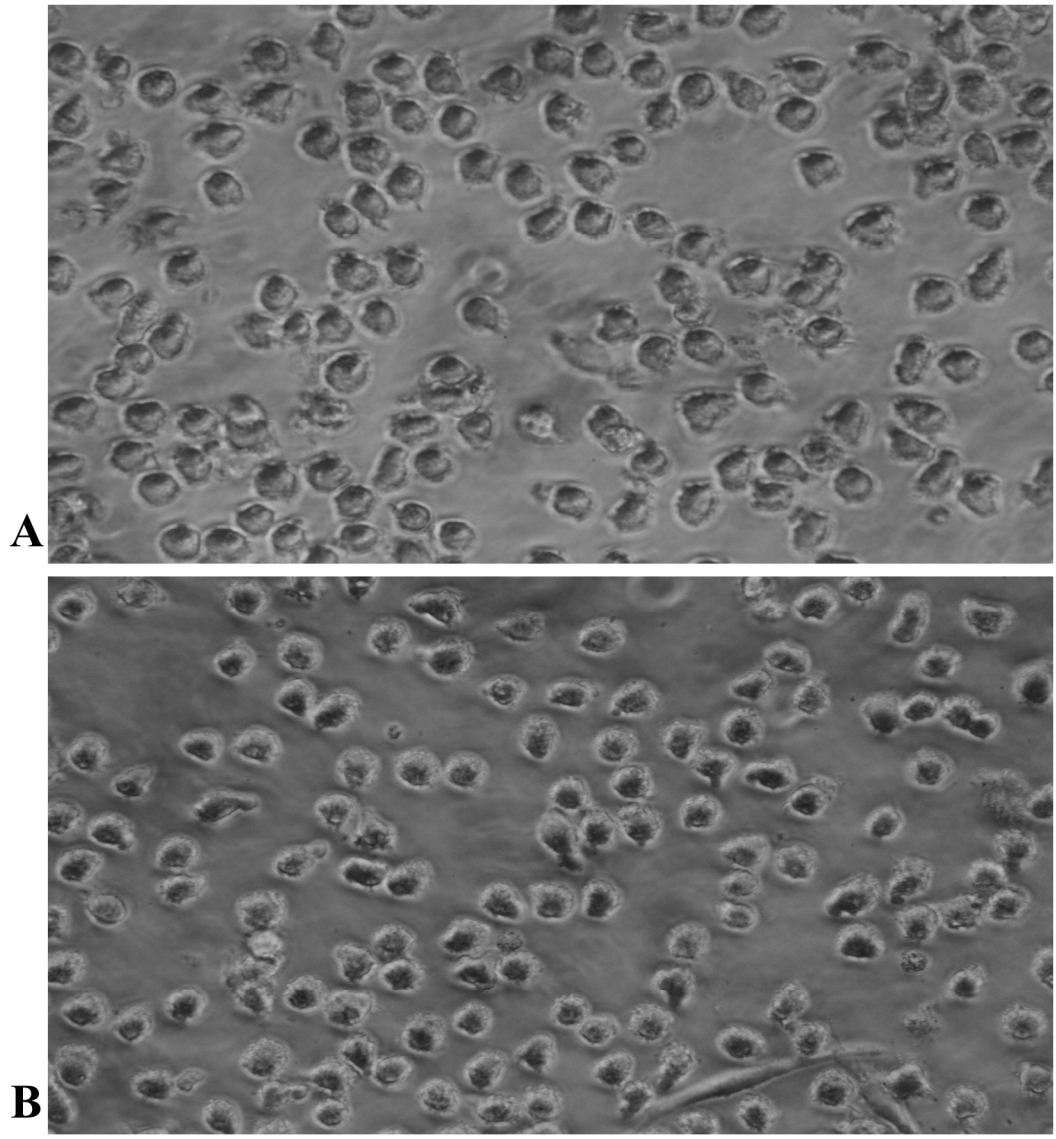


Fig. 1

Immature DCs without rosiglitazone (A) and immature DCs with rosiglitazone (B). Several cells show a dendritic profile.

DCs obtained from CD34+ cells after 14 days culture

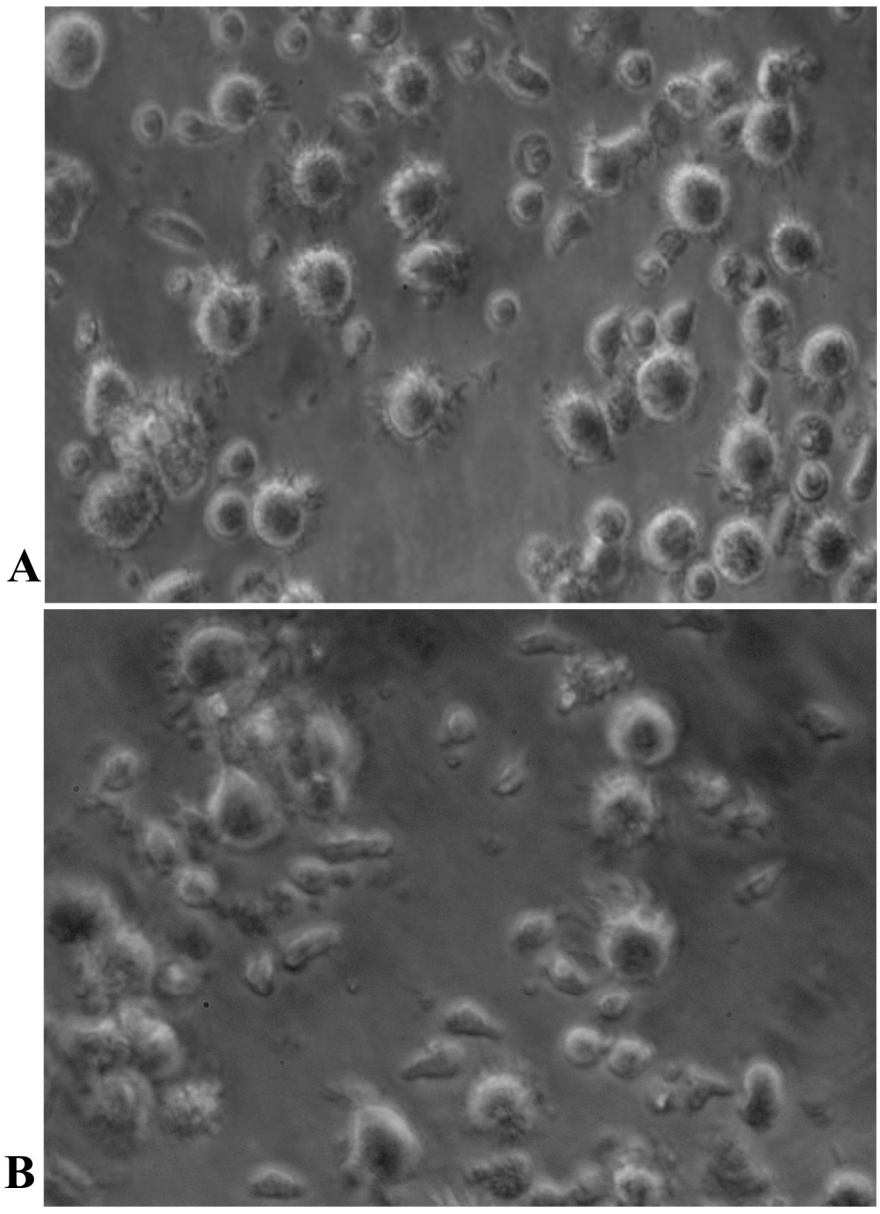


Fig. 2

DCs differentiated from CD34+ cells without (A) and with addition of rosiglitazone (B) to the culture medium.

DCs obtained from CD133+ cells after 17 days culture

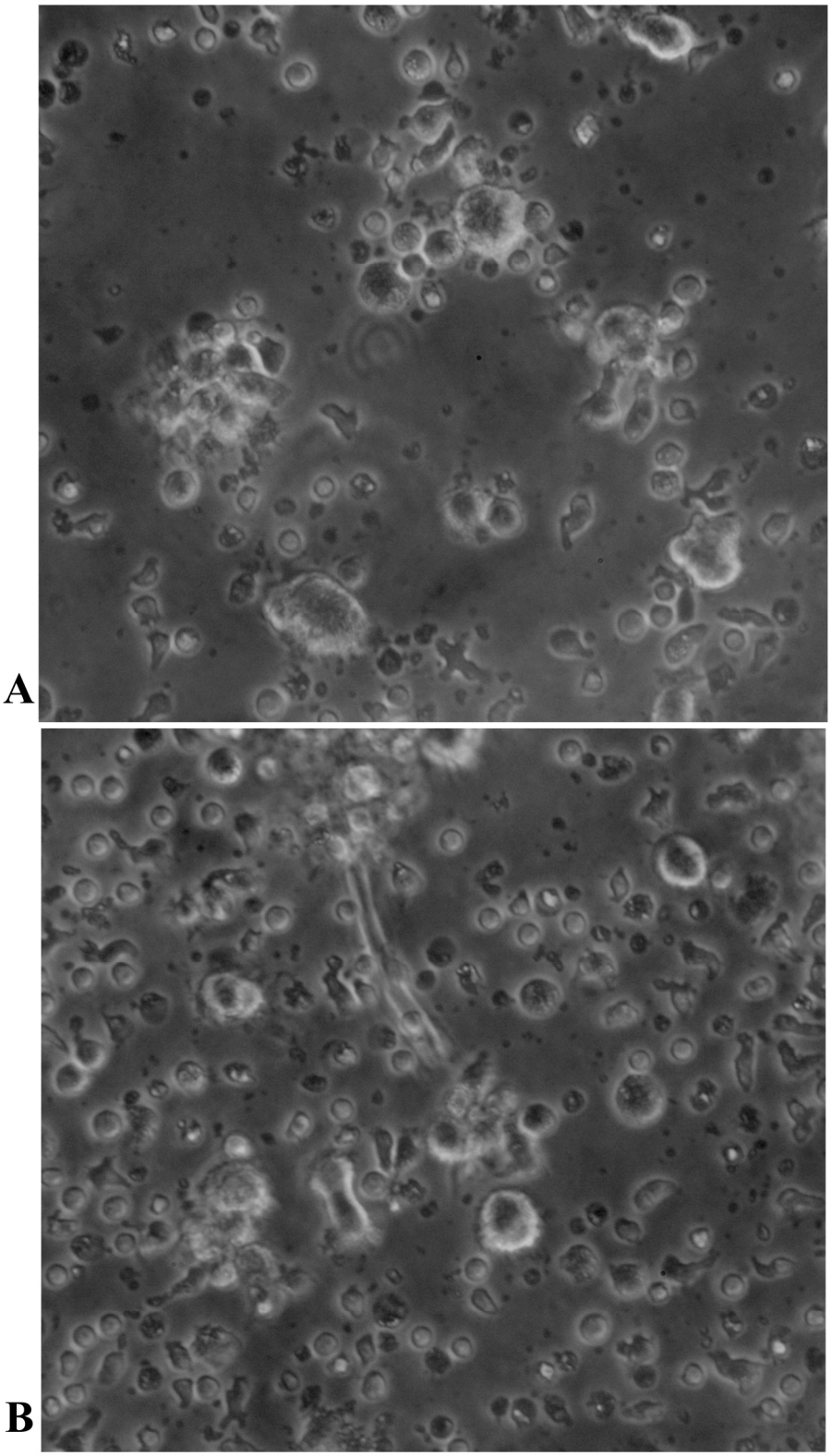


Fig. 3

Large DCs obtained without rosiglitazone (A) and with rosigitazone (B), among medium and small size cells.

the culture expressed CD54 (figs. 5 and 6, upper middle panels); the fluorescence intensity of the analyzed cells ranged from light to moderate, whether with or without rosiglitazone, and was most intense in paranuclear position. All cells with a larger size were langerin/CD207 positive (figs. 5 and 6, central middle panels), the fluorescence appeared in part granular; the signal ranged from light to bright and did not appear to vary depending on rosiglitazone addition. Most cells expressed DC-SIGN, with a light to bright signal apparently not affected by rosiglitazone (figs. 5 and 6, bottom middle panels).

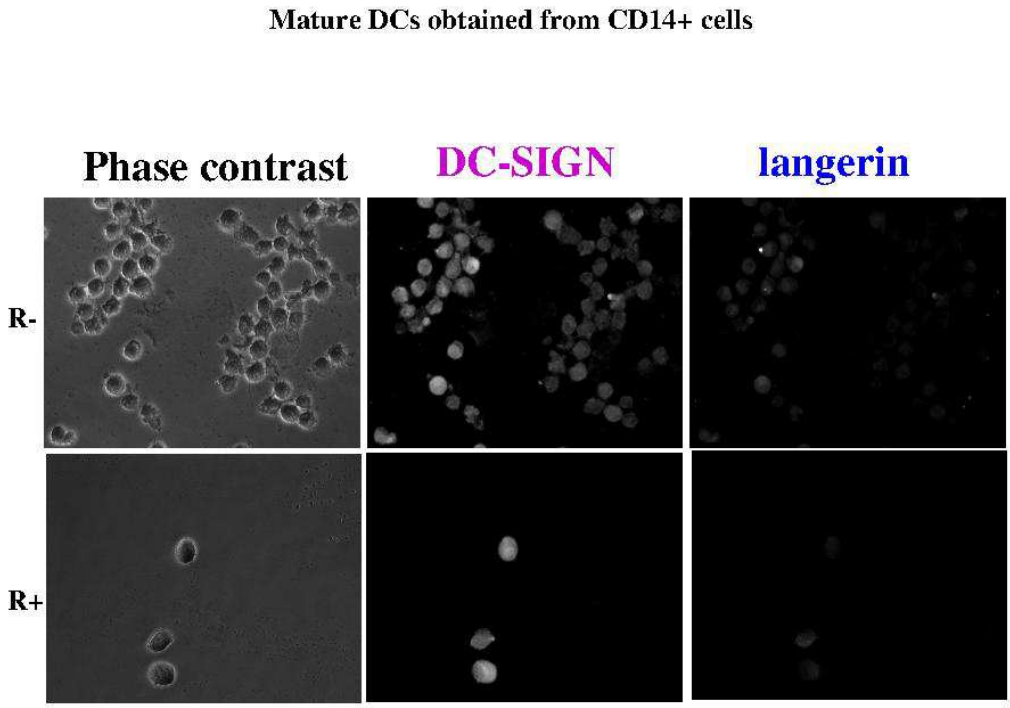


Fig. 4

Immunophenotype of DCs differentiated from CD14+ precursors without (R-) and with rosiglitazone (R+), as evaluated in immunofluorescence. Virtually all cells expressed DC-SIGN while only a minority was labelled for langerin/CD207, which co-existed with DC-SIGN expression. The results were not affected by addition of rosiglitazone during generation of DCs.

Fig. 5 - DCs obtained from CD34+ precursors

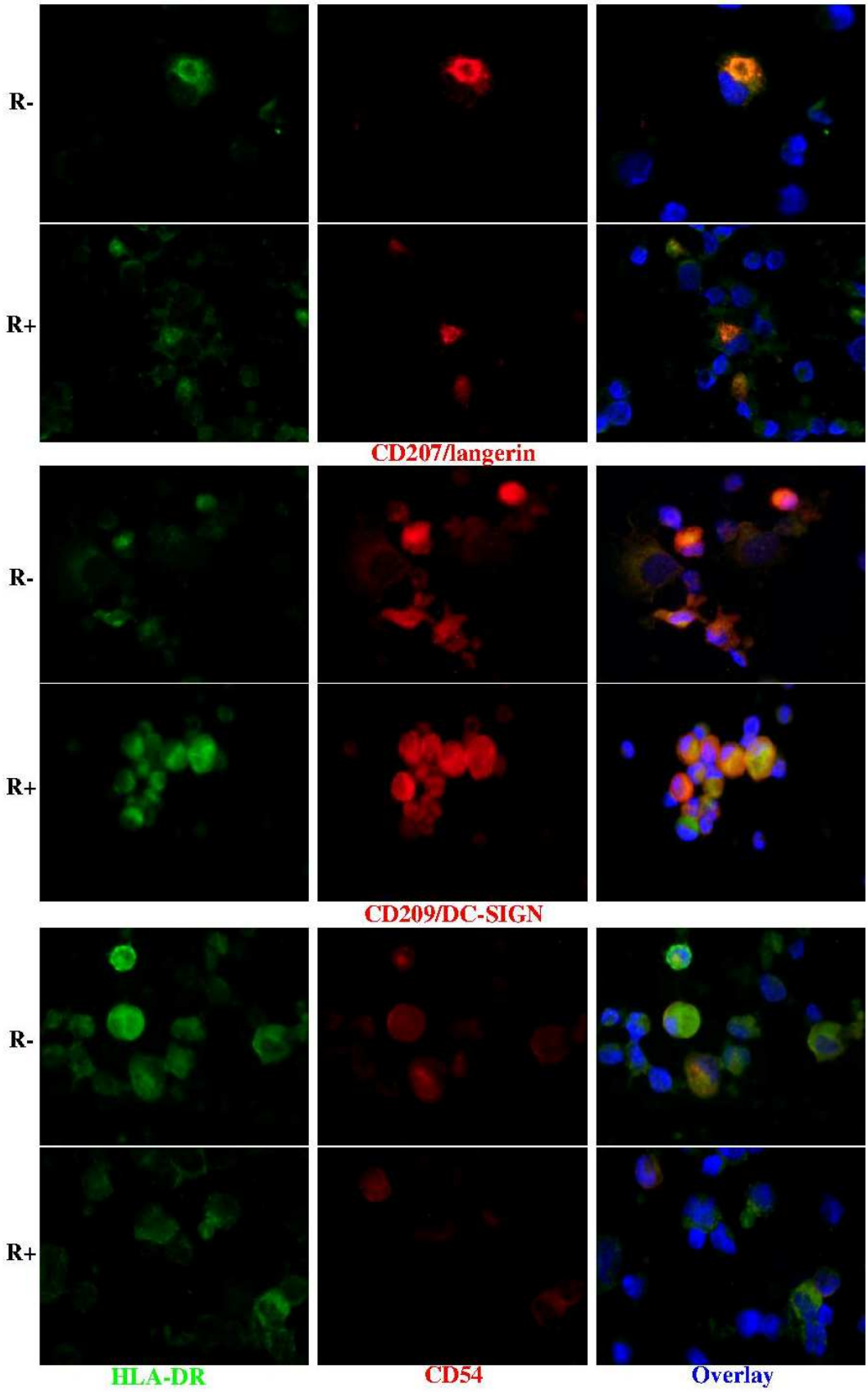
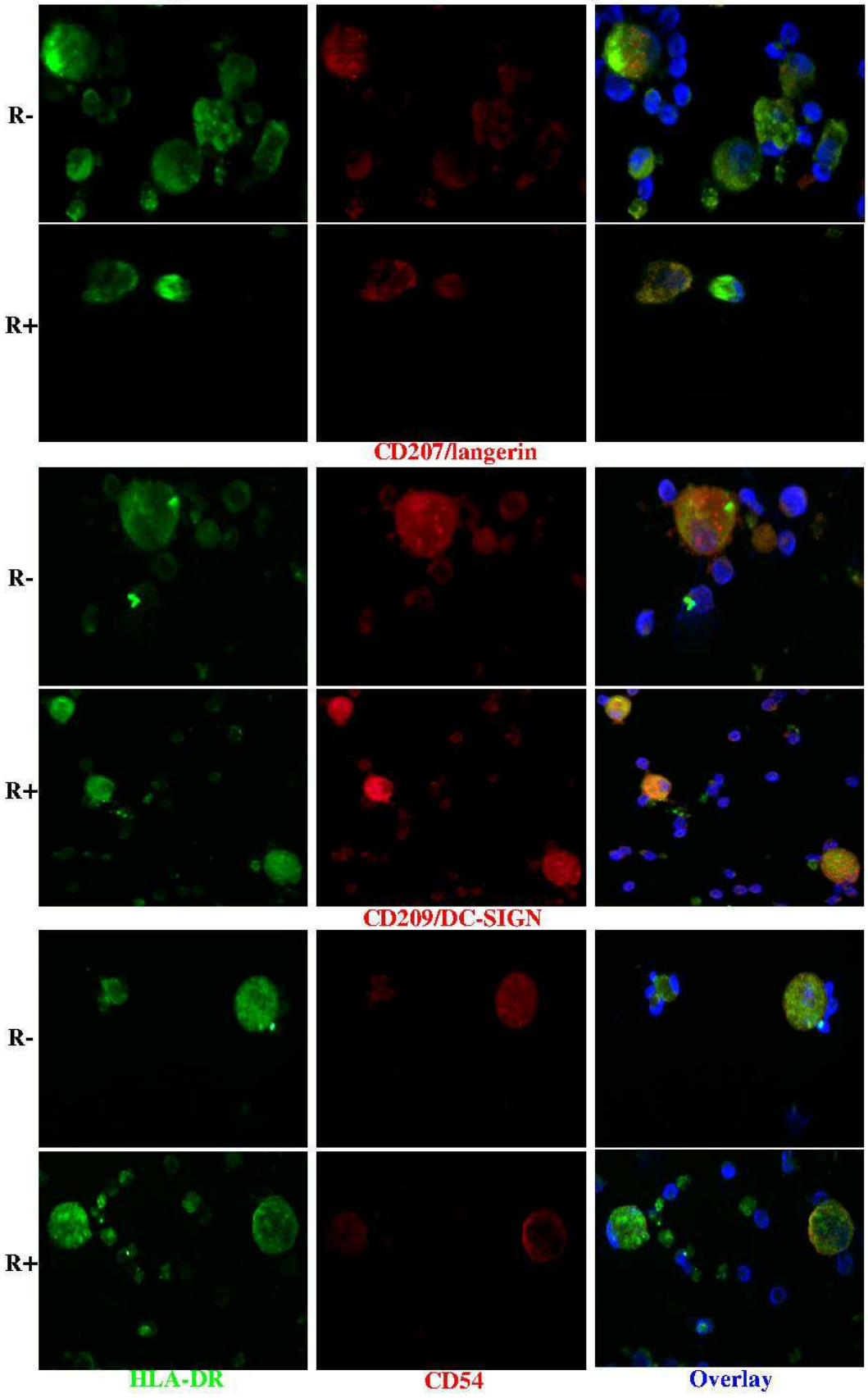


Fig. 6 - DCs obtained from CD133+ precursors



Flow cytometry

Dendritic cells from CD14+ precursors

Dendritic cells obtained from CD14+ precursors were analyzed in the immature and mature state; in both conditions they were HLA-DR+, whether with or without rosiglitazone. The cells were homogeneous for scatter parameters (both forward and side scatter). About a half of the HLA-DR+ cells expressed CD1a, in similar percentage before and after induction of maturation and with or without rosiglitazone.

The percentage of DCs expressing CD207 was slightly higher among immature than mature cells both with and without rosiglitazone. After permeabilization the percentage of DCs expressing CD207 increased over that of unfixed, non-permeabilized cells (fig. 7). The observed differences were not significant.

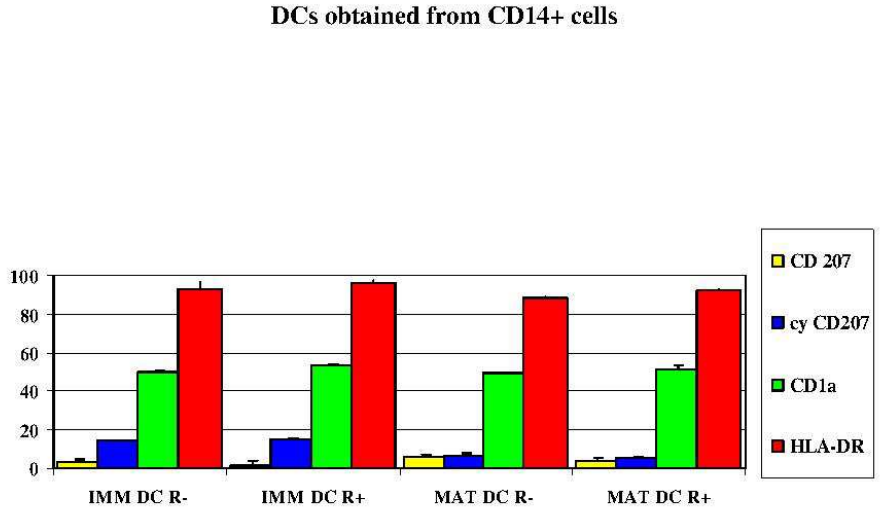


Fig. 7

Percentage of DCs differentiated from CD14+ cells that expressed the indicated antigens, as evaluated by flow cytometry; yellow columns indicate CD207 positivity of unfixed cells, blue columns indicate CD207 positivity upon fixation and permeabilization. The expression of langerin/CD207 was limited to a low number of cells. Mean ± SE, N = 4.

Dendritic cells from CD34+ precursors

The cells obtained from CD34+ precursors could be divided into two populations, one CD33+ CD45+ and the other CD33- CD45+. This latter population was excluded from analysis, because the lack of CD33 prevented to identify those cells as DCs: they represented up to 30-40% cells at the end of culture, as estimated by flow cytometry. Two distinct populations were observed among CD33+ CD45+ cells, which differed in size and cellular complexity. The latter is representative of both cell surface roughness and cytoplasm compartmentalization. The cells with greatest size and complexity were CD1a+ and CD207+. The antigen expression of DCs differentiated from CD34+ precursors precursors is reported in figs. 8, 9 and in fig. 10, it varied among donors for several antigens.

From any donor, all DCs obtained from CD34+ cells were HLA-DR+. Between 50% and 70% of these cells were classified as LC like on the basis of scatter parameters (high forward scatter and high sid scatter). The majority of the latter cells were CD1a+ and almost a half were also CD207+. Rosiglitazone led to increase - but insignificantly - the cells expressing langerin/CD207 on the cell surface (*i.e.*, labelled unfixed) and led to increase the cells labelled for CD207 upon fixation and permeabilization (fig. 10B). A high percentage of LC-like cells expressed CD11c, lower but far from negligeable percentages were found for the maturation marker CD83 and the co-stimulatory molecule CD80 (fig. 10A, C). A part of DCs and of LC-like cells expressed CD209. No significant differences were observed depending on the presence or absence of rosiglitazone.

DCs obtained from CD34+ precursors without rosiglitazone

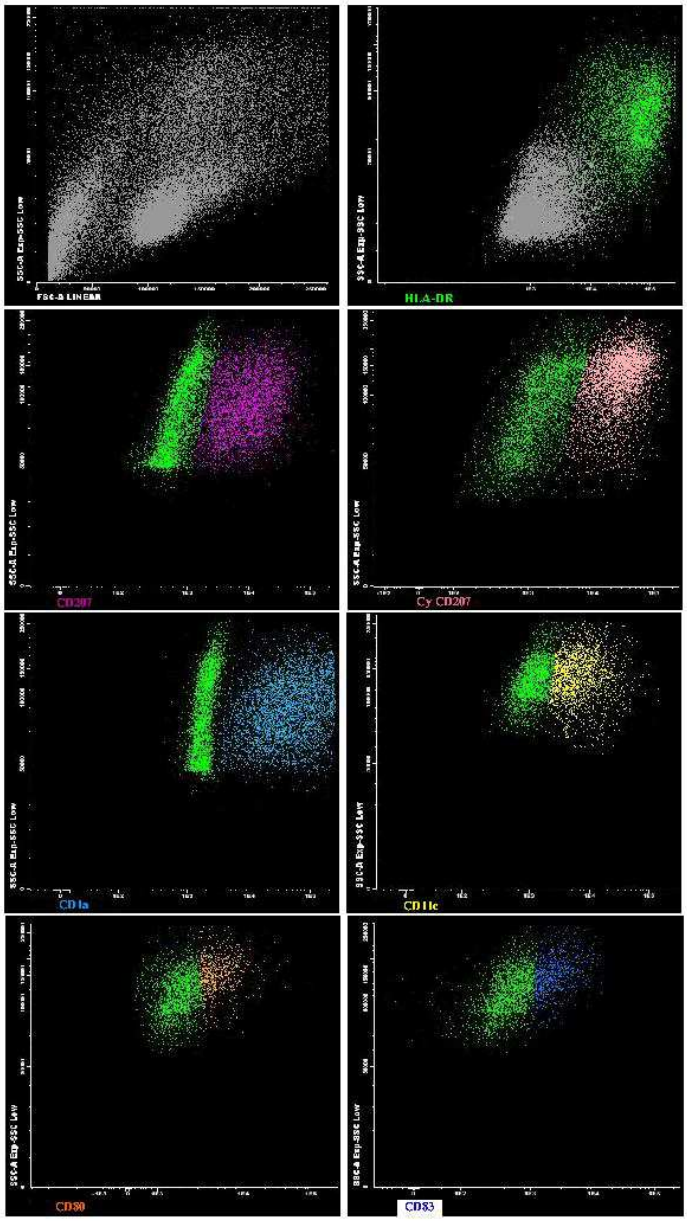


Fig. 8

Flow cytometry analysis of antigen expression by CD34+ cells upon differentiation in DCs, including LCs, without addition of rosiglitazone during culture. Dot plot of a representative experiment. The markers evaluated are shown below in each dot plot.

All HLA-DR positive cells were considered for analysis. These cells are shown in the right panel of the first line. In the following panels, green dots indicate cells labelled for HLA-DR but not for the indicated second marker.

DCs obtained from CD34+ precursors with rosiglitazone

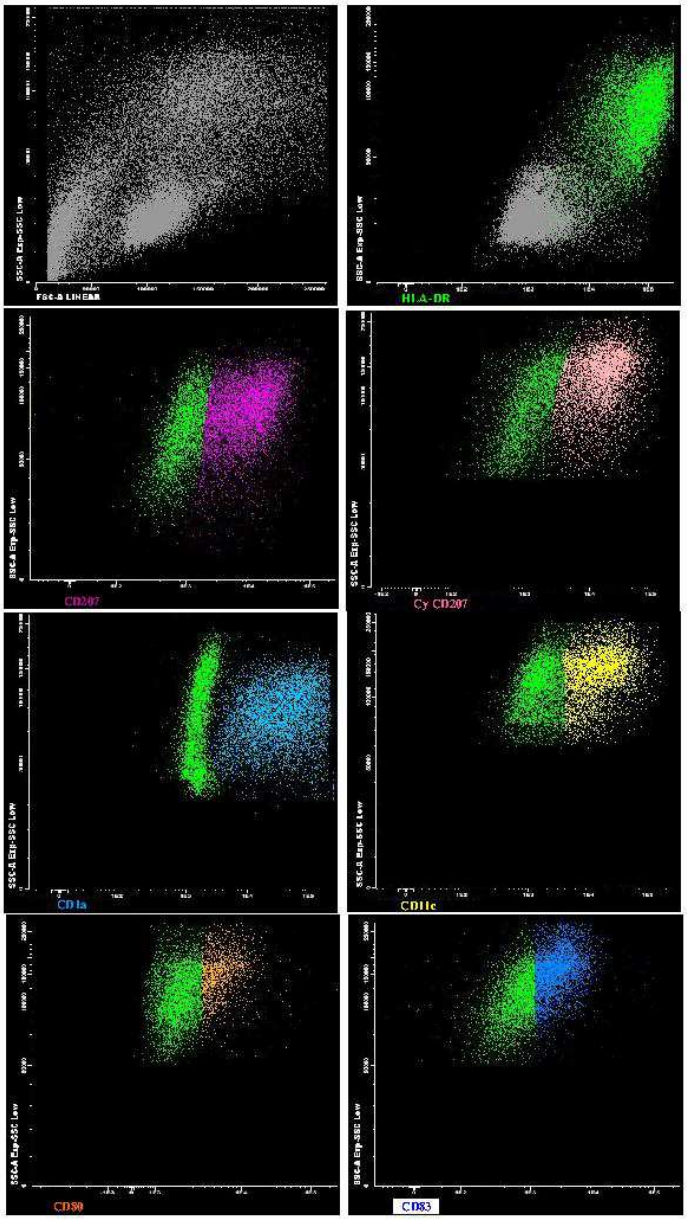


Fig. 9

Flow cytometry analysis of antigen expression by CD34+ cells upon differentiation in DCs, including LCs, with rosiglitazone added to the culture medium. Dot plot of a representative experiment. The markers evaluated are shown below in each dot plot.

All HLA-DR positive cells were considered for analysis. These cells are shown in the right panel of the first line. In the following panels, green dots indicate cells labelled for HLA-DR but not for the indicated second marker.

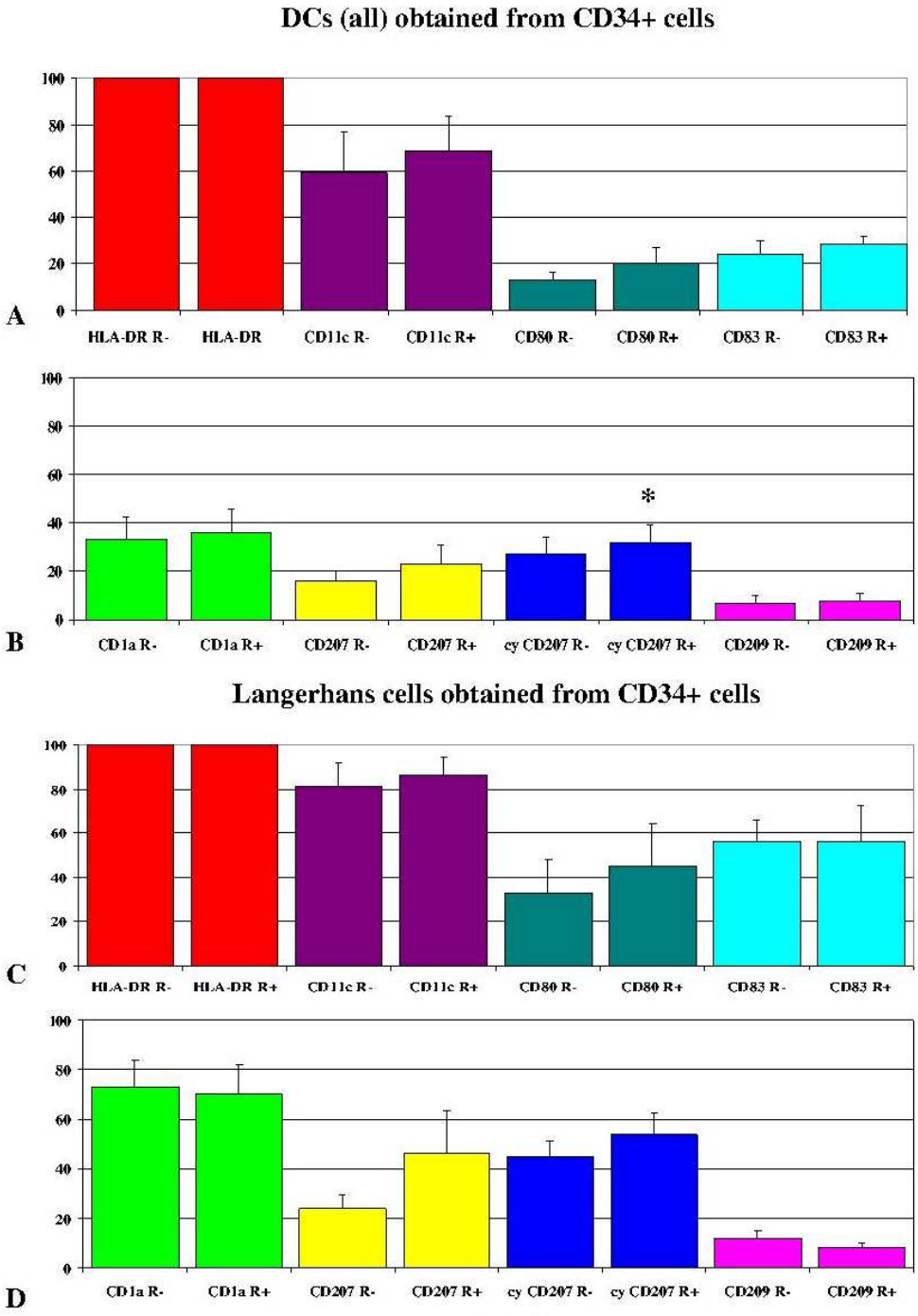


Fig. 10

Flow cytometry analysis of DCs differentiated from CD34+ precursors. (A, B): Total DCs. (C, D): Cells with scatter features of LCs. The tested antigens are indicated in the X axis caption; yellow columns indicate CD207 positivity of unfixed cells, blue columns indicate CD207 positivity upon fixation and permeabilization. The percentage of langerin/CD207 positive cells was significantly higher among cells grown with than without rosiglitazone (*$p<0.05$). Mean ± SE, N = 4.

Dendritic cells from CD133+ precursors

Also the DCs obtained from CD133+ precursors could be divided into a CD33+ CD45+ and a CD33- CD45+ population and only the first one (about 60-70% harvested cells) was subjected to further analysis. They were all HLA-DR+; 53-94% cells were LC-like, both with and without rosiglitazone, as appreciated by scatter parameters and the expression of CD1a and CD207 (figs. 11, 12). The percentage of cells expressing CD207+ was significantly higher among cells grown with than without rosiglitazone; this was true for both membrane and total (*i.e.* including intracytoplasmic) labelling; the amount of cells labelled upon permeabilization was not higher than that of cells labelled without fixation nor permeabilization. All LC-like cells expressed CD11c and the vast majority expressed the maturation marker CD83 and the co-stimulatory molecule CD80; a considerable percentage of DCs and LCs expresses DC-SIGN; no significant differences were observed for these molecules depending on the presence or absence of rosiglitazone (fig. 13).

The difference in the expression of CD80 between cells obtained from CD34+ and from CD133+ precursors was significant, the higher expression being by cells obtained from CD133+ cells, with or without rosiglitazone.

DCs obtained from CD133+ precursors without rosiglitazone

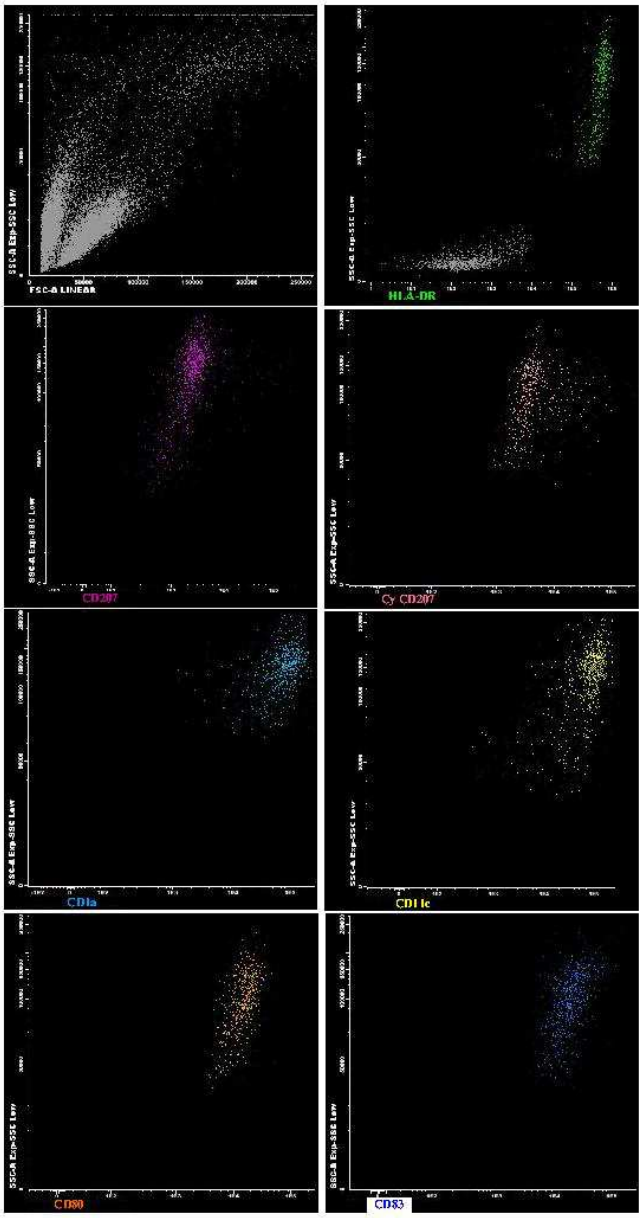


Fig. 11

Flow cytometry analysis of antigen expression by CD133+ cells upon differentiation in DCs, including LCs, without addition of rosiglitazone during culture. Dot plot of a representative experiment. The markers evaluated are shown below in each dot plot.

All HLA-DR positive cells were considered for analysis. These cells are shown in the right panel of the first line. In the following panels, green dots indicate cells labelled for HLA-DR but not for the indicated second marker.

DCs obtained from CD133+ precursors with rosiglitazone

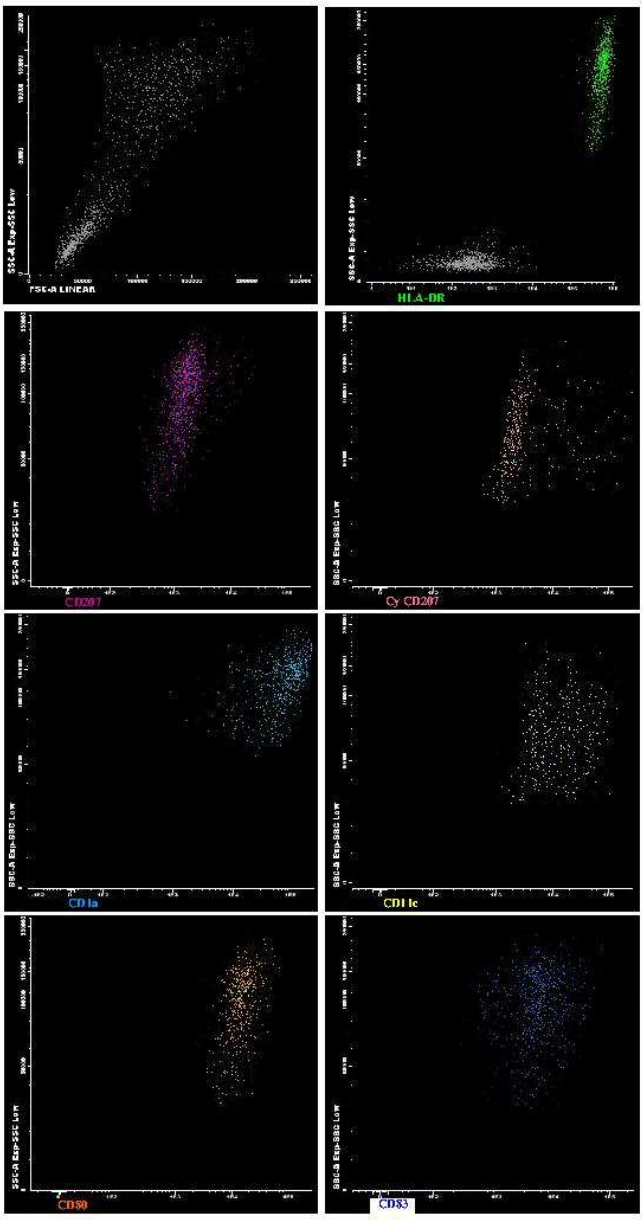


Fig. 12

Flow cytometry analysis of antigen expression by CD133+ cells upon differentiation in DCs, including LCs, with rosiglitazone added to the culture medium. Dot plot of a representative experiment. The markers evaluated are shown below in each dot plot.

All HLA-DR positive cells were considered for analysis. These cells are shown in the right panel of the first line. In the following panels, green dots indicate cells labelled for HLA-DR but not for the indicated second marker.

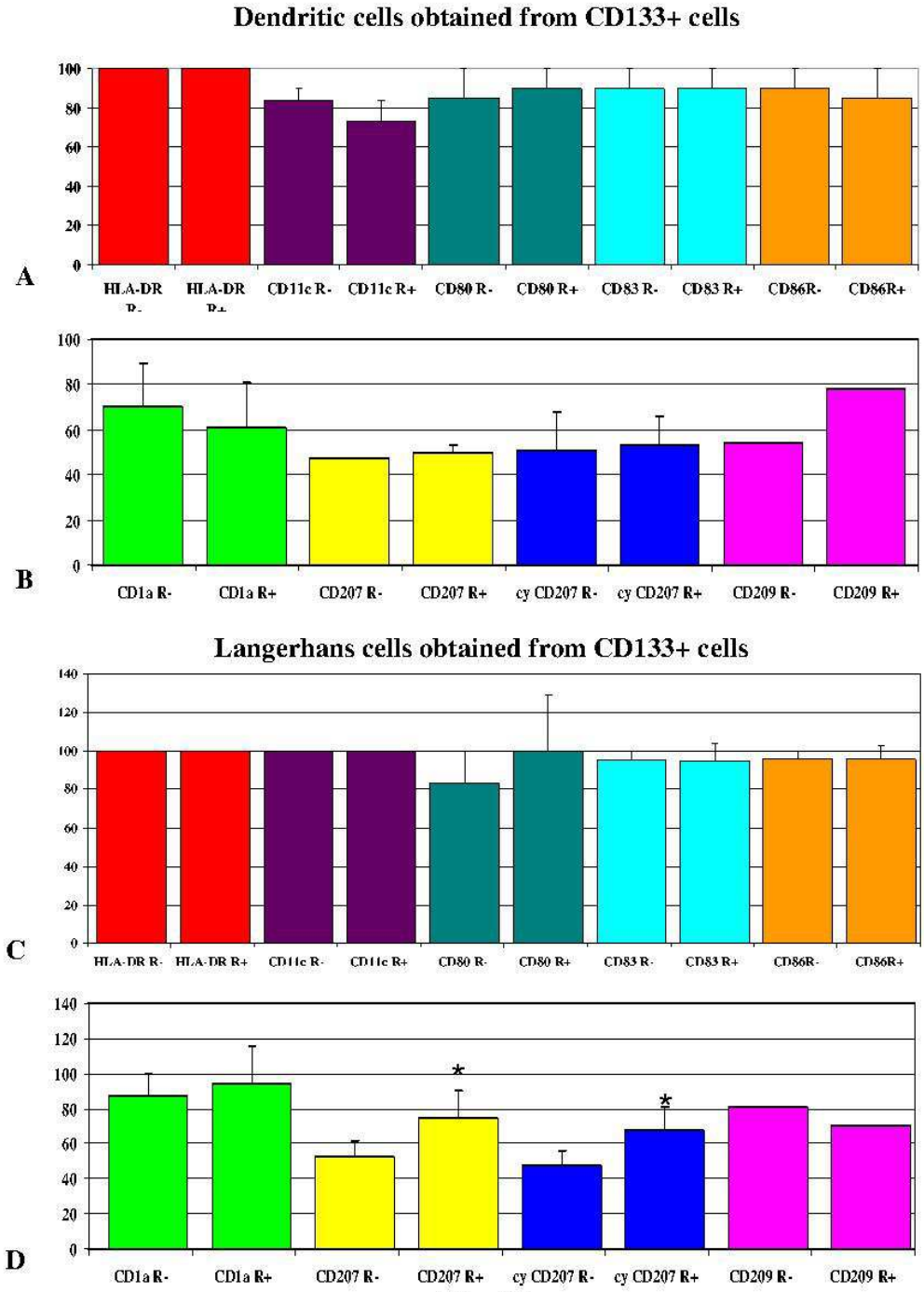


Fig. 13

Flow cytometry analysis of DCs differentiated from CD133+ precursors. (A, B): Total DCs. (C, D): Cells with scatter features of LCs. Captions colours as in fig. 9. The percentage of langerin/CD207 positive cells was significantly higher among cells grown with than without rosiglitazone (*$p<0.05$). Also, the percentage of CD80+ cells was significantly higher ($p<0.01$) than in cells generated from CD34+ precursors (with or without rosiglitazone). Mean ± SE, N = 4 except for DC-SIGN.

Gene expression

PPAR-γ gene expression differed greatly depending on donor and precursor cell type, while no significant differences were found between cells grown with or without rosiglitazone.

Newly isolated CD14+ cells expressed a low number of copies of PPAR-γ mRNA. The expression increased during culture, with a maximum after maturation *i.e.* 9 days culture (fig. 14A).

Freshly isolated CD34+ precursors showed a much lower expression of PPAR-γ gene than CD14+ cells and the expression remained low for all the culture time (fig. 14B).

Also freshly isolated CD133+ precursors showed a low number of PPAR-γ mRNA copies, but higher than that observed for both CD14+ and CD34+ cells. This number increased with culture, variably among experiments *i.e.* donors; the response to rosiglitazone varied greatly with donors, the mRNA increased in the first 14 d of culture in three experiments and did not increase in two experiments (fig. 14C). In all but one experiments, *i.e.* with four donors, the expression of PPAR-γ mRNA upon 7 d culture was higher than that of newly isolated CD14+ cells.

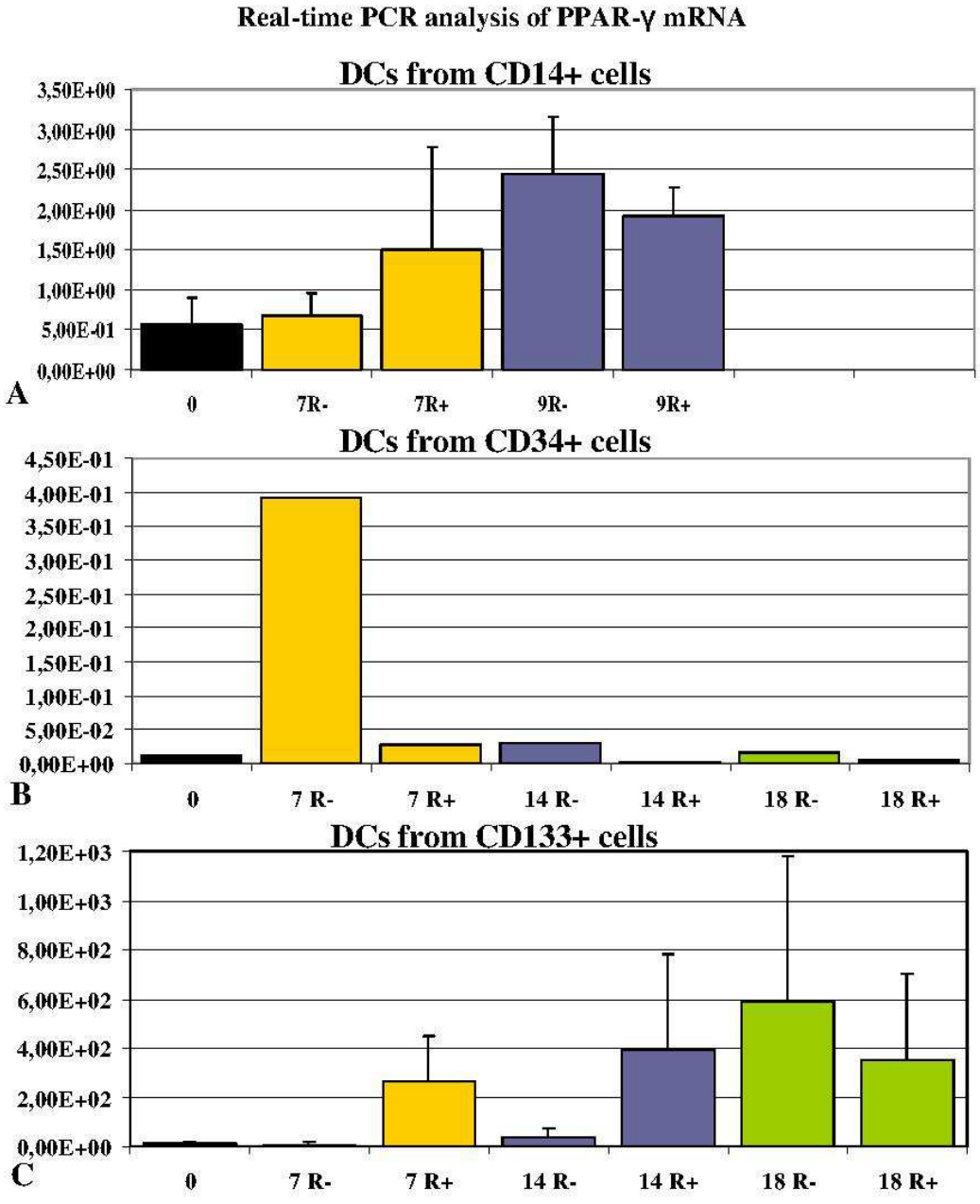


Fig. 14

The graphs show the the number of the PPAR-γ mRNA copies in DCs generated from different precursors and analyzed at different times of culture (indicated by the numbers on the X axis captions), without and with rosiglitazone (R- and R+ respectively). The graduation on the Y axis differ among the graphs, depending on the intensity of expression of PPAR-γ mRNA. (A) Mean ± SE, N = 2. (B) Results of a single experiment. (C) Mean ± SE, N = 5.

Electron microscopy

Upon culture of CD14+ precursors, i.e. monocytes, immature dendritic cells were characterized by large size, dendritic shape, nucleus with shallow indentations, loose chromatin with small peripheral clumps, many mitochondria, well developed rough and smooth endoplasmic reticulum, variably extended Golgi apparatus and a few lysosomes. Upon maturation, dense chromatin restricted to a thin, uniform peripheral rim, and the cytoplasm came to contain many lysosomes appearing as electron dense, round bodies and thin curved cisternae with electron dense content, sometimes with cylindrical shape (figs. 15 to 18).

Upon culture of CD34+ and of CD133+ precursors, including a final maturation step, the harvested cells included large, dendritic cells; medium size, mildly dendritic cells; and small, roundish cells. The largest cells, in particular, had and oval nucleus with multiple, usually hollow, occasionally deep indentations, and a uniformly dispersed chromatin; they contained many mitochondria, well developed rough and smooth endoplasmic reticulum, extended Golgi apparatus, and lysosomes. The intermediate sized cells also had an oval nucleus, with deep indentations, and many, small, uniformly distributed clumps of chromatin besides an irregular peripheral rim of condensed chromatin. They contained several mitochondria, little rough and abundant smooth endoplasmic reticulum, a small Golgi apparatus and a few lysosomes. The smallest cells had a round, lightly scalloped nucleus, with huge clumps of dense chromatin both attached to the nuclear envelope and free in the nucleoplasm; the cytoplasm was poor in organelles, but for many free ribosomes (figs. 19 to 24).

Addition of rosiglitazone to culture led to modifications of the nucleus and cytoplasm of large dendritic cells, in that the condensed peripheral chromatin appeared as a thin uniform rim instead of small clumps and the cytoplsm was enriched in many lysosomes appearing as electron dense, round bodies and in thin, curved cisternae with

electron dense content, sometimes with cylindrical shape. Some cells also contained wide lysosomes with inhomogeneous content sometimes including recognizable remnants of organelles, hence expressing auto- or heterophagocytosis. These modifications were seen also in immature dendritic cells generated from CD14+ precursors.

Straight, short structures with parallel membranes and a fain, unstructured central density, as wide as Birbeck granules, were recognized occasionally in large dendritic cells in all culture conditions (figs. 16C, 24A).

Immature DCs obtained from CD14+ precursors without rosiglitazone

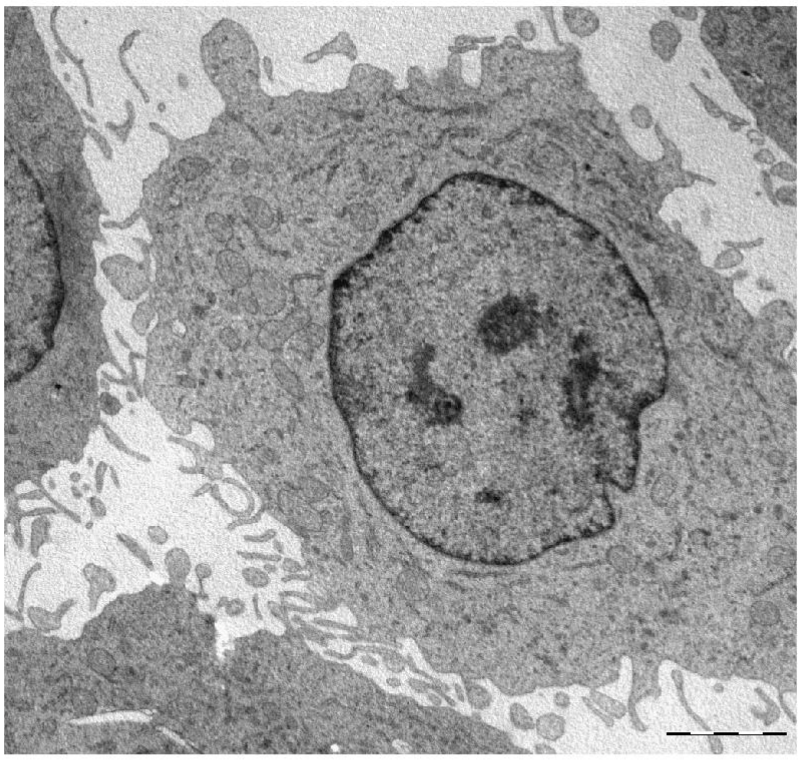


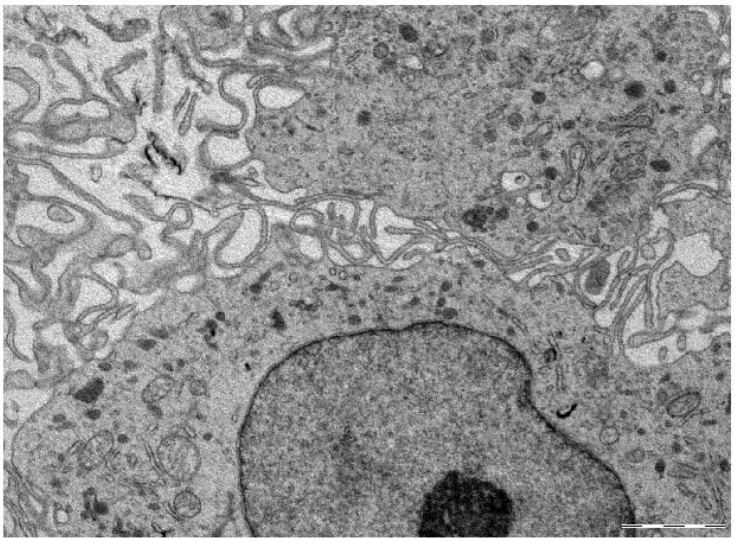
Fig. 15

Large cell with dendritic shape and indented nucleus, loose chromatin except for a peripheral rim, many mitochondria, well developed rough and smooth endoplasmic reticulum. Bar = 2 μm.

Immature DCs obtained from CD14+ precursors with rosiglitazone

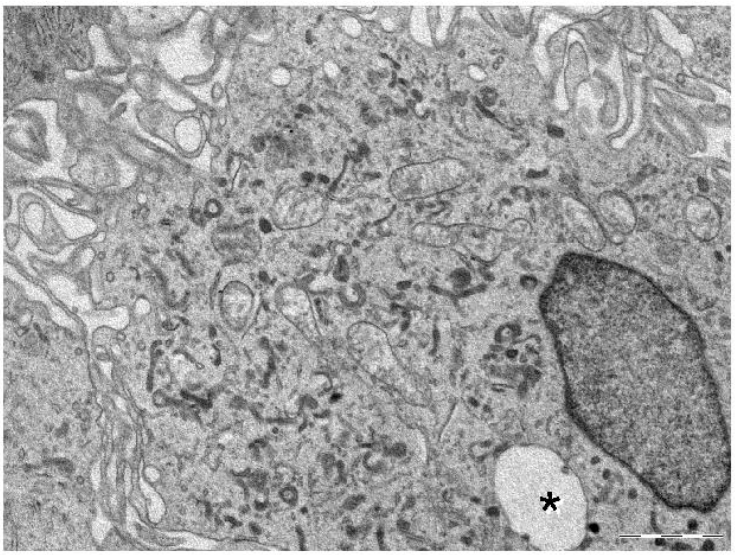
Fig. 16

A



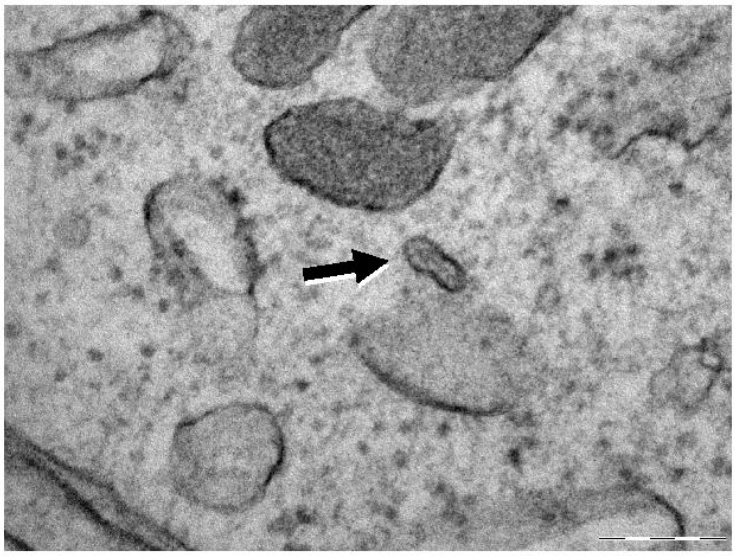
Large cells with dendritic shape and nucleus with smooth profile. Bar = 2 µm.

B



Detail of a dendritic cell; the electron dense structures are part of the lysosomal compartment; the asterisk marks a multivesicular body. Bar = 2 µm.

C

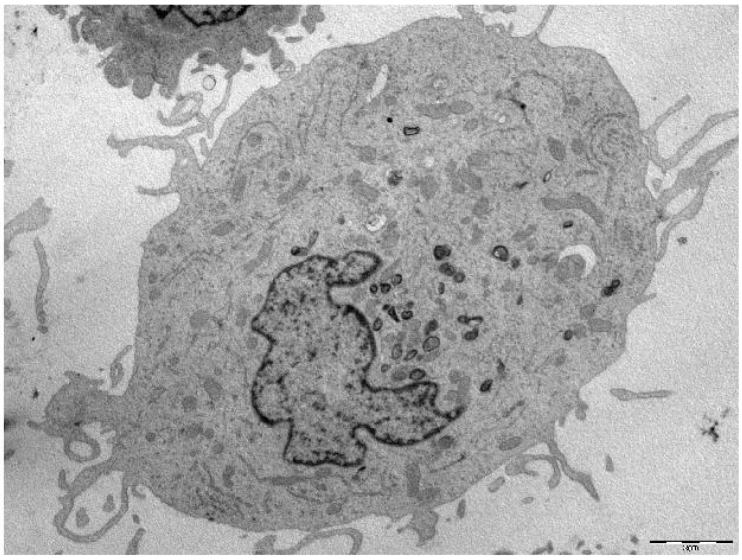


Short rod-like structure with parallel membranes and unstructured central density, similar to a Birbeck granule. Bar = 200 nm.

Mature DCs obtained from CD14+ precursors without rosiglitazone

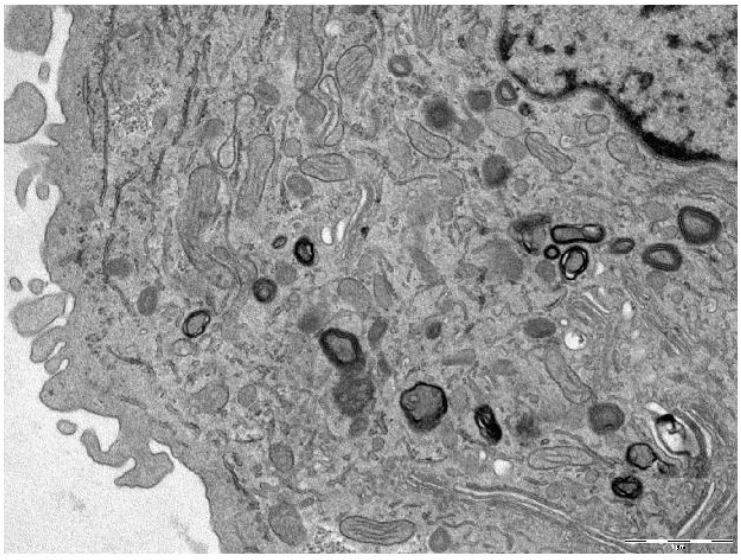
Fig. 17

A



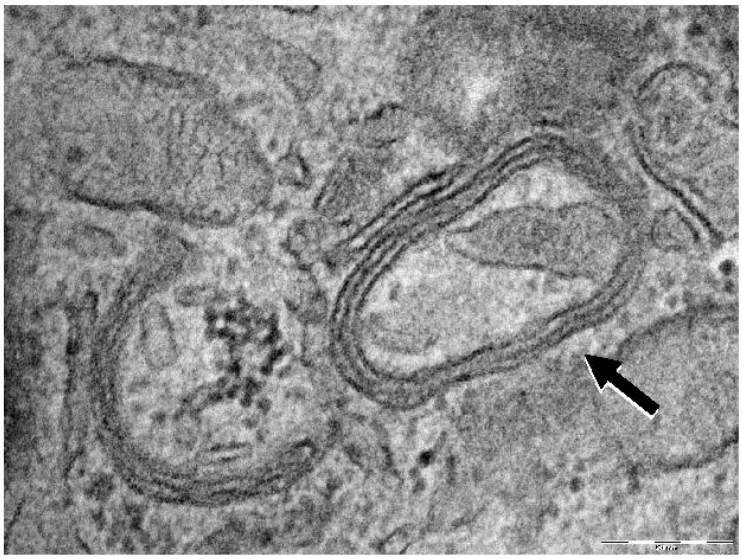
Large cell with dendritic shape and loosely indented nucleus; the electron dense structures are part of the lysosomal compartment. Bar = 2 µm.

B



Detail of a dendritic cell, with rough and smooth endoplasmic reticulum and round electron dense bodies. Bar = 1 µm.

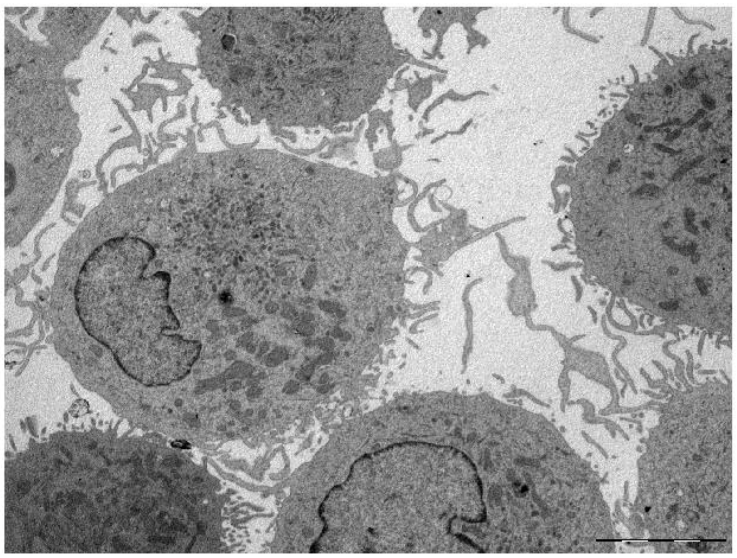
C



Curved cisternae with parallel membranes electron dense content. Bar = 200 nm.

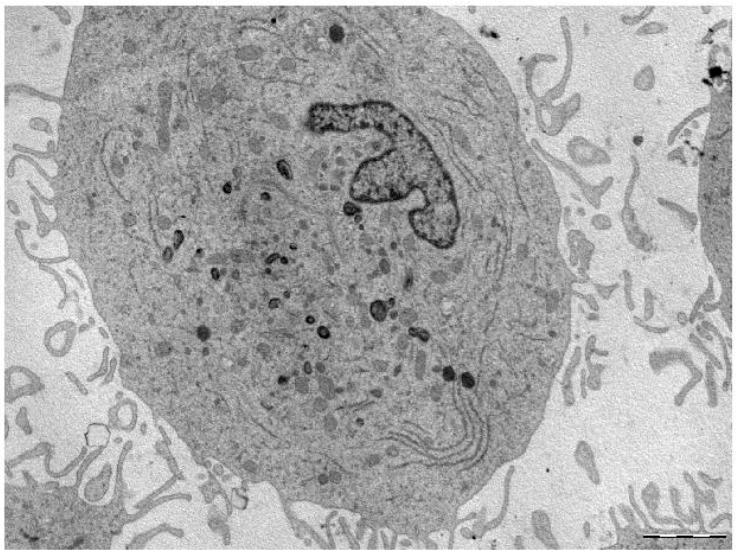
Mature DCs obtained from CD14+ precursors with rosiglitazone

Fig. 18



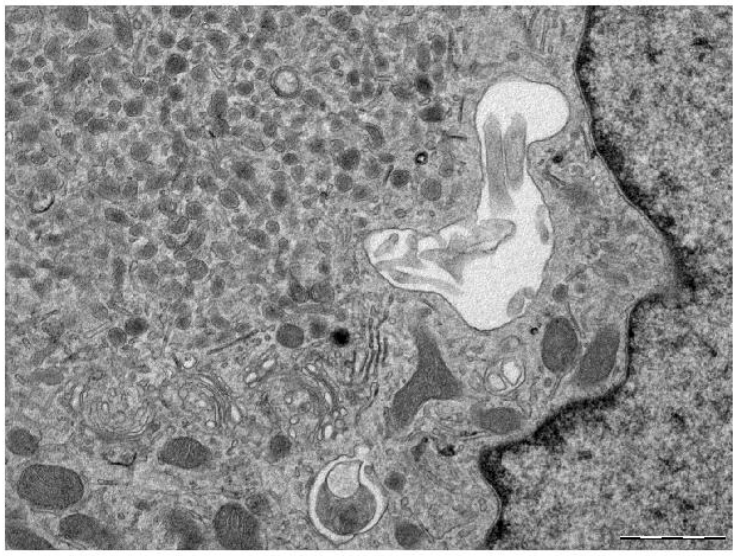
A

Overview of dendritic cells. Bar = 5 µm.



B

Detail of a dendritic cell. Bar = 2 µm.

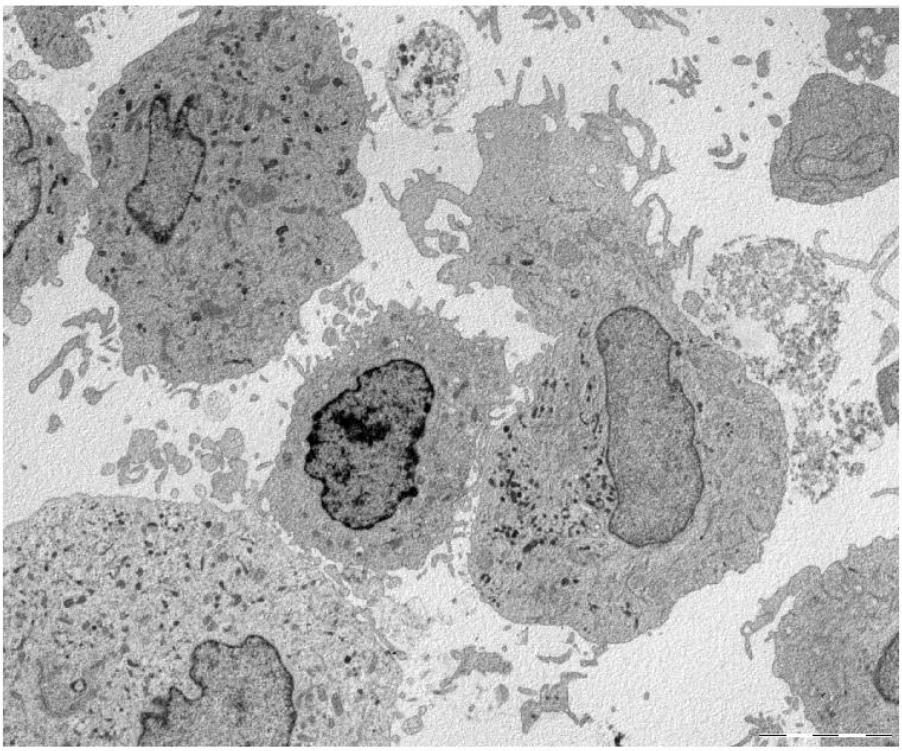


C

Detail of a dendritic cell especially rich in lysosomes. Bar = 1 µm.

DCs obtained from CD34+ precursors without rosiglitazone

A



B

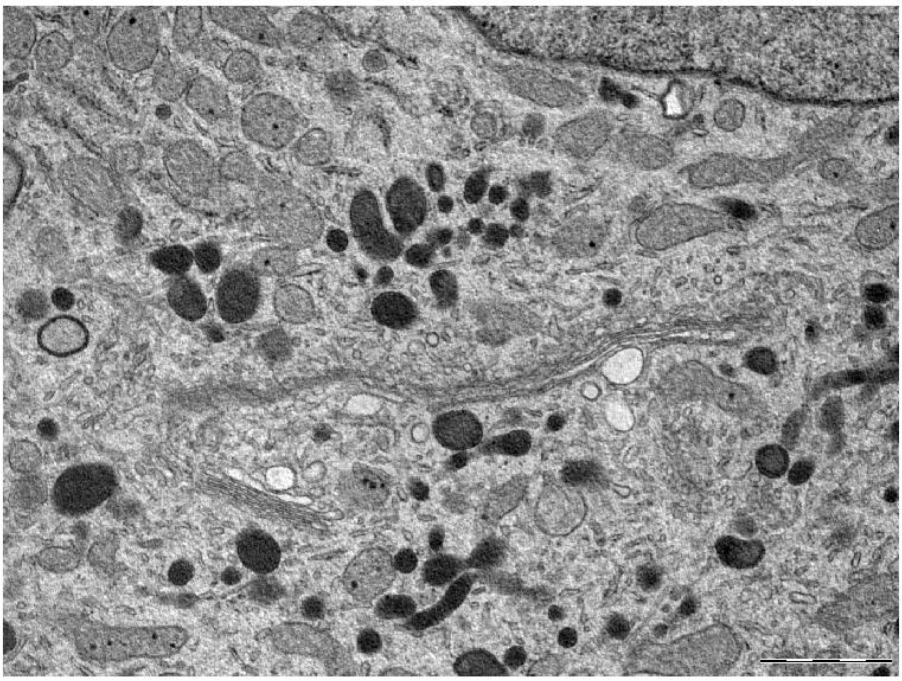


Fig. 19

(A) Dendritic cells of different size. Bar = 5 μm. (B) Detail of paranuclear cytoplasm of a dendritic cell, with Golgi apparatus and many lysosomes. Bar = 1 μμ.

Cells obtained from CD34+ precursors without rosiglitazone

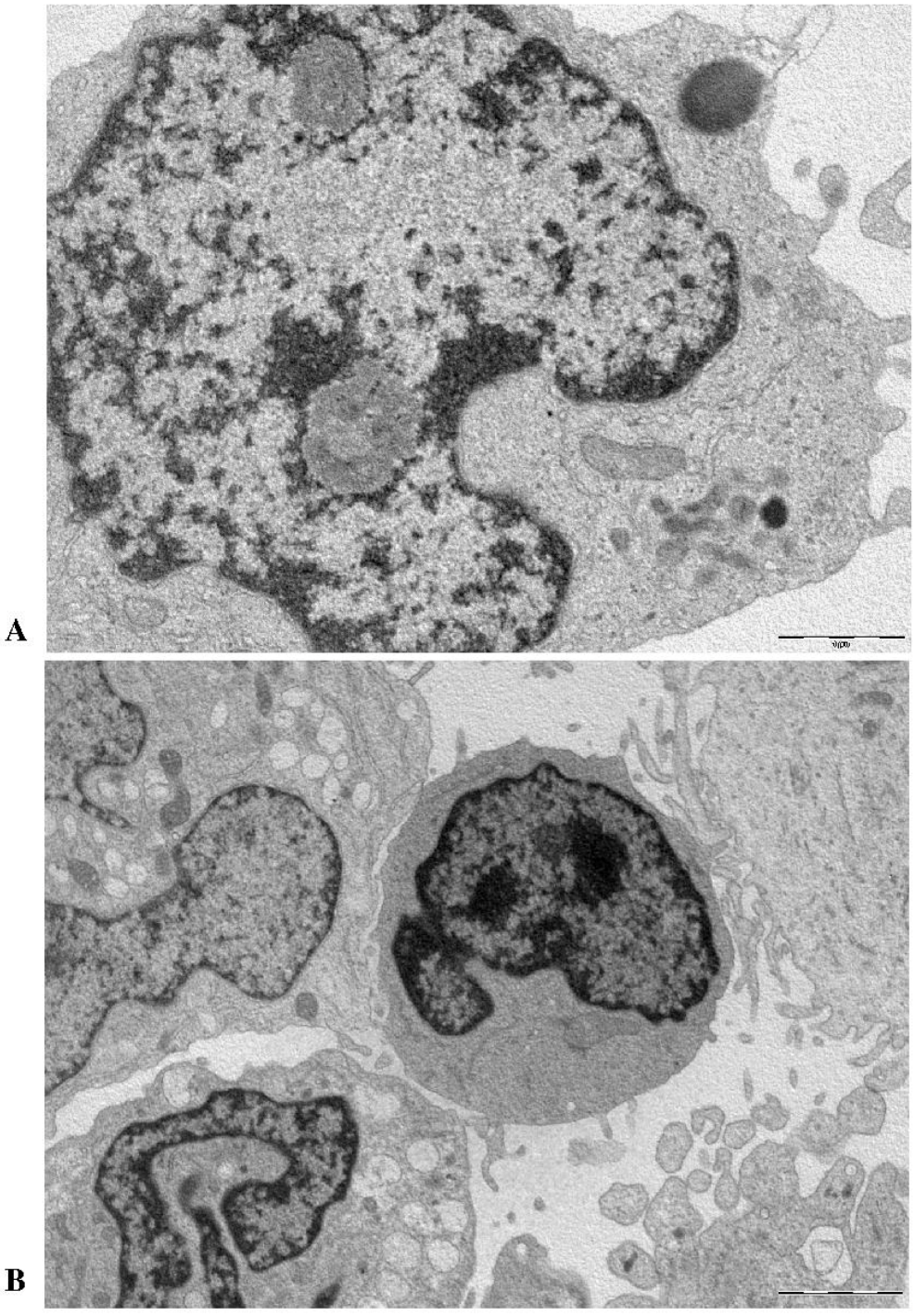


Fig. 20

(A) Small,, poorly dendritic cell with clumped chromatin and few electron dense lysosomes. Bar = 1 µm. (B) Small cell with clumped chromatin and few organelles. Bar = 2 µm.

DCs obtained from CD34+ precursors with rosiglitazone

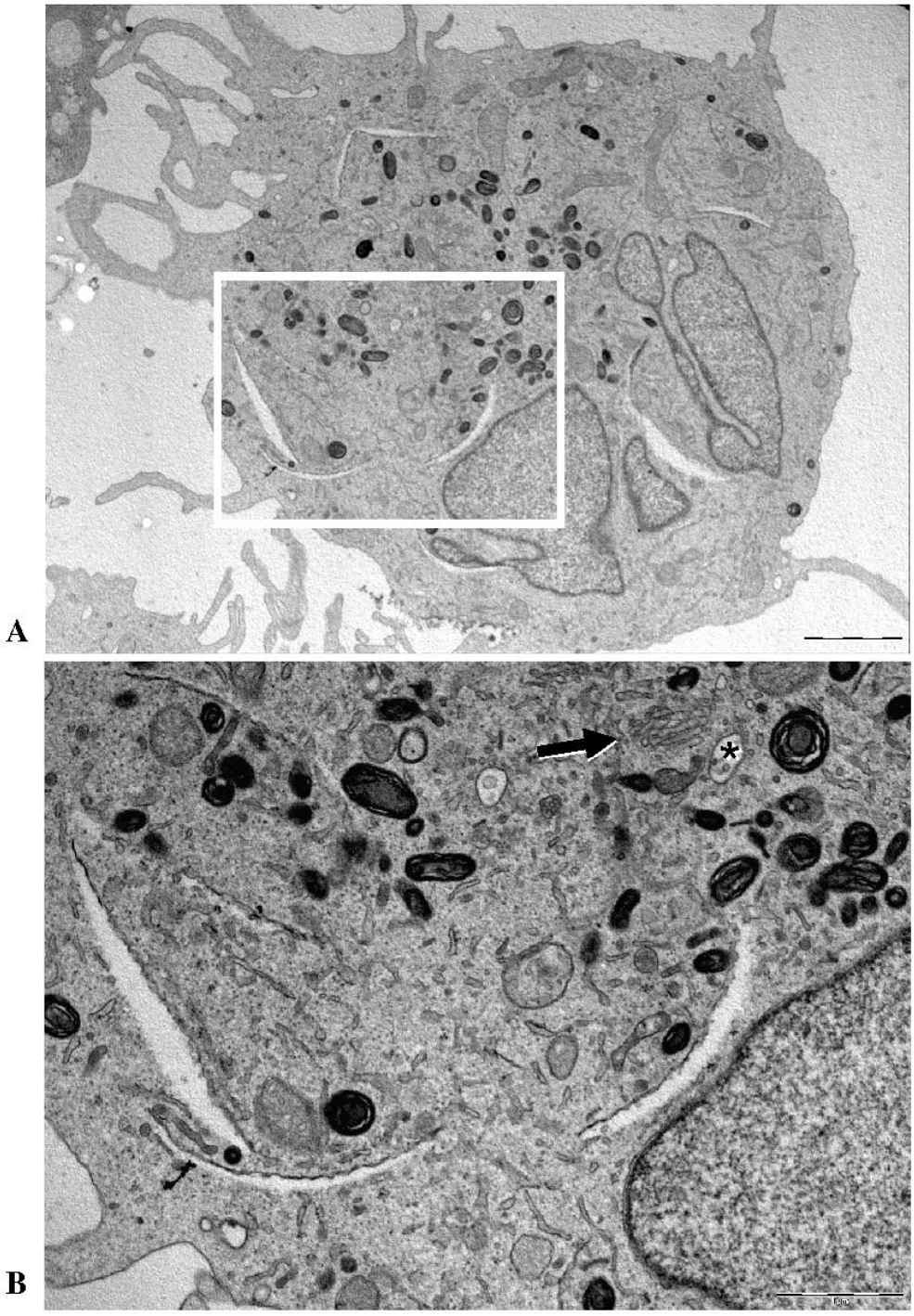


Fig. 21

(A) Dendritic cell. The white spaces in the cytoplasm are cholesterol clefts, sometimes found in cells in culture. Bar = 2 µm. The boxed area is enlarged in (B). (B) Detail of the cytoplasm with lysosomes, multivesicular bodies (asterisk), smooth endoplasmic reticulum and Golgi apparatus (arrow). Bar = 1

DCs obtained from CD133+ precursors without rosiglitazone

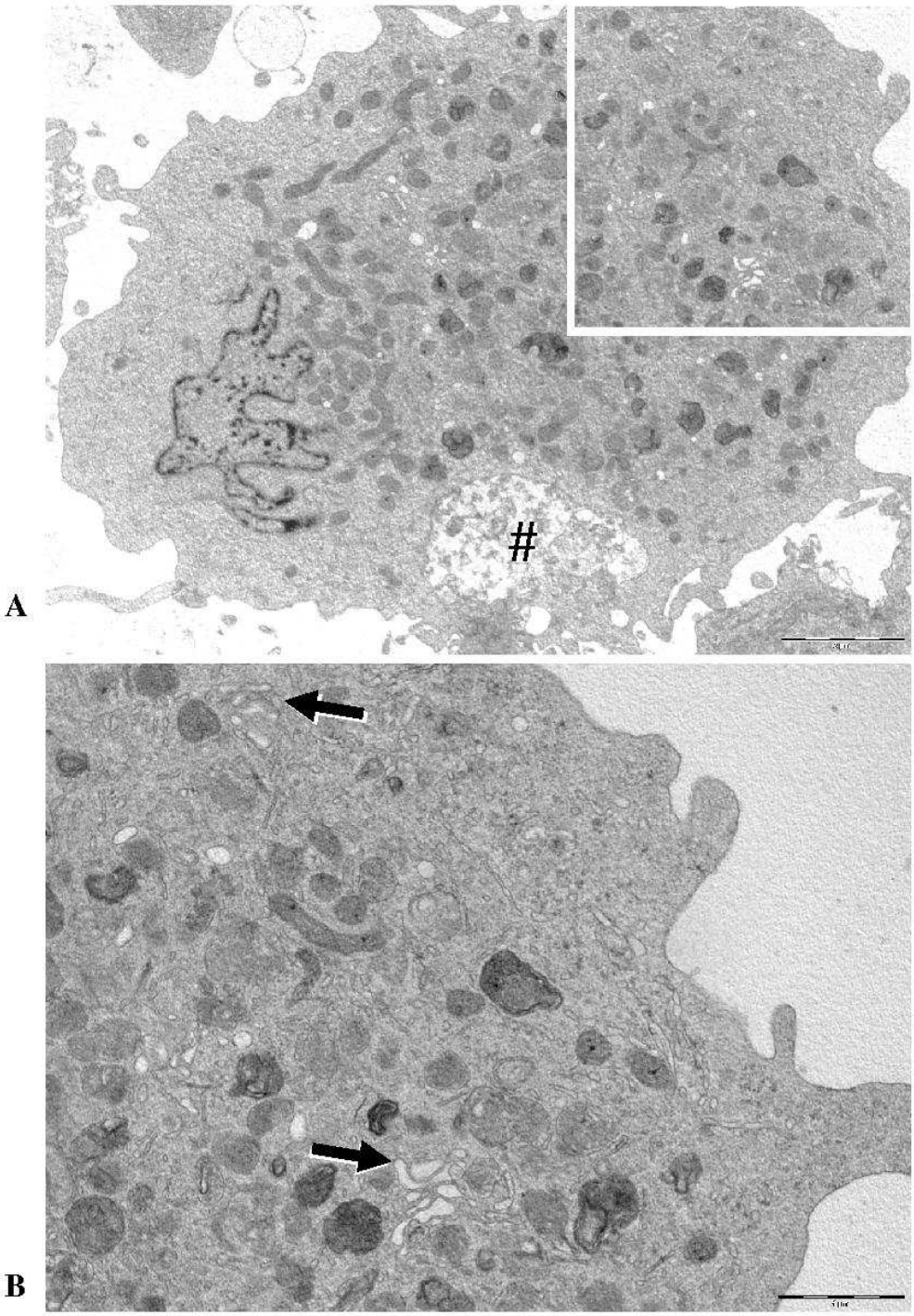


Fig. 22

(A) Large dendritic cell rich in organelles and with a huge lysosome (hashtag). Bar = 2 µm. The boxed area in enlarged in (B). (B) Detail of the cytoplasm with lysosomes and Golgi apparatus (arrows). Bar = 1 µm.

DCs obtained from CD133+ precursors with rosiglitazone

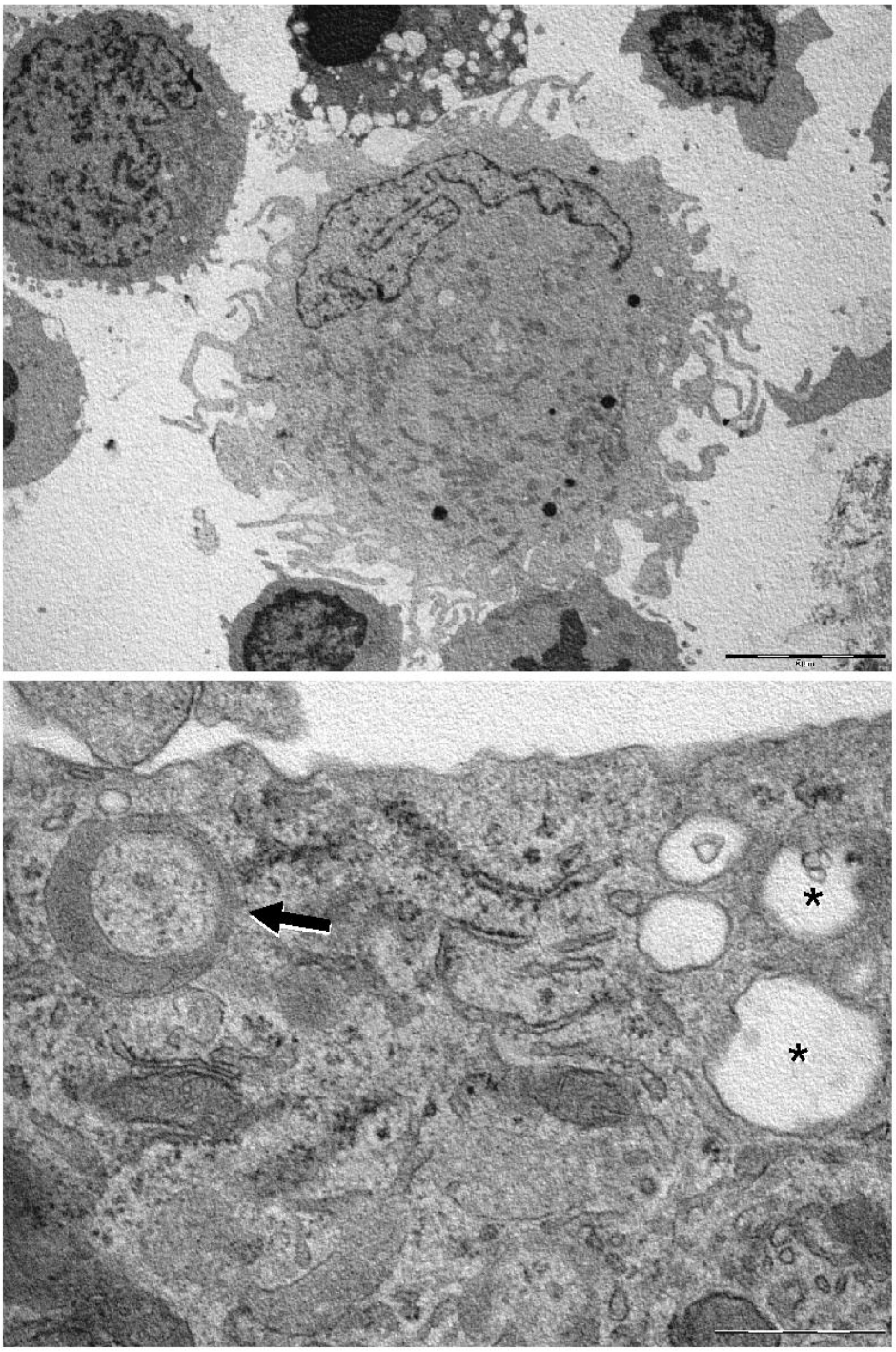


Fig. 23

(A) A large dendritic cell (center), an intermediate size dendritic cell (top left) and some small cells (only in part included in the photomicrograph). Bar = 5 µm.(B) Detail of a dendritic cell with rough and smooth endoplasmic reticulum, a curved cisterna (arrow) and multivesicular bodies (asterisks). Bar = 500 nm.

DCs obtained from CD133+ precursors with rosiglitazone

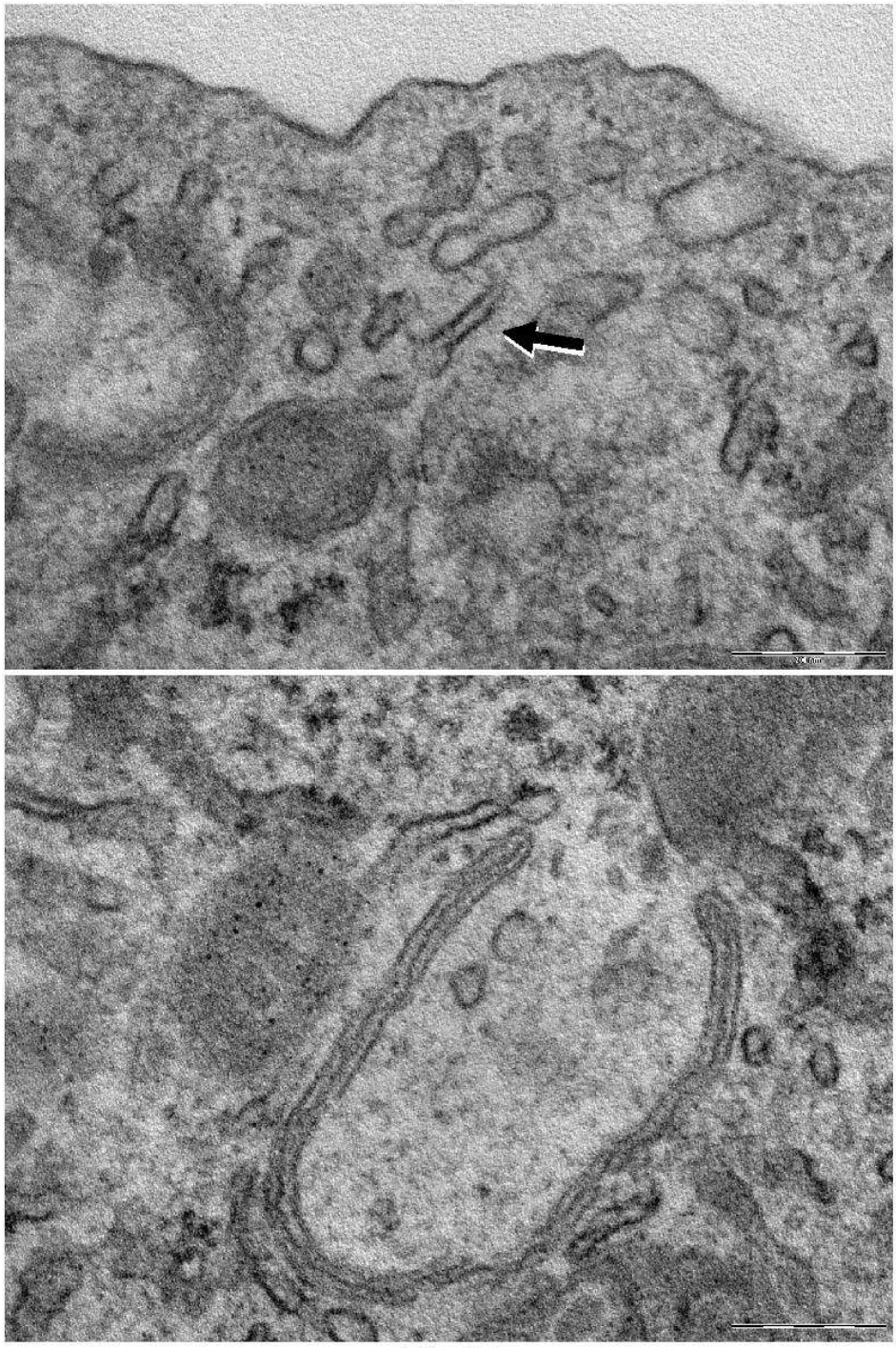


Fig. 24

(A) Short rod-like structure with parallel membranes, unstructured central density and terminal dilation, similar to a Birbeck granule. Bar = 200 nm. (B) Detail of a curved cisterna. Bar = 200 nm.

Mixed lymphocyte reaction

The proliferation of CD4+ lymphocytes after 5 d co-culture with DCs was significantly higher from that of lymphocytes alone. The proliferation was significantly higher if the DCs had been treated without rosiglitazone during differentiation and maturation than if they had been grown with rosiglitazone. The measure of proliferation by dilution of CFSE allows to separate the response of different subtypes of lymphocytes. This led to recognize that the proliferative response of CD8+ lymphocytes to DCs was much lower than that of CD4+ lymphocytes; it appeared less marked when DCs had been differentiated in the presence of rosiglitazone but the difference was not significant, possibly because of the low numbers of proliferating cells in either condition (figs. 25, 26).

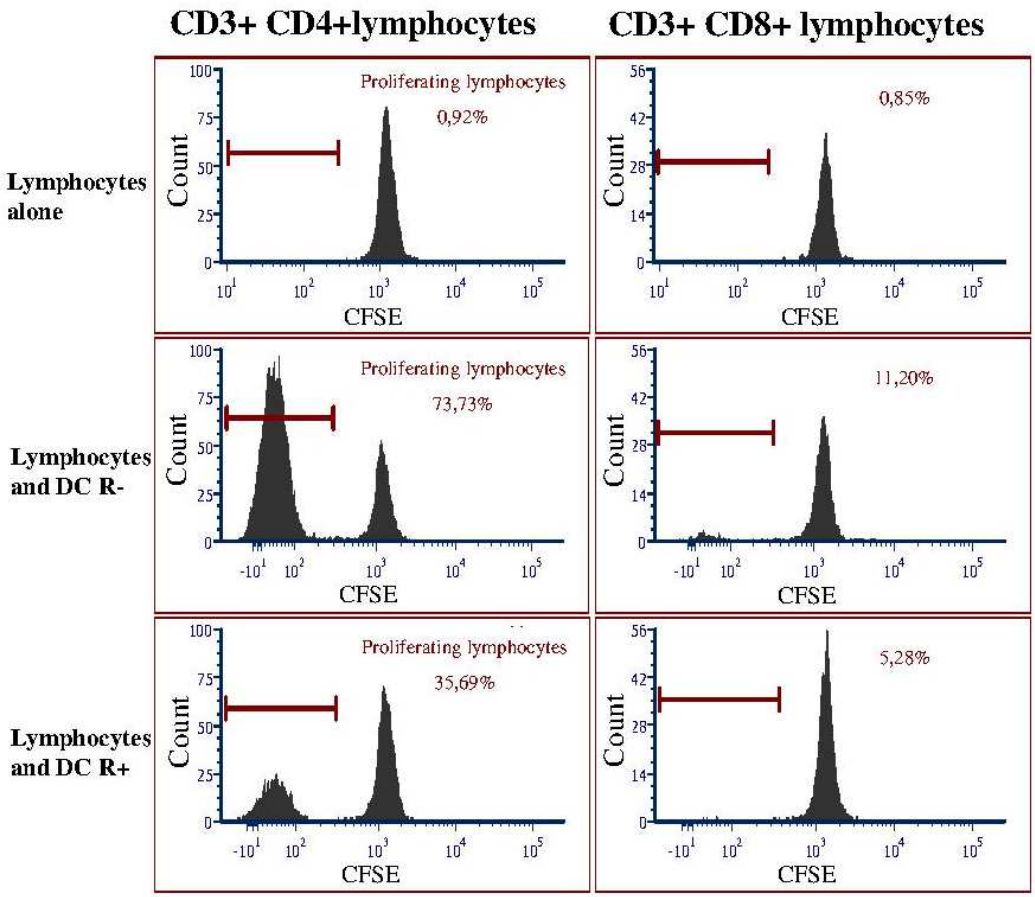


Fig. 25

MLR. T lymphocyte proliferation in response to DCs generated from CD133+ precursors without (R-) or with rosiglitazone (R+). The peak on the right in all graphs indicates the population of CFSE loaded lymphocytes at the beginning of co-culture. The peak on the left (where present) indicate the lymphocytes that have proliferated after 5 d of co-culture with DCs and therefore have undergone a dilution of the label. The proliferation of CD4+ lymphocytes increased significantly after 5 days with and without rosiglitazone, compared to lymphocytes alone ($p<0,001$), and the proliferation of CD4+ lymphocytes stimulated by DCs grown without rosiglitazone was even higher ($p<0,001$) than the proliferation induced by DCs grown with rosiglitazone. The proliferation of CD8+ lymphocytes was virtually negligible. Percentages of proliferating lymphocytes are reported for each experimental condition. Similar results were obtained with the lymphocytes of a different donor, see fig. 26.

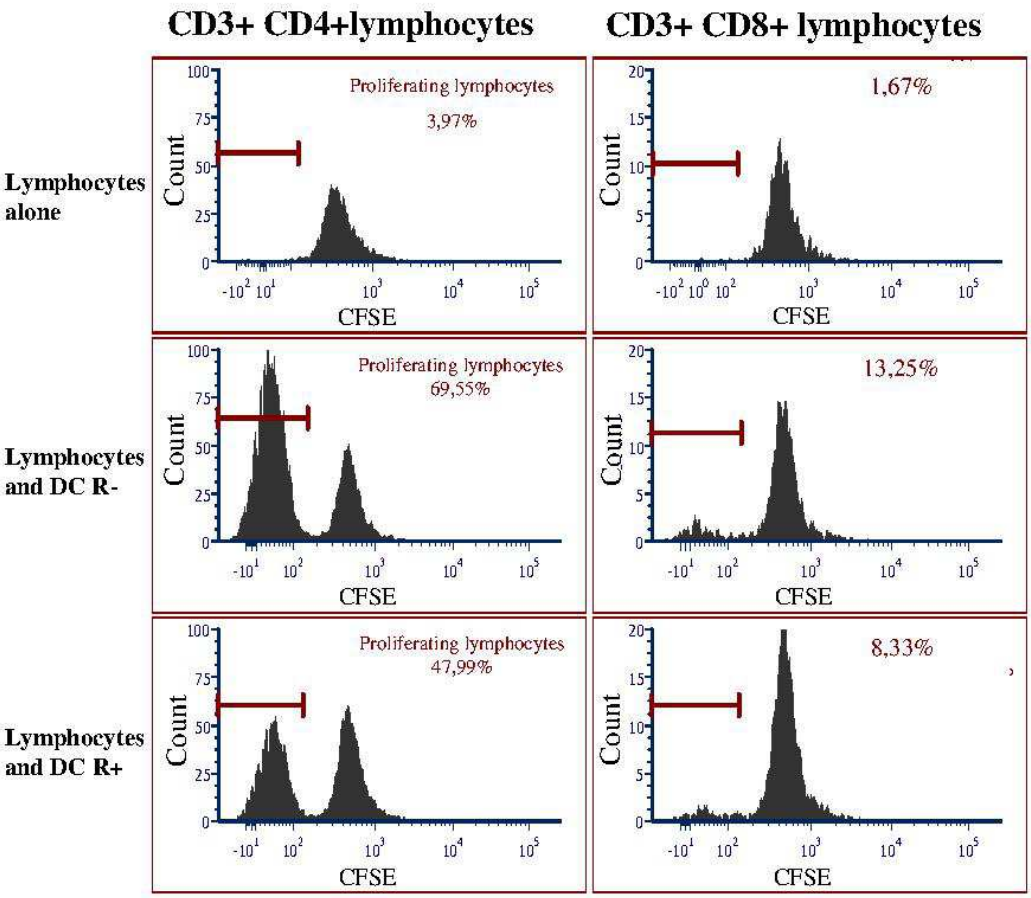


Fig. 26

MLR. T lymphocyte proliferation in response to DCs generated from CD133+ precursors without (R-) or with rosiglitazone (R+). In respect to fig. 23, the DC population was the same while the responding lymphocytes were from a different donor. The results give the same indications in both cases: CD4+ cells responded with significant proliferation ($p<0,001$), CD8+ cells proliferated to a negligible extent, and the proliferation was higher with DCs not treated with rosiglitazone during differentiation. Percentages of proliferating lymphocytes are reported for each experimental conditions.

Interaction of nanoparticles with dendritic cells generated from CD14+ monocytes

About one third immature DCs (29-38%) had internalized 10 µg/ml fluorescent silica NPs after 4 e 24 h of incubation, the percentage of immature DCs internalizing fluorescent NPs raised to 94-98% upon incubation with 100 µg/ml fluorescent NPs. At each time point, the difference between NP concentrations was significant, while those between time points for each concentration were not significant (figs. 27A, 28).

Also the mean fluorescent intensity per labelled cell was significantly higher upon incubation with 100 µg/ml than with 10 µg/ml NPs; this intensity increased significantly between 4 and 24 h incubation with 10 µg/ml NPs, while it remained stable (at high values) upon incubation with 100 µg/ml (figs. 27B, 28).

Electron microscopy showed that addition of gold or silica nanoparticles led to their endocytosis and to the appearance of NPs within membrane-bound compartments, made of both small endocytic vesicles and larger structures with features of endosomes and lysosomes (figs. 29, 30).

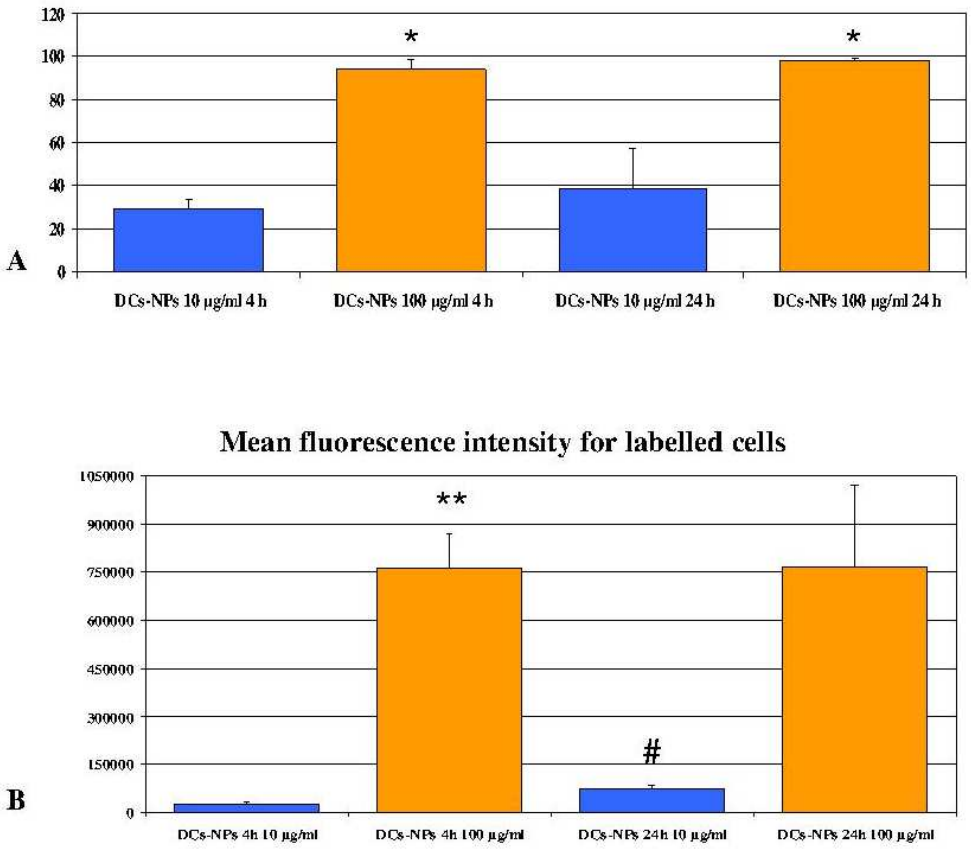


Fig. 27

Immunofluorescence analysis of DCs upon exposure to fluorescent silica NPs. The concentration of NP and the time of exposure are specified in the bottom legend of each graph. (A) Percentage of DCs containing fluorescent SiO_2NPs. At each time point, the difference between NP concentrations was significant (*$p<0.05$), while that between time points for each concentration was not significant. Mean ± SE, N = 2 (10 µg/mL NPs) or 4 (100 µg/mL) independent experiments. (B) Fluorescence intensity of DCs, arbitrary units. The mean ± SE were calculated assuming each cell as a sample unit; N = 15~35 depending on experiment. The difference between NP concentrations was significant (**$p<0.01$) at 4 h, while the difference between 4 and 24 h was significant (**$p<0.01$) for 10 µg/ml NPs (indicated with #).

Immature DCs incubated with fluorescent silica

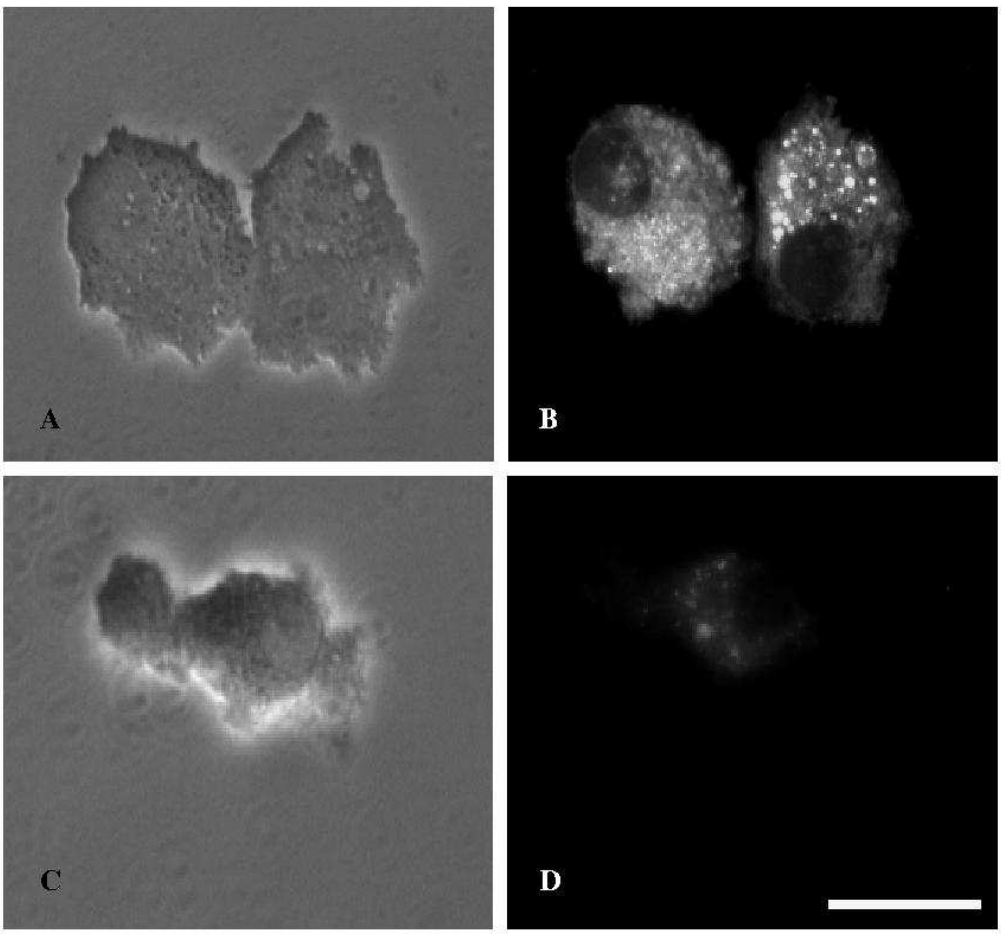


Fig. 28

Immature DCs incubated with fluorescent silica NPs for 24 h.

Phase contrast microscopy (A, C) and fluorescence microscopy (B, D) of the same microscopic fields. (A, B): (C, D): DCs incubated with 10 µg/ml fluorescent silica NPs. The number of intracellular fluorescent bodies, *i.e.* lysosomes, and the intensity of fluorescence were directly correlated with the concentration of NPs. Bar = 10 µm.

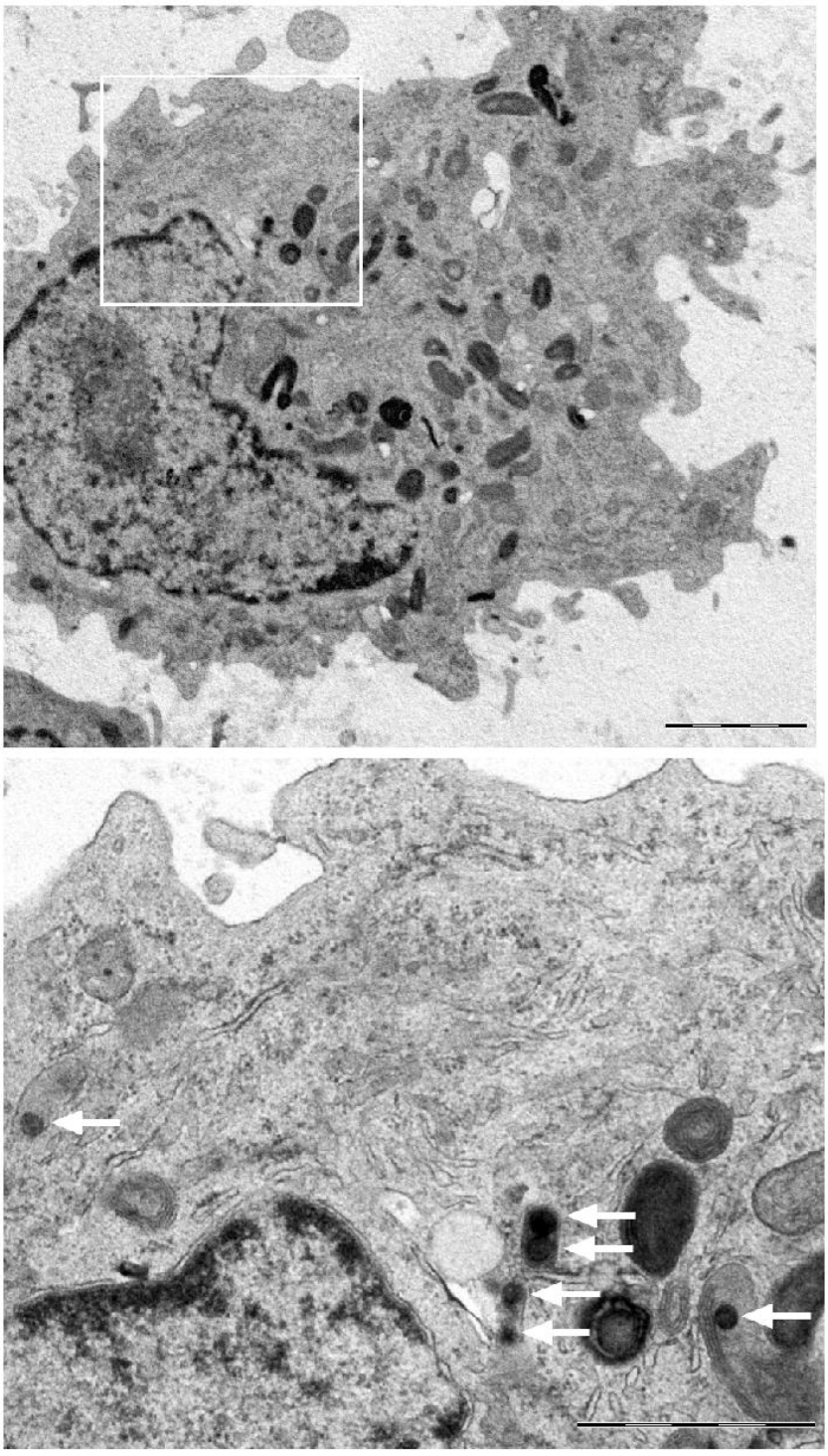


Fig. 29

DCs generated from CD14+ precursors and incubated with SiO_2NPs for 48 h. Endocytosed NPs appeared in small vesicles and in larger vacuoles mainly in the Golgi area, representing late endosomes and lysosomes. (A) Overview. Bar = 2 µm. (B) Detail of the boxed area in (A). The arrows indicate SiO_2NPs. Bar = 1 µm.

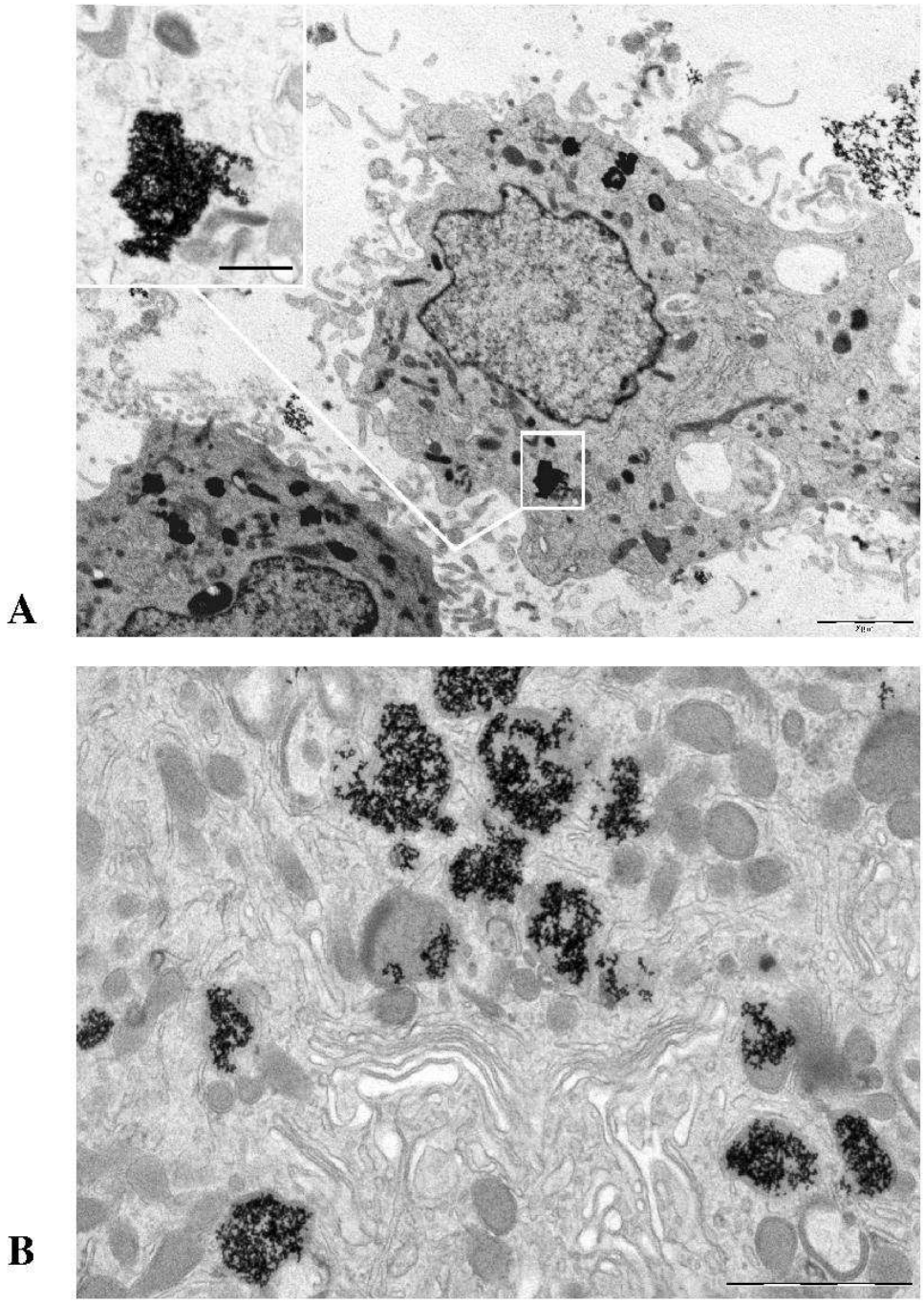


Fig. 30

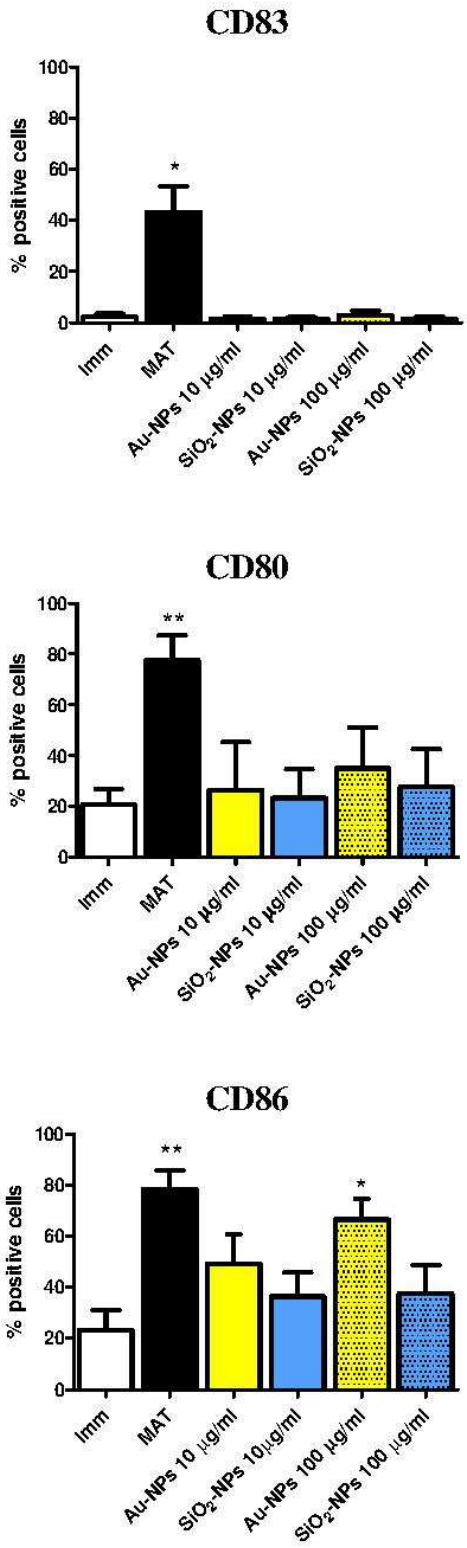
DCs generated from CD14+ precursors and incubated with AuNPs for48. Endocytosed NPs were located in small endocytic vesicles and in late endosomes and lysosomes. (A) Overview; the boxed area is magnified in the inset and shows an irregularly shaped lysosome containing AuNPs. Bar 1 = µm, inset bar = 400 nm (B) Detail. Bar = 1 µm.

The immunophenotype of immature DCs incubated with SiO_2NPs and AuNPs

(10 µg/ml and 100 µg/ml) was compared with that of untreated immature DCs (negative control) and of DCs stimulated with maturation-inducing cytokines (positive control). The results showed that NPs did not influence maturation markers expression of DCs, except for CD86 which was significantly increased only following 100 µg/ml AuNPs (23 ± 8% and 66 ± 8% for DCs without NPs and DCs with 100 µg/ml AuNPs, respectively) (fig. 31).

Dendritic cells treated with SiO_2NPs or AuNPs did not stimulate lymphocyte proliferation significantly, at variance with DCs maturated with inflammatory cytokines (fig. 32).

Fig. 31



Expression of maturation markers of DCs upon exposure to inorganic NPs. DCs were generated from CD14+ precursors and treated with SiO_2NPs and AuNPs, 10 e 100 µg/ml each type of NP. Imm: immature DCs; MAT: DCs stimulated with matutation cytokines (positive control); SiO_2NPs or AuNPs 10 µg/ml and SiO_2NPs or AuNPs 100 µg/ml: DCs incubated with SiO_2NPs or AuNPs at 10 and 100 µg/ml, respectively. Mean ± SE, N = 6; *P<0.05 and **P<0.01 vs Imm.

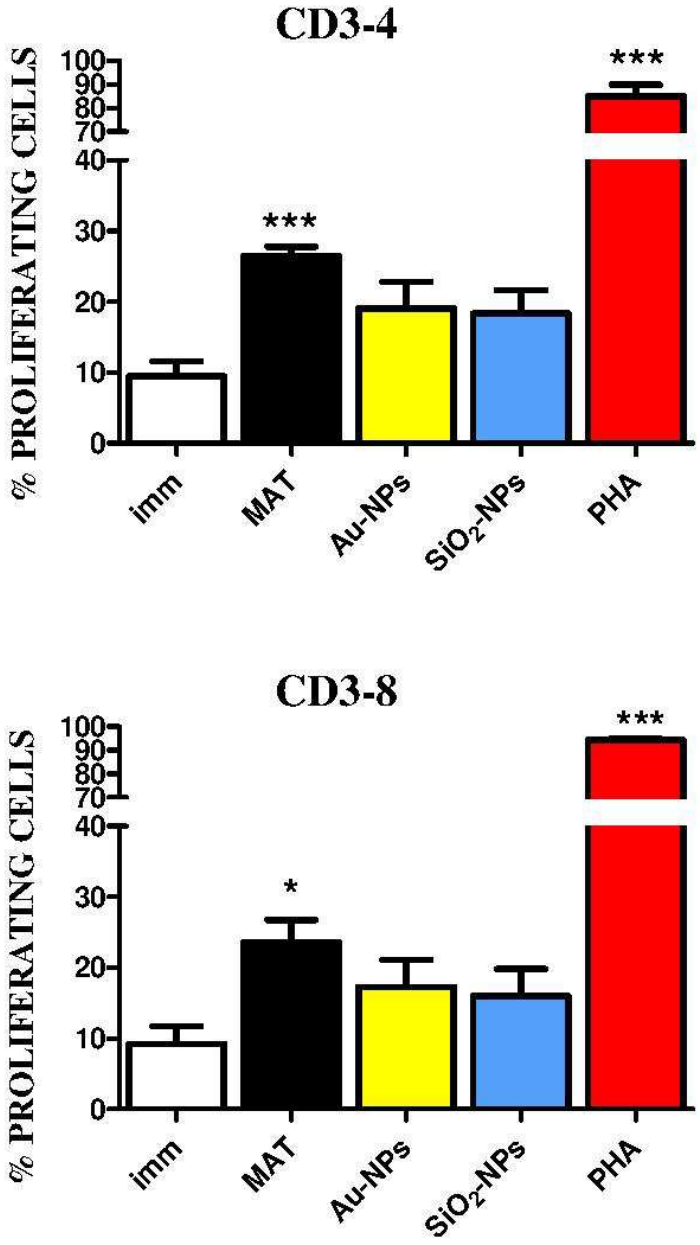


Fig. 32

MLR. T lymphocyte proliferation in response to DCs generated from CD14+ precursors and treated with NPs: Imm: lymphocytes in co-culture with immature DCs; MAT: lymphocytes in co-culture with DCs matured with inflammatory cytokines; AuNPs: lymphocytes in co-culture with immature DCs pre-incubated AuNPs; SiO_2NPs: lymphocytes in co-culture with DCs pre-incubated with SiO_2NPs; PHA: lymphocytes stimulated with 5 µg/ml phytohaemoagglutinin, without DCs (positive control). Mean ± SE, N = 4; *P<0,05 and ***P<0,001 vs Imm.

Discussion

The results of this study have shown that it is possible to generate DCs from different precursors obtained from adult human blood. The generated progeny included cells with features of a specific DC type, *i.e.* LCs; the percentage of this last cell type varied depending of the precursors. The generated cells were able to stimulate mixed lymphocyte reaction, hence they were functionally efficient. The PPAR-γ agonist rosiglitazone influenced the morphology and immunophenotype of DCs, apparently leading to more mature features, but appeared to reduce the capacity of DCs to stimulate MLR when DCs were generated from a specific precursor, namely CD133+ cells which are believed to be the most immature among the tested cells. CD133+ precursors expressed mRNA for PPAR-γ at appreciable level, indeed they were the precursors better endowed with this mRNA among those studied here.

Large DCs with mature features at electron microscopy, *i.e.* rich in organelles and especially lysosomes, contained inclusions resembling Birbeck granules but with a rudimentary central density. Moreover, they contained flat, curved cisternae that were delimited by parallel membranes, had a central density over most of there extension, and were slightly dilated and with uniform electron dense content in some areas. The images resemble those of extended Birbeck granules elicited by anti-CD1a treatment of human epidermal LCs (Hanau et al., 1988).

At variance with what is usually observed in vivo, the cells with LC features, namely a typical scatter pattern at flow cytometry, the expression of CD1a and langerin/CD207 at immunophenotypical analysis, and cytoplasmic inclusions similar to Birbeck granules as above described, also expressed DC-SIGN/CD209 which instead is typical of connective tissue, non-Langerhans DCs (Nestle et al., 1993; Palucka and Banchereau, 2012; Klechevsky and Banchereau, 2013). This was true whichever the

starting precursor and suggest that the culture conditions cannot reproduce completely those occurring *in vivo*.

Most if not all cells identifiable as LCs, or at least LCs-like, expressed langerin/CD207 at the cell surface as well as in the cytoplasm, since the number of those cells was not appreciably increased by cell permeabilization, as estimated by flow cytometry. Immunocytochemistry confirmed the presence of a labelled granular compartment within langerin/CD207 expressing cells, as expected (Romani et al., 2003). In DCs generated from CD34+ precursors in the presence of rosiglitazone, the number of cells expressing langerin/CD207 was higher upon labelling of fixed, permeabilized cells than unfixed cells, which suggests higher intracellular than membrane localization of this adhesive molecule in such cells, possibly correlated to its cycling through different compartments, and an influence of PPAR-γ stimulation on that cycling.

The small number of cells with LC-like features starting from CD14+ precursors is in substantial agreement with literature reports that LCs are not, or only in limited measure generated from CD14+ monocytes (Geissmann et al., 1998). Also in line with previous reports (Szatmari et al., 2004; Varga and Nagy 2008) is the appearance of PPAR-γ mRNA in cells generated from CD14+ precursors, more intensely if the culture was treated with rosiglitazone which hints to a possible positive feedback. Connective tissue cells (and also keratinocytes) can indeed secrete molecules acting as PPAR-γ stimulants in vivo (Kozak et al., 2002; Nakahigashi et al., 2012; Frolov et al., 2013; Itaka et al., 2015; Mashima and Okuyama, 2015; Powell and Rokach, 2015; Han et al., 2017; Moore and Pidgeon, 2017) and so regulate final differentiation and function of DCs.

Treatment of CD133+ cells with rosiglitazone during culture led to the generation of many cells, only some which with features of LCs. In previous study from

this laboratory on CD133+ cells from cord blood it had been found that a small number of well differentiated LCs were generated while other cell types degenerated into detritus (Bonetti et al., 2014). Therefore the results upon culture of CD133+ precursors from adult blood were in part at variance with those obtained in same laboratory starting from CD133+ precursors of cord blood. It is not possible exclude that this depends on technical reasons because in the meantime from previous research the cytokines have been bought new, hence they were from different lots and in some cases different producers, and also rosiglitazone had to be obtained new, from a different producer. However, it is reasonable to conceive that the differentiation potential and response to PPAR-γ agonists differ between foetus and adult.

The effect of rosiglitazone on the differentiation of DCs appeared contradictory: it stimulated the morphological and immunophenotipical differentiation and reduced the MLR stimulating ability of the generated DCs. The possibility that this depended on piggyback transfer of rosiglitazone to lymphocytes by DCs is highly improbable because DCs were extensively washed before transferring into co-culture wells for MLR. In this study, the effect of rosiglitazone during DC differentiation was evaluated for cells derived from CD133+ precursors. It had already been demonstrated by Nencioni (2002) and Appel (2005) that DCs generated from CD14+ precursors in the presence of PPAR-γ agonists had impaired T cell stimulating activity. It may be correlated with this effect of PPAR-γ stimulation the fact that in a mouse model of atopic dermatitis PPAR-γ agonists led to decreased severity of the disease and to selective inhibition of the maturation in vitro of DCs derived from untreated animals (Jung et al., 2011).

The study of the differentiation and functional potential of different DCs precursors was extended to check the interaction of DCs derived from CD14+ precursors with inorganic NPs. These DCs were chosen because they are quicker to

differentiate, require lower amounts of cytokines and can be generated from more numerous precursors, given the frequency of CD14+ cells in peripheral blood. Gold and silica NPs entered cells by endocytosis, accumulated in vacuolar (endo-lysosomal) compartments in the cytoplasm and did not appear to influence DC maturation except for a minor effect of AuNPs (these results were object of a publication: Fogli et al., 2017). The results indicate that NPs are well tolerated by DCs and can represent a simple, cost-effective, easy to synthesize and effective method to deliver active molecules (including antigens and drugs) to DCs in view of cancer and immune-mediated diseases therapy. However the final destiny of NPs, which are not digested by cell enzymes, requires further studies.

During this study a reason of trouble was the high variability of the results among experiments. For each experiments a single donor was used and each donor contributed to only one experiment. On account of the Italian law on privacy and personal data protection no information was available on the donors (not even sex or age), the only information being that they were healthy subjects acceptable as blood donors for transfusion. This drawback is intrinsic to studies with human cells and it could not be avoided in this research either.

The use of CFSE for evaluating the proliferation of the lymphocytes in MLR allowed to recognize a different effect of DCs generated in vitro on CD4+ and CD8+ lymphocytes, when DCs were generated from CD133+ precursors: these cells stimulated CD4+ lymphocytes, but were virtually ineffective on CD8+ lymphocytes, at variance with what was found for DCs generated from CD14+ cells, as shown in fig. 32: mature DCs not exposed to NPs and used as control for NP experiments stimulated the proliferation of both CD4+ and CD8+ lymphocytes. Since the cells generated from CD133+ precursors were more similar to LCs than those generated from CD14+

precursors, the finding may indicate a different capacity of different types of DCs to interact productively with different subsets of T lymphocytes.

Dendritic cells play a important role in the immune system, in fact they recognize, bind and process antigens and present them to T cells to initiate an immune response; they cells also can cause anergy and trigger active tolerance. Despite the fact that DCs have been studied over years, many points regarding their differentiation and function remain obscure. This study has shown that the DCs can be generated in vitro from different precursors, that the choice of precursors influences the phenotype and functional ability of the generated DCs, and that the features of generated cells do not mach exactly those of cells seen in vivo especially LCs.

These findings may be relevant to the question whether, to induce, enhance or alternatively lower an immune response, it would be better to differentiate dendritic cells from haematopoietic precursors *in vitro* and inject them into a patient upon appropriate treatment, or find ways to influence the DCs of a patient *in vivo*, as also proposed in the clinics (Gerlini et al., 2012b). The latter strategy would obviate the imperfect control of the differentiation of DCs *in vitro* and the risk of raising cells prone to induce responses inappropriate to the single case which they would be supposed to cure.

Acknowledgements

The authors gratefully acknowledge Dr Stefano Bacci, Dr. Paola Di Gennaro and Dr. Sergio Fabbri for advice on cell and molecular biology techniques; Mrs. Laura Calosi, Mr. Daniele Guasti and Mr. Stefano Catarinicchia for technical help in microscopic analyses; ,Associazione Italiana contro le Leucemie-Linfomi e Mieloma and the University of Florence for financial support.

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