Chapter 13

Development of Myeloid Dendritic Cells under the Influence of Sexual Hormones Visualized using Scanning and Transmission Electron Microscopy

Josef Neumüller, Sylvia Emanuela Neumüller-Guber, Johannes Huber, Adolf Ellinger and Thomas Wagner

Additional information is available at the end of the chapter

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Abstract

Dendritic cells (DCs) are antigen-presenting cells, which are mediated by MHC-class II molecules reacting with T-helper cells, eliciting a broad spectrum of immune reactions at cellular and humoral levels depending on their subtypes. DCs are also able to cross-present peptides from intracellular proteins as well as from intracellular pathogens via MHC-class I molecules by inducing MHC-class I–restricted cytotoxic T cells, which are also able to destroy cells undergoing malignant transformation. DCs originate from $CD34^+$ hematopoietic stem cells but can also develop from monocytes. The local or systemic milieu of cytokines and steroid hormones significantly influences the generation of particular DC subtypes such as the classical myeloid DCs such as cDC1 and cDC2 as well as the plasmacytoid DCs. These subtypes are able to induce specific Th1- and Th17-dependent, Th2-dependent, or regulatory immune responses, respectively. Immature DCs take up extracellular pathogens that are presented by MHC molecules that are upregulated during maturation. Immature and mature DCs can be characterized by morphological and biochemical features that are outlined in this article. In addition, DCs are under control of sexual hormones. Estrogen receptor ligands are potent modulators of hemopoiesis and immune function in health and disease, influencing key cytokines promoting the maturation of DCs. DC differentiation is mainly regulated by binding of estradiol to ERα. Estrogen promotes the differentiation of immature DC subsets derived from bone marrow precursors or from myeloid progenitors. In contrast to estrogen, progesterone inhibits DC maturation, causing a decreased immunity in pregnancy or in postmenopausal women, where elevated levels of progesterone result in the production of Th2 cytokines. The influence of estrogen and progesterone on DC maturation has been demonstrated in own *in vitro* experiments using fluorescence microscopy and cell sorting and, above all, by visualization using SEM and TEM. At the end of

this article, pits and falls concerning the treatment of malignancies with living DC vaccines are discussed.

Keywords: Dendritic cells, MHC antigens, antigen presentation, cross-presentation, cytokines, transcription factors, steroid hormones, anti-tumor vaccine

1. Introduction

Dendritic cells (DCs) are antigen-presenting cells (APCs) interacting with CD4-positive T-Helper (Th) cells via peptide-presenting MHC (HLA) class II molecules and the α/β or γ/δ T-cell receptor and via costimulatory molecules. These interactions provide initiation or modulation of specific kinds of immune reactions triggering effector cells via direct cell contacts as well as by intercellular communications involving a complex cytokine network.

In the first part, the differentiation of myeloid DCs, lymphoid DCs as well as of Langerhans cells (LCs) are explained, including their morphological and immunological characteristics based on their local or systemic sites of activity. Local areas mainly concern the secondary lymphoid organs such as lymph nodes and spleen as well as the mucosa-associated lymphoid tissues (MALT), including the Waldeyer's tonsillar ring and the mucosa lining the respiratory and the gastrointestinal tract. Although the objective of this article is rather morphological, we think that an immunological overview is helpful for understanding the functional interrelations of DCs not only with responding immune cells but also with other cells in the vicinity of locally acting DCs. This will be concisely discussed in the light of our present scientific knowledge.

In the second part, we show own results concerning ultrastructural details from the maturation of myeloid DCs under *in vitro* conditions using TEM and SEM. We started the differentiation of myeloid DCs outgoing from $CD34^+$ hematopoietic stem cells (HSCs) of human umbilical cord blood. In the course of differentiation in a conditioned cell culture medium, the HSCs differentiated into immature DCs and finally into mature myeloid DCs.

In the third part, the maturation of myeloid DCs under the influence of sexual hormones is demonstrated using scanning electron microscopy (SEM), showing images taken from our own investigations. We visualized the mDC maturation under the influence of β-estrogen and progesterone by SEM after flow cytometry and cell sorting.

In the fourth part, the impact of mDCs for anti-tumor therapies is discussed. In this respect, different DC isolation protocols (from HSCs or from monocytes) as well as DC anti-tumor targeting methods are presented. Several efforts to produce an efficacious anti-tumor vaccine using primed autologous DCs were undertaken and clinical trials were carried out with varying success. Some clinical trials were promising, but other clinical applications were disappointing since recurrence of the respective tumor could not be prevented.

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2. Functional aspects of DCs in the immune system

During the past years, the view about the differentiation lines of DCs has been changed significantly [1]. In this respect, different efforts were made to find an appropriate nomenclature. Some researchers use a development-orientated nomenclature concerning the development dependency from flt-3L or from macrophage colony–stimulating factor (M-CSF). Another feature of DCs concerns their maturation either from HSCs or from monocytes. Above all, specialized DCs are located in the intestine and other MALTs or in the integument. The DCs of the skin can be divided into epidermal Langerhans cells (LCs) characterized by cytoplasmic Birbeck granules containing Langerin and dermal Langerin-positive as well as Langerin-negative DCs [2]. Langerin (CD207) is a transmembrane protein belonging to the family of C-type II Ca^{2+}-dependent lectins with an extracellular carbohydrate-binding domain specific for mannosyl residues and an intracellular domain with a proline-rich motif. It is taken up from the cell surface by clathrin-dependent receptor-mediated endocytosis and traffics via early endosomes (EEA1) to the endosomal recycling compartment (ERC) but does not reach lysosomes for degradation. Langerin is also localized at the inner band of Birbeck granules [3]. These organelles have a unique ultrastructural morphology and consist of superimposed and zippered membrane components [3]. A second type of nomenclature distinguishes classical or conventional DCs (myeloid DCs) from plasmacytoid DCs based on the distinct morphology, markers, and gene expression profiles. Furthermore, one can distinguish between resident DCs moving directly to lymphatic compartments and migratory DCs entering to a respective tissue and traveling via lymphatic vessels to lymph nodes. Both cell types originate from blood cell precursors. More recently, newer criteria including not only developmental but also functional characteristics refer to regulatory mechanisms such as the initiation of immune tolerance, the role of hematopoietins such as G-CSF, M-CSF, flt-3L, GM-CSF and their receptors responsible for the generation of monocyte-dependent or independent DCs, as well as the significance of various transcription factors, for example, PU.1, mafs, ID2, IRFs, E2-2, and NF-κB, that are responsible for DC development according transcriptional programs [1, 4].

2.1. Antigen presentation

The most important DC function is the uptake of antigenic material via phagocytosis or pinocytosis by immature DCs and its processing into peptides and presentation via the peptide binding groove of MHC (in human HLA) molecules by mature DCs. Uptake of antigenic material is mediated by four alternative pathways: endocytosis, pinocytosis, phagocytosis and macropinocytosis. How the antigenic material is taken up by DCs depends on its features such as the form of the antigen, its solubility, or whether it is part of an immune complex or still associated with a pathogen [5]. During maturation, DCs not only change their dendritic morphology and their ability to take up antigenic material but also upregulate the expression of MHC-class I and MHC-class II molecules. In addition, the mature phenotype is characterized by specific differentiation markers [6]. Antigen presentation is initiated by direct contact with respective subtypes of T-Helper cells via binding to the T-cell receptor (TCR) complex, the CD4 molecule, as well as to additionally required costimulatory molecules expressed at the cell surface of both involved cells [7]. Antigen processing involves an interaction of the endocytic

and lysosomal pathway in the MHC-class II compartment (MIIC) and the presentation by MHC-class II molecules. This process requires an activation of the lysosomal machinery [8] and a stepwise association of the degradation products with the MHC-class II binding groove. This complex process has been summarized in an excellent review by Seliger et al. [9]. Exogenous antigens are internalized via the endocytic pathway. HLA class II heterodimers assemble in the endoplasmic reticulum (ER) with the invariant chains (Ii) to form nonameric a/b–Ii complexes [(ab)3Ii3], targeted to MIIC. The invariant chain Ii protects the binding groove against association with intracellular degradation products shredded by proteasomes. The HLA-class II–associated Ii is degraded in distinct steps, leaving class II–associated Ii peptide (CLIP) within the HLA-class II binding groove. CLIP can then be exchanged for antigenic peptides, a process catalyzed by HLA-DM molecules. The HLA-DM–dependent peptide loading is regulated by HLA-DO molecules. Peptide-loaded HLA-class II molecules are then transported to the cell surface for presentation to $CD4^+$ T cells.

At the EM level, multivesicular and multilamellar bodies are characteristic organelles related to the MCII compartment [10–15]. Although MHC-class II molecules are abundantly expressed at the plasma membrane of DCs, they are also present in immature DCs where they have only a short half-life since they are directed in a recycling pathway. They are actively sorted to luminal vesicles of multivesicular bodies after ubiquitination and subsequently transferred to lysosomes. An increase of MHC-class II molecules at the plasma membrane could be explained by a recruitment of antigen-loaded MHC-class II molecules to the cell surface during maturation by allowing intraluminal vesicles to fuse back with the delimiting membrane of multivesicular bodies. However, there is strong evidence that the enhanced cell surface expression of antigen-presenting MHC-class II molecules is due to their increased synthesis but to a reduced lysosomal degradation [16]. Nevertheless, the stable expression of peptide-loaded MHC-class II molecules requires the intersection of the endocytic and the lysosomal pathway as explained above.

2.2. Cross-presentation

As most of nucleated cells, DCs also express classical MHC-class I molecules presenting intracellular peptides prepared by proteasomes and cytosolic proteases. The peptides are transported by the TAP transporters (heterodimeric multimembrane-spanning polypeptides belonging to the ABC transporter family) from the cytosol into the ER, where their assembly with MHC-class I molecules takes place, assisted by various chaperones, such as calnexin, calreticulin, ER60, and tapasin [9].

In DCs, two alternative pathways for cross-presentation have been reported: the “cytosolic” and the “vacuolar” pathway. In the cytosolic pathway, phagocytosed proteins are transported to proteasomes, where they are degraded and bound to MHC-class I molecules in the ER and directed to the plasma membrane for antigen presentation. This retrotranslocation machinery involves the ER membrane proteins sec61 and p97 ATPase [17]. Alternatively, after degradation by proteasomes, ingested proteins can be re-transported to phagosomes, where they are associated with MHC-class I molecules. In the vacuolar pathway, exogenous proteins are transported to phagosomes, where degradation, alternative to the proteasomes, takes place.

Consecutively, appropriate peptides are bound to the antigen-binding groove of MHC-class I molecules [18, 19].

Bacterial and cellular antigens are also efficiently cross-presented in association with heat-shock proteins such as hsp 70, hsc 70, and hsp 90 and the ER chaperones grp94 /gp96 and their bacterial homologues. Peptide complexes are ingested after binding to specific receptors such as CD91 (the α_2-macroglobulin receptor) and the scavenger receptors LOX-1 and SR-A [5, 20–23].

Only the MHC-class I–restricted $CD8^+$ cytotoxic T-cell (CTL) response can destroy intracellular pathogens such as viruses and intracellular parasitic bacteria as well as cells undergoing malignant transformation. Only professional APCs such as DCs and some macrophages express an efficient combination of co-receptors and MHC-class I molecules to stimulate naive $CD8^+$ T cells [24]. However, cross-presentation of intracellular antigens after degradation of intracellular peptides belonging to internal cell structures and compartments is important for the maintenance of peripheral tolerance to self-antigens (cross-tolerance) [5, 25]. Cross-presentation is also important for vaccination against tumors, which is discussed at the end of this article.

2.3. Types of DCs and their characteristics

The enormous progress in DC research has been achieved mainly in the mouse system. Although many homologies exist between the human and murine immune system, there are also significant differences in the expression of differentiation markers, transcription signatures, and cytokine release. In the mouse system, DCs can be subdivided into classical (cDCs) and plasmacytoid (pDCs) DCs. However, under inflammatory conditions, a new subtype of so-called inflammatory DCs appears. DCs acting in the murine immune system, cDCs, can be further subdivided into "CD8-like" DCs with a $Clec9A^+$/$CD141^+$ phenotype and CD11b-like" DCs with a $CD1c^+$ phenotype. Both of these subsets include resident DCs that are located in secondary lymphoid organs such as lymph nodes, tonsils, and spleen as well as migratory DCs, which are present in peripheral tissues as well as in non-lymphoid organs (skin, liver, lung, kidney, intestine, and other organs). After antigen contact, cells of this subtype migrate via lymphatic vessels into local lymph nodes [19]. In addition, special DC types are present in the skin: the epidermal LCs and dermal DCs, as mentioned above.

In vitro, human DCs can be either derived from $CD34^+$ HSCs, or isolated from bone marrow, umbilical cord blood, or purified monocytes (Fig. 1a and b). In this respect, monocytes represent an immature cell type with a potential to differentiate either into macrophages or into DCs. *In vivo*, similar differentiation lines can be demonstrated, whereas the decision to follow a distinct differentiation line is made in the bone marrow. Surface markers, transcription signatures, as well as growth factors driving differentiation have been summarized in several reviews [6, 26–30].

Depending on their stimulation, DCs are able to promote different T-cell responses. In contrast to the murine immune system, in human system, pDCs (bearing MHC-class II antigens, CD4, CD123, CD80, and CD86) can provoke a Th2 and Th0/Tr1 response, whereas mDCs (bearing

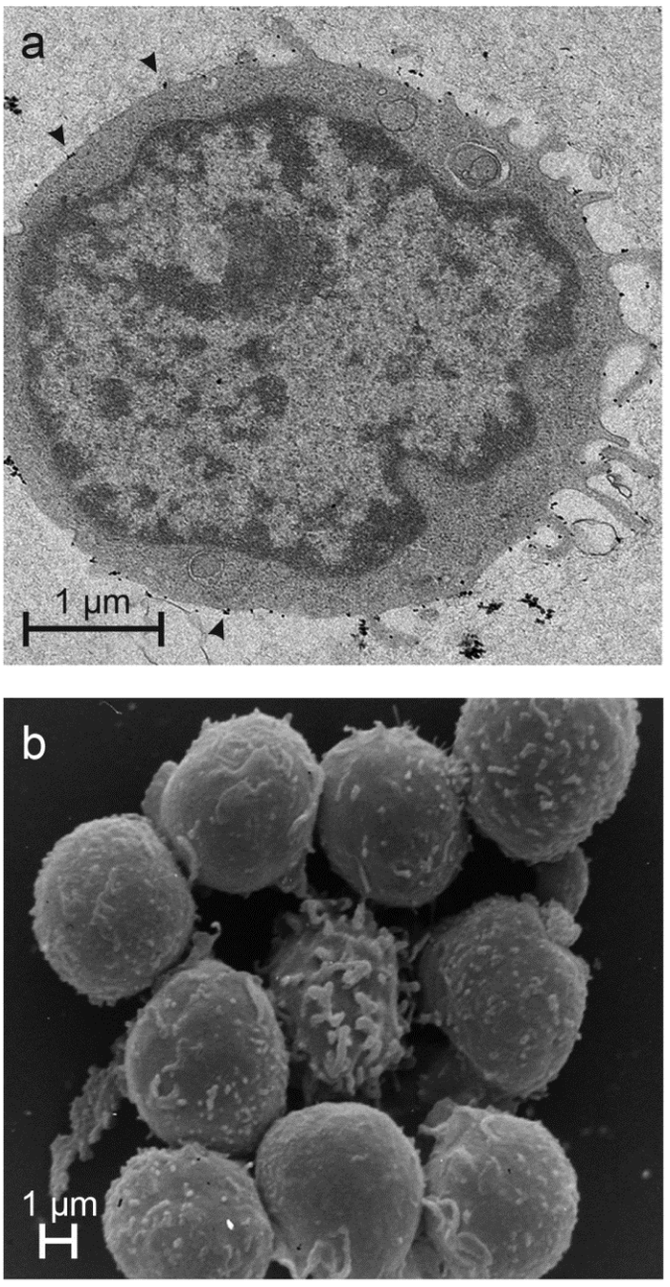

Figure 1. $CD34^+$ HSCs, isolated with paramagnetic particles, coated with an anti-CD34 monoclonal antibody shown by TEM (a) and SEM (b). In Figure 1a, arrowheads mark binding of anti-CD34 antibody-coated amorphous paramagnetic particles (average size: 50 nm).

MHC-class II antigens CD11c, CD80, and CD86) can provoke a Th1 response [6]. Naive $CD4^+$ T cells can differentiate into Th1, Th2, Th17, and regulatory T cells (Tregs). While Th1 cells secrete mainly IFN-γ to eliminate intracellular antigens, Th2 cells produce IL-4, IL-5, and IL-13 to eliminate helminthes and extracellular pathogens [31]. The IL-17-synthesizing Th17 cells are functional in the clearance of extracellular bacteria and fungi [32]. Tregs secrete anti-inflammatory cytokines such as IFN-β and IL-10 and are essential for the maintenance of self-

tolerance and for the prevention of autoimmunity [4, 33]. After activation of the TCR, the signals for $CD4^+$ T-cell differentiation are mediated by binding of specific cytokines to their cognitive receptors that are associated with Janus kinases (Jaks). After phosphorylation of the intracellular domains of the cytokine receptor and activation of Jaks, STATs are also phosphorylated, inducing the expression of genes responsible for the initiation of differentiation by the action of master regulators responsible for the synthesis of Th subtype–specific cytokines [4, 34]. STATs are signal transducers and activators of transcription pathways initiating the expression of master regulator transcription factors belonging to a complex network of other transcription factors. Different STATs are required for Th-specific differentiation lines: STAT4 and 1 for Th1 cells, STAT6 and 5 for Th2 cells, STAT3 for Th17 cells, and STAT5 for Tregs [4].

According to a recent excellent and comprehensive review [35], we can summarize that DCs belong to the entire phagocytic system including monocytes/macrophages in different compartments such as lymphoid and non-lymphoid tissue and blood. In this nomenclature, DCs are subdivided into classical $CD141^+$ myeloid DCs (cDC1), $CD1^+$ myeloid DCs (cDC2), and plasmacytoide DCs (pDCs). DCs can also differentiate from monocytes (moDCs). In the murine system, different development pathways for moDCs and DCs derived from HSCs have been demonstrated. However, because of the research at the transcriptional level, it became evident that these restrictions are not so strong in both humans and mice as previously suggested.

Human DCs can be classified according to Haniffa et al. [35].

2.3.1. $CD141^+$ myeloid DCs (cDC1)

CD141+ myeloid DCs (cDC1)secrete TNFα, CXCL10, and IFNγ, but little IL-12p70. They cross-present necrotic cell–derived and soluble antigens via CLEC9A, but cross-presentation is not totally restricted to this cell type. CD141 (BDCA-3, thrombomodulin), an integral membrane protein, is expressed not only at the cell surface on DCs but also on endothelial and mesothelial cells as well as on monocytes. By binding to thrombin, it serves as its cofactor and reduces blood coagulation by converting thrombin to an anticoagulant enzyme and participates in inhibition of fibrinolysis. In addition, it regulates C3b inactivation by coagulation factor 1 [36].

2.3.2. $CD1c^+$ myeloid DCs (cDC2)

CD1c+ myeloid DCs (cDC2) are characterized by a high expression of the toll-like receptors (TLRs) 1, 2, 4, 5, and 8. They are able to present glycolipid antigens and react with mycobacteria via CD1a and c. They also exhibit reactivity against fungi by involving the receptors Dectin-1 (CLEC7A) and Dectin-2 (CLEC6A), the receptors DEC205 (CD205; CLEC13B), and the macrophage mannose receptor (CD206; CLEC13D). Upon stimulation, these cells secrete IL-1β, TNFα, IL-8, and IL-10. In blood, cDC2 cells can abundantly secrete the enzyme retinaldehyde dehydrogenase (RALDH)2 dependent on the level of vitamin D3. They are able to initiate Th2, Th1, and also Th17 response dependent on stimulation by respective antigens.

CD1c (BDCA-1) acts as antigen-presenting protein binding self- and non-self–lipid and glycolipid antigens and presenting them to T-cell receptors on natural killer T cells [37].

2.3.3. Plasmacytoid DCs (pDCs)

Plasmacytoid DCs (pDCs) strongly express TLR7 and TLR9, enabling the recognition of autologous nucleic acids and viral antigens. Challenging pDCs with viral antigens results in a potent secretion of class-I interferons, mainly IFNα. In response to inflammation, pDCs are recruited to tissues and lymph nodes. They display two main functions: they can polarize Th1 and Th2 response and play an important role in immune tolerance but also in autoimmune disease by initiation of a Treg response. The functional capacity of pDCs increases gradually during fetal life. In this respect, it could be demonstrated that preterm neonates possess an impaired BDCA4 expression and produce lower levels of IFNα. Ultrastructurally, they show an immature morphology [38]. In addition, the *in vitro* maturation of blood DC depends on the upregulation of BDCA3 on pDCs [39].

3. Maturation of myeloid DCs (mDCs)

When challenged by immune stimulatory and pathogenic antigens, mDCs mature into regulatory or stimulatory DCs. The development of regulatory DCs requires tolerogenic stimuli via anti-inflammatory cytokines and mediators such as TGF-β, IL-10, and PGE_2. Regulatory DCs provide induction of Tregs, inhibiting the proliferation of $CD4^+$ $CD8^+$ T-cells and producing anti-inflammatory cytokines such as IL-10 and TGF-β, as well as immune-regulatory molecules such as indoleamine 2,3-dioxygenase (IDO) and programmed cell death protein 1 (PD-1), which suppress activation and proliferation of effector T cells and efficiently induce Tregs [36]. In contrast, stimulatory DCs develop under the influence of proinflammatory signals: inflammatory cytokines, chemokines, pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharide (LPS), flagellin, lipoteichoic acid, peptidoglycan, and viral double-stranded RNA (dsRNA) [40–42]. Additional stimulators are damage-associated molecular patterns (DAMPs) representing host molecules that can initiate and perpetuate inflammatory response that are not caused by infectious agents. DAMPs can be proteins like heat shock proteins or breakdown products of the extracellular matrix such as hyaluronan fragments or other molecules such as ATP, uric acid, heparin, sulfate, or DNA [43–46].

3.1. Immature mDCs

Immature mDCs circulate either in the peripheral blood or migrate into infected tissues where they take up pathogens or cell debris. This uptake is mediated by type C lectin receptors such as CD206, DEC205, or CLEC4A or in the case of PAMPs by toll-like receptors. Antigenic material is ingested by phagocytosis or pinocytosis and directed to the intracellular degradation machinery [36].

In our own experiments, we differentiated mDCs from $CD34^+$ HSCs of human cord blood: Fig. 1a and b. We followed a cultivation protocol established by Strunk et al. [47]. Shortly, mono-

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nuclear cells were obtained by centrifugation over the flotation medium Ficoll®-Paque Premium (Sigma-Aldrich, Vienna, Austria). DCs were incubated with paramagnetic particles coated with a monoclonal antibody against CD34, isolated using a magnetic cell separator (MACS®, Miltenyi Biotech, Bergisch Gladbach, Germany), and cultivated in a conditioned RPMI-1640-medium containing 20% FCS, 100 ng/ml GM-CSF, 20 ng/ml SCF, 20 ng/ml TNF-α, and 0,5ng/ml TGF-β1. $CD34^+$ stem cells differentiated into immature mDCs showed an LC-like phenotype ($HLA\text{-}DR^{dim}$, $CD1a^+$, and $Langerin^+$) and grew in clusters. Cultures containing clusters were layered over 7.5% bovine serum albumin solution in phosphate-buffered saline (BSA/PBS, Sigma, St. Louis, MO), where they could be collected and transferred to fibronectin-coated chambered coverslips. Further differentiation to mature DCs has been achieved by addition of 10 μg/ml LPS + 50 ng/ml TNF-α to the culture medium or by supplementing the medium with steroid hormones as outlined later on.

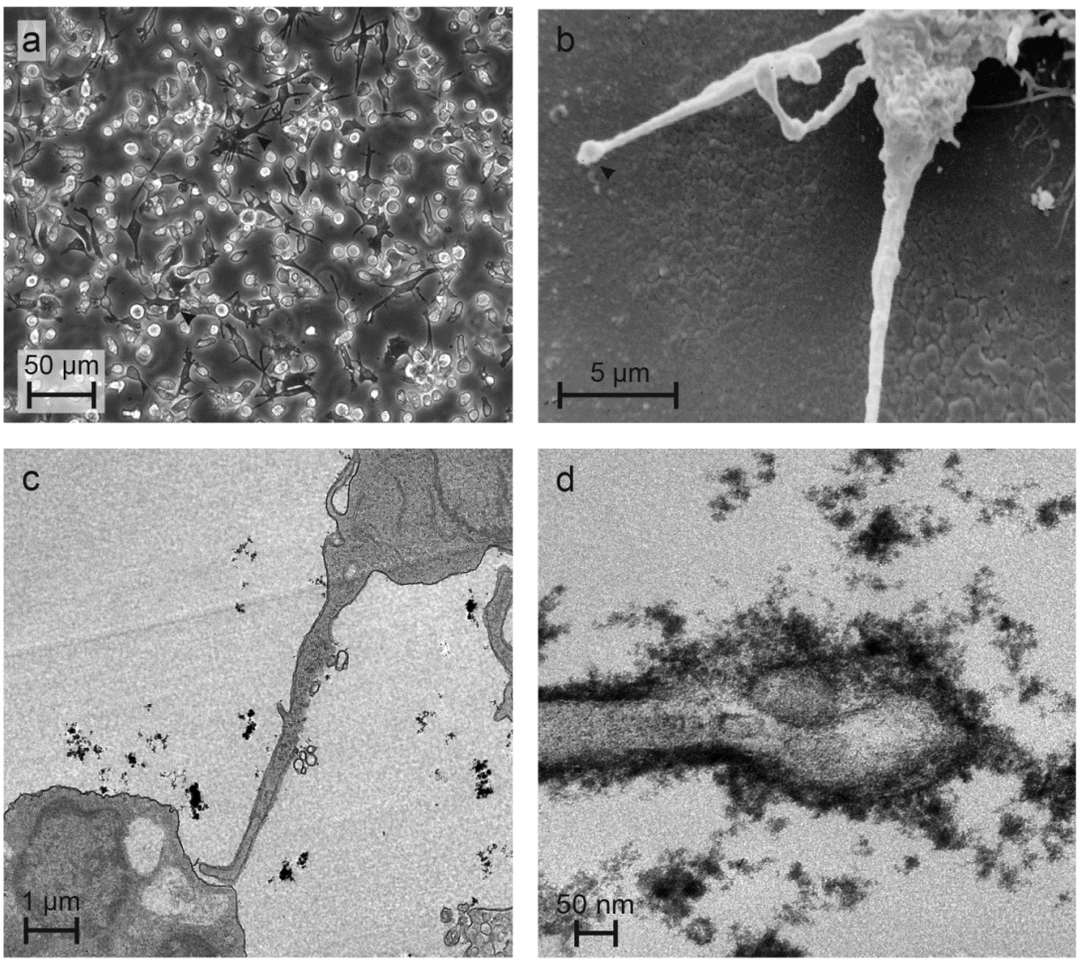

Figure 2. Immature dendritic cells growing in conditioned cell culture medium on the surface of fibronectin-coated chambered coverslips. Fig. 2a shows the appearance of the DC morphology in phase contrast. Fig. 2b and c show extended cell projections with knob-like structure in SEM (Fig. 2b) and TEM (Fig. 2c and at higher magnification in Fig. 2d). Note the prominent coat of the glycocalyx in Fig. 2d. In Fig. 2c paramagnetic particles are visible, which are still present after cell separation but partially detached from the cell surface.

Immature mDCs preferentially extended large dendritic cell processes (Fig. 2 ac) which a knob-like structure as shown in Fig. 2d where we suppose a concentration of the mentioned receptors. In this respect, DCs, located in the lamina propria of the small intestine, extending long cell processes that survey antigens in the lumen of the gut have been demonstrated [48–50].

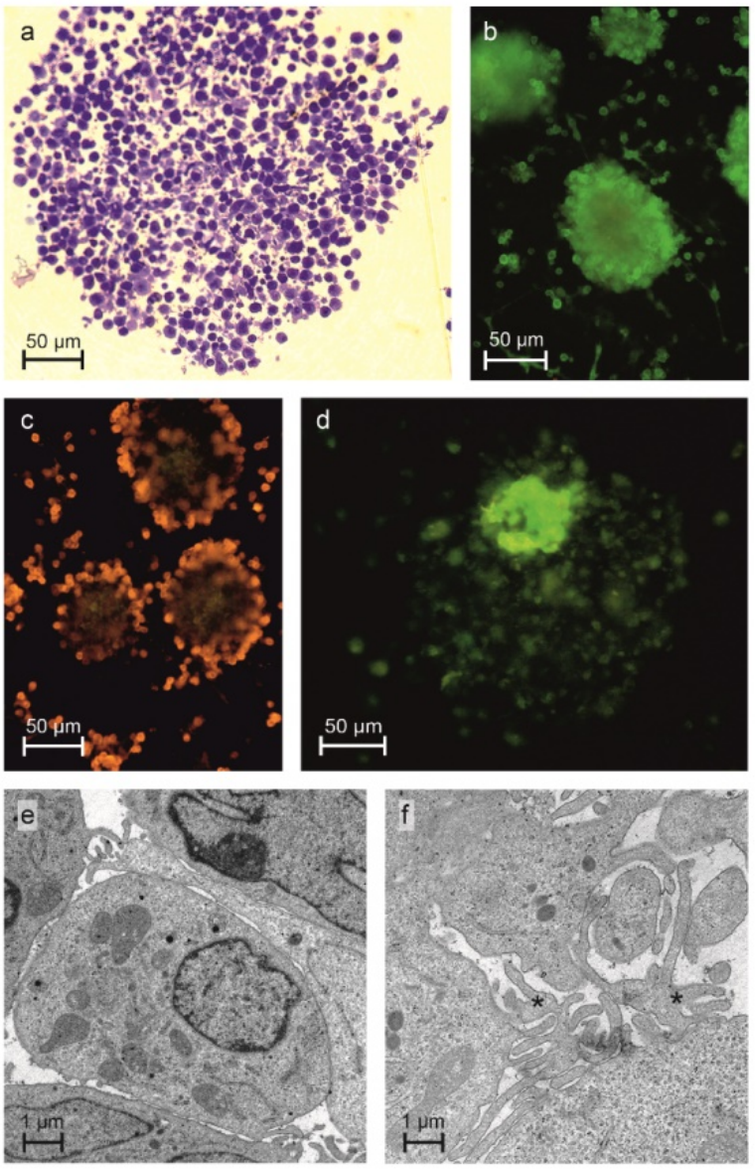

Figure 3. Cultivation and characterization of immature mDC clusters using light microscopy (Fig. 3a), fluorescence microscopy (Fig. 3b, c, and d), and TEM (Fig. 3e and f). Fig. 3a shows a low-magnification image of an adhered cell cluster taken from a 1 µm semithin section stained with toluidine blue. Fig. 3b shows staining with an FITC-conjugated monoclonal antibody against CD1a, and Fig. 3c, a cell surface staining with a PE-conjugated monoclonal anti-HLA DR antibody. In Fig. 3d, only the center of the cluster is positive for E-cadherin cell surface expression using an FITC-conjugated monoclonal antibody against E-cadherin. TEM images show cells with relatively smooth surface in the center of a cluster (Fig. 3e) as well as cells at the periphery of clusters forming interdigitating cell projections, which are marked by stars (Fig. 3f).

After collection of clusters and transfer to fibronectin-coated polystyrol dishes, they adhered again to the growing surface (Fig. 3a). At this stage, they displayed still an immature phenotype till day 7 of cultivation. Under the influence of maturation signals, they differentiated starting from the margins of clusters (Fig. 3). Using fluorescence microscopy, it could be shown that CD1a staining was present at the surface of all mDCs (Fig. 3b), while HLA-DR expression could be found only at the periphery of clusters (Fig. 3c). E-cadherin could also be demonstrated but only in the center of the clusters (Fig. 3d). TEM imaging of the clusters revealed that in their center the surface of cells remained relatively smooth (Fig. 3e), while at the periphery, many interdigitating cell projections were formed (Fig. 3f).

In TEM, immature mDCs display characteristics of high activity as they develop an abundant rough ER and a prominent Golgi apparatus as well as an extended trans-Golgi network (TGN) (Fig. 4a and b). The MIIC compartment is characterized by the presence of abundant lysosomes and multilamellar bodies (Fig. 4c and d). Also autophagosomes engulfing several multilamellar bodies could be demonstrated (Fig. 4e). Interestingly particular endosomes aligned in a pearl-like structure could be shown (Fig. 4f). We regard these structures as special organelles of the endosomal pathway. In a previous publication we could identify similar structures in endothelial progenitor cells also derived from $CD34^+$ HSCs of human cord blood [51, 52]. In this respect, immature mDCs are highly active in antigen uptake and processing.

Regarding *in vitro* conditions, above all under the influence of TGF-β1, mDCs develop a characteristic LC-like ultrastructural morphology by displaying Birbeck granules and Birbeck-like organelles already described in early publications [47, 53, 54], which we could also verify in our experiments (Fig. 5). Birbeck granules store langerin, a mannose-binding lectin.

3.2. Mature mDCs

The maturation of mDCs is accompanied by morphological and functional changes: Mature mDCs lose their capacity to adhere to plastic surfaces. The few long cell processes of immature DCs are replaced by numerous short filopodia (Figs. 6a and b) and veil-like membrane projections while the uptake of antigenic materials is downregulated in favor of antigen presentation as well as of cross-presentation [55]. In this respect, only mature DCs are entirely able to cooperate with lymphocytes (Fig. 6b) by presenting antigens via MHC-class I and II molecules [56]. Maturation of mDCs requires the expression of costimulatory molecules such as CD80, and CD86 and the integrin receptor LFA-1 (CD11a). In addition, the cytokine tumor necrosis factor alpha (TNF-α), LPS, and the CD40 ligand (CD40L) have been found to induce DC-maturation [56]. The degeneration of aged mature mDCs is shown in Figs. 6c and d.

It has been demonstrated that CD83 is expressed only in mature DCs [55]. CD83 is a 45-kD, type-1 membrane glycoprotein belonging to the Ig superfamily. CD83 is released from activated cells, and the soluble form of CD83 has a strong immunosuppressive effect. The immunoregulatory function of CD83 implicates its use for therapy of cancer and autoimmune diseases [57].

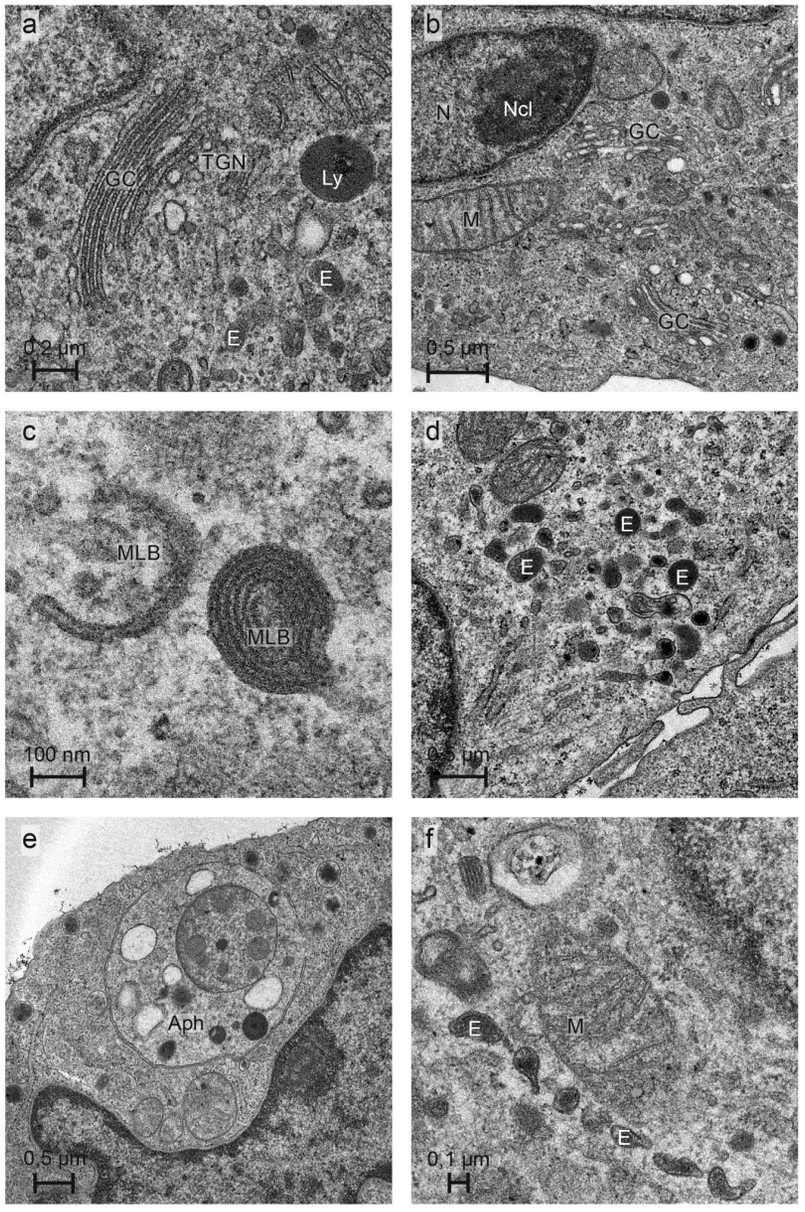

Figure 4. Particular ultrastructural characteristics of immature mDCs visualized by TEM: Well-expressed Golgi complexes (GC) and trans-Golgi networks (TGN) as well as endosomes (E), lysosomes (Ly), mitochondria (M), and an active nucleus (N) with a distinct nucleolus (Ncl) are visible in Figs. 4a, b, d, and e. Multilamellar bodies (MLB) shown in Fig. 4c are part of the MIIC compartment. An autophagosomes (APh) with degraded material is shown in Fig. 4 e. In Fig 4f, a pearl-like structure of aligned endosomes (E) is visible.

4. How sexual hormones influence the development and maturation of mDCs

In vitro, mDCs can be derived from $CD34^+$ HSCs from bone marrow but also from umbilical cord blood. Triggered by the cytokines GM-CSF and TNFα, HSCs differentiate into inter-

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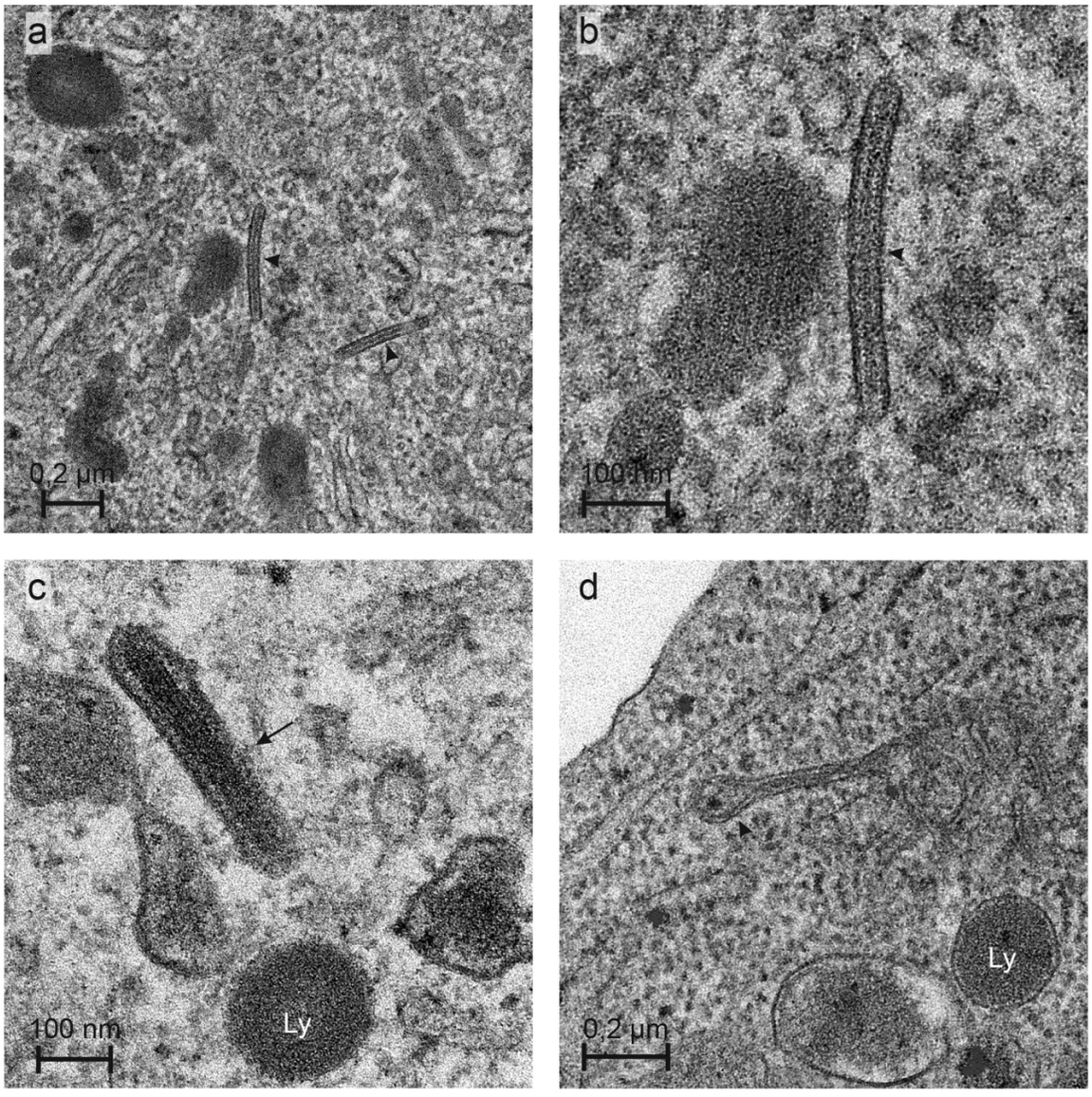

Figure 5. Formation of Birbeck granules and related structures visualized by TEM: Classical Birbeck granules are shown in Figs. 5a and b (arrowheads). They are rod-shaped (Fig. 5a and b) or drumstick-like (Fig. 5d; arrowhead).organelles with a central linear density and a striated appearance. Occasionally, organelles resembling endothelial Weibel-Palade bodies with central microtubular structures (arrow) occur (Fig. 5c). In addition, lysosomes (Ly) are visible.

mediate $CD14^+/CD1a^-$ precursors, which develop consecutively into mature DCs but also to LC-like cells. Bone marrow-derived $CD34^+/CD10^+/Lin^-$ cells mature directly into DCs under the influence of a cytokine cocktail containing IL-1, IL-7, TNFα, GM-CSF, SCF, and Flt3-ligand [30]. ER ligands are potent modulators of hemopoiesis and immune function in health and disease influencing key cytokines such as GM-CSF and Flt3 ligand that promote the maturation of mDCs. However, myeloid progenitors differentiate variably under the influence of 17β estradiol with each of the two cytokines: While GM-CSF promotes the development of LC-like mDCs, Flt3 ligand decreases the number of plasmacytoid, lymphoid, and myeloid DCs in a dose-dependent manner. DC differentiation is mainly regulated by binding of estradiol to

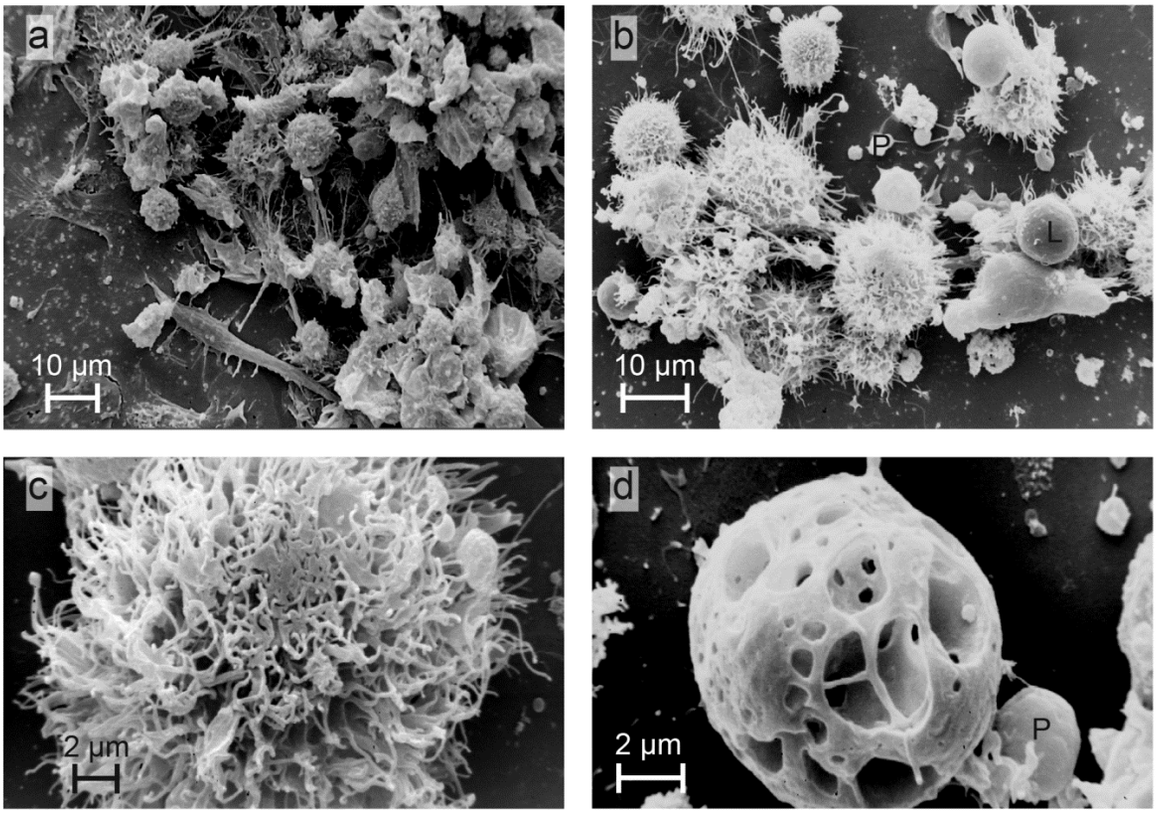

Figure 6. Ultrastructural characteristics of mature and apoptotic/necrotic myeloid DCs investigated by SEM. Figs. 6a and b show clusters of mature DCs with numerous microvillus-like cell projections. Adhesion of lymphocytes (L) and platelets (P) is visible in Fig. 6b. In Fig. 6a, still some immature DCs with long cell projections are visible. Fig. 6c shows abundant cell projections as a sign of beginning degeneration, distinctly pronounced in Fig. 6d.

ERα. Therefore, endogenous or ER ligands produced by pharmaceutical companies may differentially affect DC development in health and disease concerning the DC-mediated immunity [58]. ERα and β belong to the nuclear receptor superfamily encoded by the ESR1 and ESR2 genes, respectively. Different types of ER receptor isoforms are generated by homeotypic or heterotypic dimerization of both chains [59, 60]. Activation of ERs exhibits an epigenetic modification of DNA by chromatin-modifying co-regulators and other transcription factors that initiate DNA transcription [61]. It has been shown that estrogen preferentially promotes the differentiation of immature $CD11c^{+}$ $CD11b^{int}$ DCs from bone marrow precursors [62] as well as the GM-CSF-mediated differentiation of a $CD11b^{+}$ subset from myeloid progenitors via the interferon regulatory factor (IRF4). This transcription factor is induced by GM-CSF [63].

In contrast to ER activation, therapeutic selective ER modulators such as tamoxifen and raloxifene have been shown to impair DC differentiation and activation. As competing with estrogen, both drugs possess antagonistic effects on ER function. They were not able to provide DC differentiation and have, therefore, no effect as ER agonists. Tamoxifen has beneficial effects in respect to the treatment of breast cancer, while it increases the risk for endometrial cancer, whereas raloxifene can be successfully used against osteoporosis since it exerts similar

effects than estrogen. However, tamoxifen and raloxifene medication can lead to a partial immune suppression at the level of antigen presentation and T-cell activation by downregulating the expression of MHC-class II and costimulatory molecules. Unfortunately, this downregulation can interfere with cellular immune therapy against cancer such as vaccination with DCs. [64].

In pregnancy, an immune-modulatory function of progesterone has been discussed [65]. It could be demonstrated that pregnancy-associated elevated levels of progesterone induced the production of IL-10 by mature DCs derived from human blood monocytes, leading to a Th2 immune response. In this respect, the regulation of the Th1- and Th2- related cytokines is important for a successful pregnancy [66]. Impaired DC function has also been reported in the mouse immune system. In this respect, progesterone-treated DCs were characterized by a decreased expression of Ia molecules (MHC-class II), CD80, and CD86 as well as by an increased production of IL-10, and a decreased secretion of IL-12. In addition, an impaired stimulatory capacity for $CD4^+$ helper cells was demonstrated [67].

In vitro experiments with metroxyprogesterone acetate, an anti-contraceptive drug, could impressively demonstrate that TLR3 agonist stimulation decreased the expression of CD40 and CD80 and impaired the initiation of T-cell proliferation [68].

In general, most of functions of the innate and adaptive immune system decline with aging. Therefore, vaccination response against pathogens is also frequently impaired. Postmenopausal women produce higher levels of proinflammatory cytokines such as MCP1, TNFα, and IL-6, which has been implicated in the development of several diseases such as osteoporosis (by activation of osteoclasts) but also in diabetes, atherosclerosis, and cardiovascular diseases. Estrogen (E_2) and progesterone are the master cytokines of the immune system in the female reproductive tract (FRT). Although there are still gaps in our knowledge of the immune system of the FRT, above all concerning the mucosal parts of FRT, there is good evidence for successful treatment of postmenopausal women using an appropriate hormone therapy [69, 70].

4.1. Investigations performed in our laboratory

We visualized the maturation of mDCs cultivated using the differentiation protocol as described above under the influence of 10^{-5}M β-estradiol or alternatively to 10^{-5}M progesterone. After 1 week of cultivation, clusters of immature DCs were transferred to new chambered coverslips containing the same conditioned medium containing the respective hormones and cultivated for another week. Using flow cytometry and cell sorting (Fig. 7a and b), immature (Fig. 7c) and mature mDCs (Fig. 7d) were collected and prepared for SEM. In addition, the percentage of mDCs positive for CD1a, CD14, CD83, CD1a/CD83, and CD14/CD83 was determined.

4.1.1. Immunological characterization with fluoro-labelled antibodies

Immunological characterization with fluoro-labelled antibodies was performed by staining of cells using a fluorescein-isothiocyanate (FITC)-conjugated monoclonal antibody against CD1a (BD Pharmingen™, Vienna, Austria), clone HI149, mouse IgG1, a phycoerythrin (PE)-

conjugated monoclonal anti-HLA DR antibody (BD Biosciences, Vienna, Austria), clone L243, mouse IgG_{2a}, and a FITC-conjugated monoclonal antibody against E-cadherin from BD Biosciences, clone 36/E cadherin, mouse IgG_{2a}. Fluorescence microscopy was performed using an inverted Nikon Eclipse TE-300 Microscope (Nikon Coop., Tokyo, Japan) with conventional filter packs for blue and green light excitation. Micrographs were taken with a Nikon Coolpix 5000 digital microscope camera.

4.1.2. For TEM investigations

For TEM investigations cells were attached to poly L-lysine coated coverslips, washed twice with Ca^{++}- Mg^{++}-free PBS, and fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer at pH 7.2 for 60 minutes at 4°C. After washing with cacodylate buffer, cells were postfixed with 1% aqueous OsO_4 for 90 minutes at 4°C. Cells were dehydrated stepwise with ethanol and embedded in Epon. After polymerization, 70 nm ultrathin sections parallel to the growing plane were performed using a diamond knife. Acquisition of digital images was performed using a bottom-mounted CCD camera (Gatan Inc., Munich, Germany) in a Tecnai 20™ transmission electron microscope (FEI company, Eindhoven, The Netherlands).

4.1.3. For SEM investigations

For SEM investigations, cells were treated according to the TEM preparation protocol till fixation and then subjected to a critical point drying procedure and sputtered with gold. Micrographs were taken using a Nikon F3 reflex camera (Nikon Coop.) mounted on a Stereoscan S90 scanning electron microscope (Cambridge Instrument Company, Cambridge, UK).

4.1.4. Flow cytometry and cell sorting

Flow cytometry and cell sorting was performed using a FACSort machine (Becton Dickinson) which utilizes a "catcher tube", a mechanical sorting device, located in the upper portion of the flow cell. By moving in and out of the sample stream, it allows to collect a population of gated cells. In our investigations, sorted $CD1a^{bright}/CD83^{dim}$ immature mDCs (Fig. 7c) and sorted $CD1a^{dim}/CD83^{brigh}$ mature mDCs (Fig. 7d) were shown in SEM. Fig. 7a shows the gating process of mature DCs by forward and side scatter and Fig. 7b, the gating of $CD1a^{dim}/CD83^{brigh}$ mDCs at the fluorescence dot blot (FL1: labeling with a FITC-conjugated anti-CD1a monoclonal antibody; FL2: labeling with a PE-conjugated monoclonal antibody against CD83) using the Attractor software (Becton Dickinson). In addition, the software allows neglecting cell debris and, therefore, cell quantification in terms of absolute counts using reference beads. For demonstration of mature mDCs, a monoclonal PE-conjugated antibody against CD83 (Clone HB15e (RUO) from mouse, IgG1 κ chain) has been used.

4.1.5. Results from our own studies

It could be demonstrated that already at day 7 of cultivation above all the percentage of CD1a and CD14 positive DCs decreased under the influence of progesterone, while it increased

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under the influence of β-estradiol (Table 1a). At day 14 of cultivation, a similar situation occurred but CD83 in combination with CD1a and CD14 was markedly reduced due to progesterone treatment but distinctly increased by β-estradiol (Table 1b). In addition, the maturation process concerning the formation of abundant cell projections was demonstrated by SEM analysis, and was still present at day 7 (Table 1a), but more pronounced on day 14 (Table 1b).

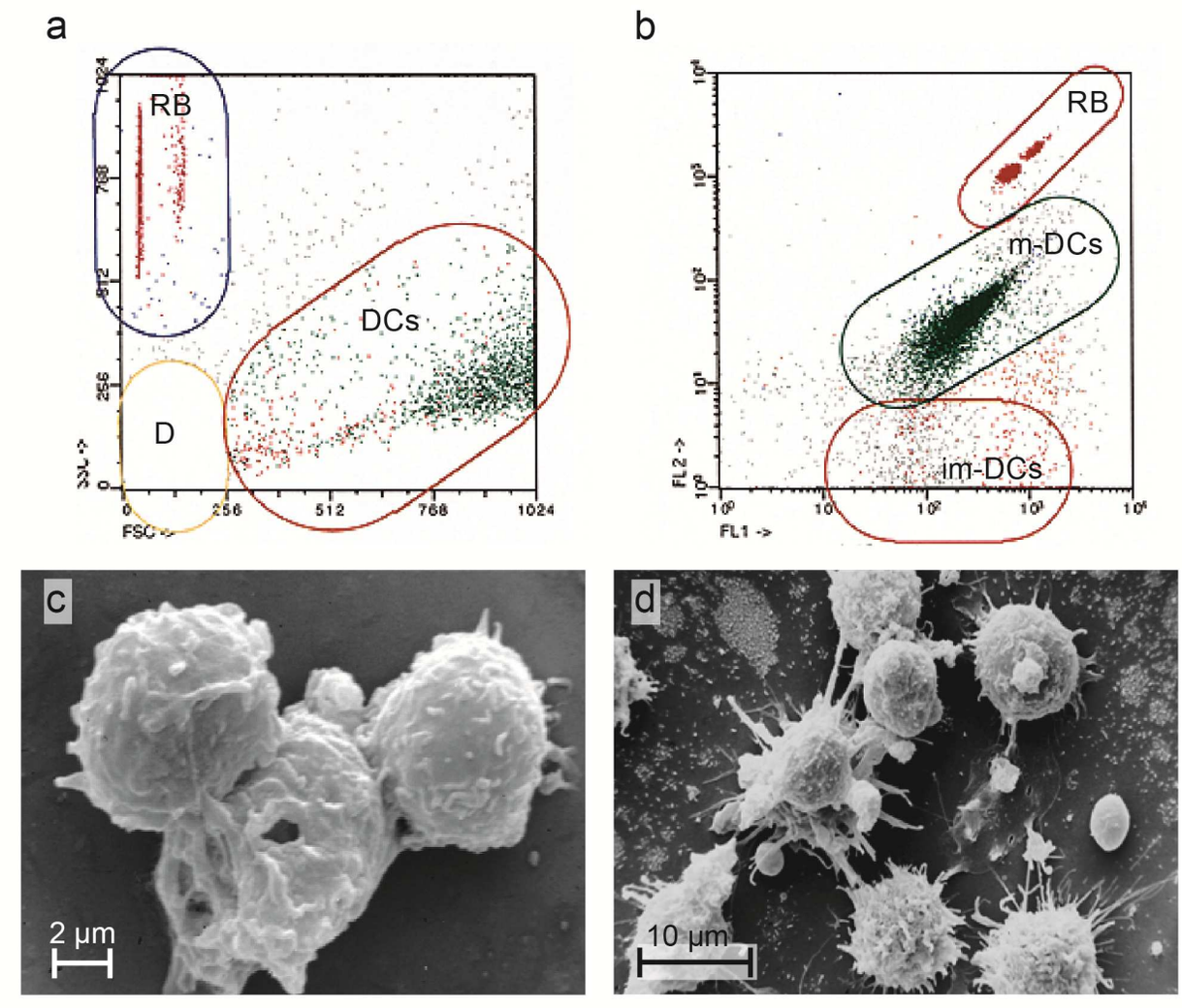

Figure 7. Visualization of immature mDCs by SEM after flow cytometric cell sorting by gating of CD1a^{bright}/CD83^{dim} mDCs (Fig. 7c) and of mature mDCs by gating of CD1a^{dim}/CD83^{brigh} mDCs (Fig. 7d). Fig. 7a shows the gating process of mature DCs using a forward and side scatter dot plot, Fig. 7b the gating of CD1a^{dim}/CD83^{brigh} mDCs at the fluorescence dot plot (FL1, determination of cell labeling with an FITC-conjugated anti-CD1a monoclonal antibody; FL2, cell labeling with a PE-conjugated monoclonal antibody against CD83 using the Attractor software, which allows neglecting cell debris and therefore also cell quantification in terms of absolute counts by using reference beads).

Hormone	Flow cytometry and SEM on day 7					
DC markers	$CD1a^+$	$CD14^+$	$CD83^+$	$CD1a^+/CD83^+$	$CD14^+/CD83^+$	SEM: mature DCs
Control (only medium)	16	23	18	6	4	16
Progesterone	7	5	17	4	3	14
β-estradiol	17	39	11	5	6	26

(a)

Hormone	Flow cytometry and SEM on day 14					
DC markers	$CD1a^+$	$CD14^+$	$CD83^+$	$CD1a^+/CD83^+$	$CD14^+/CD83^+$	SEM: mature DCs
Control (only medium)	57	30	64	45	24	29
Progesterone	26	57	56	24	10	15
β-estradiol	51	25	74	45	23	31

(b)

Table 1. a. Percentage of DC marker expression measured by flow cytometry and visualization of mature mDCs by SEM on day 7 of cultivation under the influence of steroid hormones. b. Percentage of DC marker expression measured by flow cytometry and visualization of mature mDCs by SEM on day 14 of cultivation under the influence of steroid hormones.

5. Generation of DC anti-tumor vaccines

Since two decades, mDCs, in their immature as well as mature state, have been widely used for experimental as well as for clinical purposes. The Nobel Prize Laureate in physiology or medicine Ralph Steinman was a pioneer in DC research. Sorry to say that Steinman never got to know the decision of the Nobel Prize committee since he died on October 3, 2011 [71]. He postulated that DCs could provide an immune attack on cancer, broad enough to encompass multiple targets, including mutant proteins expressed by the cancer. In addition, DCs could be able to activate and expand different arms of cell-mediated immunity such as NK cells, γδ- and αβ-T cells that can recognize different alterations in cancer cells [72]. Still in 1989 an institute for tumor therapy using DC vaccines was founded in Duderstadt near Göttingen (Germany) based on early investigations [73, 74] of the working group of Peters with the focus on gynecological-oncological diseases. Early therapeutic approaches included the following steps: (1) the freezing and storage of tumor material after operation; (2) blood drawing from the tumor patient immediately after operation; (3) isolation of mononuclear cells by gradient centrifugation; cultivation of cells in conditioned medium containing IL4 and GM-CSF in order to obtain immature mDCs; (4) confrontation of mDCs with thawed, irradiated, and homogenized tumor material; and (5) administration of mDCs by creating an intradermal blister. However, early clinical trials have frequently shown that after short recurrence of the disease a relapse occurred which was difficult to treat. The main question in this context is why the immune system fails to attack the tumor. The answer consists in the downregulation of antigen-presentation and T-cell activation of DCs in the tumor environment. The administration of

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primed DCs fails also because of the immunosuppressive action of tumor cells [75]. The reason for these pitfalls has been outlined in a recent review by Tran Janco et al. [76]. In tumors, tumor-infiltrating DCs (TIDCs) are functional but several receptors and cytokines of tumor cells compromise them leading to an environment that favors tumor progression. The expression of CD11c allows distinguishing three DC subsets: $CD11c^{bright}$, $CD11c^{intermediate}$, and $CD11c^{dim}$; $CD11c^{intermediate}$ expressing DCs are the predominant group of TIDCs. They fail to express enough quantities of costimulatory molecule, resulting in a hampered T-cell activation. In addition, they produce high levels of the immune-inhibitory molecule, programmed death 1 ligand (PD-L1). PD1 (CD279), a cell surface receptor belonging to the immunoglobulin molecule superfamily, and its ligands PD-L1 and PD-L2, impair the effector phase of immune cells. TIDCs also comprise a high number of pDCs, which promote the expansion of regulatory T cells (Tregs) characterized by high expression of the forkhead box P3 (FOXP3), a master regulator in the development of these cells. The consequence is an immune-tolerance preventing the elimination of the tumor. A further problem, concerning tumor expansion, is the secretion of the cytokine vascular endothelial growth factor (VEGF) and the chemokines CCL2, CXCL1, and CXCL5, leading to a hypervascularization of the tumor and intravasation of metastatic tumor cells. Tumor-induced transcription factors such as STAT3 induce S100A9 protein, preventing full maturation of DCs and consecutively block their responsiveness to local danger signals [77]. In this respect, the high concentration of IL-10 in the tumor cell microenvironment can also lead to STAT3 activation [76].

How could an anti-cancer therapy prevent the immune-compromising function of TIDCs? To influence the function of TIDCs, the PD1 and ligands can be inhibited by antagonist antibodies to PD1 and PD-L1 [78]. Strategies to promote DC maturation or to introduce the delivery of oligonucleotide-coated nanoparticles by DCs in order to influence immune functions at the epigenetic level by modulating the activity of miRNAs have been elaborated [79].

As mentioned above, *ex vivo* expansion of DCs originating from different sources has been used in clinical and pre-clinical trials [80]. The most commonly used approach is the differentiation of DCs from peripheral blood mononuclear cells (PBMCs) obtained via leukapheresis. After cultivation in conditioned medium containing GM-CSF and IL-4 for several days, they differentiate into immature $CD14^-/CD83^-$ DCs. Targeting these cells with maturation stimuli leads to further differentiation into mature $CD14^-/CD83^+$ DCs, that express high quantities of MHC-Class I and II as well as costimulatory molecules. A faster method includes the isolation of $CD14^+$ cells by immunomagnetic isolation and cultivation for 2 days in GM-CSF and IL-4 conditioned medium. Subsequently, the addition of proinflammatory molecules such as TNF-α, IL-1β, IL-6, and PGE_2 for further 24 hours induces maturation. Generation of DCs from $CD34^+$ HSCs requires mobilization of these cells into the peripheral blood of the patient prior to leukapheresis. Cultivation and expansion of these cells involve the cytokines GM-CSF, Flt3L, and TNF-α and results in mDCs with the typical LC-like morphology as described above. These cells are highly potent to initiate T-cell response. The most important step before vaccination is the complete maturation of DCs. In this respect, proinflammatory cytokines and PGE_2 are able to enhance the expression of costimulatory molecules, CD40L and CD70. In addition, TLR agonists can optimize DC function [80]. Loading of anti-tumor peptides to immature or mature

DCs result in an effective antigen presentation. It can be performed with homogenized and irradiated tumor material but also with known tumor-associated antigens. Loading of DCs with tumor antigens can be also carried out using recombinant bacterial or viral vectors or by RNA transfection of DCs. [81, 82]. Finally, DC transfection with mRNAs related to tumor-associated antigens can bypass the use of bacterial or viral vectors [69].

Nevertheless, an efficient DC vaccination requires strategies to overcome the immune modulating tumor environment as outlined above.

6. Conclusion

DCs are powerful mediators of innate and adaptive cellular immune response. There are different DC subtypes in respect to their localization and their functions in immune regulation or antigen presentation. The function of mDCs is highly controlled by steroid hormones. In this respect, β-estradiol supports differentiation and maturation of DCs, whereas progesterone has inhibitory effects. In the FRT, progesterone has an immune-suppressive effect during pregnancy. The estrogen deficiency after menopause also decreases the overall immune responsiveness. Also tumors of the female reproductive system are dependent on steroid hormones with implications for tumor prevention and tumor therapy. Finally, DCs can be propagated *ex vivo,* primed with tumor material, and used for vaccination against tumors. Nevertheless, the immunosuppressive tumor environment has been taken into consideration for an effective therapy.

7. Abbreviations

BDCAs, blood DC-specific antigens; BSA, bovine serum albumin; CD, cluster of differentiation; cDC1s, classical $CD1^+$ DCs; Clec9A, group V C-type lectin-like cell surface receptor mediating endocytosis but not phagocytosis; CXCL10, chemokine CXCL10; DCs, dendritic cells; E2-2, basic helix-loop-helix transcription factor (E protein); EEA1, early endosome antigen 1; ERα, estrogen receptor α; ERC, endosomal recycling compartment; EM, electron microscopy; ER, endoplasmic reticulum; ER60, chaperon thioreductase ER60; flt-3L, fms-like transcript 3 Ligand; FRT, female reproductive tract; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte macrophage colony-stimulating factor; HLA, human leukocyte antigens; ID2, transcriptional regulator belonging to the inhibitor of DNA binding (ID) family; IDO, indoleamine 2,3-dioxygenase; IFN-α, interferon alpha; IFN-β, Interferon beta; IFN-γ, interferon gamma; Ig, immunoglobulin; IL-10, Interleukin 10; IRFs, interferon regulatory transcription factors; LC, epidermal Langerhans cells; LPS, lipopolysaccharide; MACS, magnetic cell separator; maf, maf transcription factor family; MALT, mucosa-associated lymphoid tissue; M-CSF, macrophage colony-stimulating factor; MIIC, MHC-class II compartment; mDCs, myeloid dendritic cells; MHC, major histocompatibility complex; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; PAMPs, pathogen-associated

molecular patterns; PBS, phosphate-buffered saline; pDCs, plasmacytoid dendritic cells; $PGE_{2,}$ prostaglandin $E_{2;}$ PU.1, hematopoietic transcription factor PU.1; SCF, stem cell factor; SEM, scanning electron microscopy; STATs, signal transducers and activators of transcription; TAPs, transporter proteins associated with antigen processing; TCR, T-cell receptor; TEM, transmission electron microscopy; Th0, Th1, Th2, and Th17, T-helper cells 0, 1, 2 or 17; TLRs, toll-like receptors; TGF-β1, transforming growth factor β1; Treg, regulatory T-cells

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Author details

Josef Neumüller[1,2], Sylvia Emanuela Neumüller-Guber[3], Johannes Huber[4], Adolf Ellinger[1] and Thomas Wagner[5*]

*Address all correspondence to: thomas.wagner@medunigraz.at

1 Medical University of Vienna, Center for Anatomy and Cell Biology, Department of Cell Biology and Ultrastructure Research, Vienna, Austria

2 Blood Donation Center of the Austrian Red Cross for Vienna, Lower Austria and Burgenland, Vienna, Austria

3 Medical University of Innsbruck, Sektion for Virologie, Department for Hygiene, Microbiology, Social Medicine, Innsbruck, Austria

4 Medical University of Vienna, Department of Gynecologic Endocrinology and Reproductive Medicine, Vienna, Austria

5 Medical University of Graz, Department of Blood Group Serology and Transfusion Medicine, Graz, Austria

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