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Dendritic Cells

Edited by Svetlana P. Chapoval

DENDRITIC CELLS

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Meet the editor

Svetlana Chapoval, MD, PhD, is an assistant professor at the University of Maryland School of Medicine (UMSOM). She received her degrees from the Russian State Medical University and Gamaleya Research Institute of Epidemiology and Microbiology, correspondingly, both in Moscow, Russia. In 1996, she joined the laboratory of Chella David at Mayo Clinic as a postdoctoral fellow where her interest in dendritic cells (DCs) was initiated while analyzing HLA Class II polymorphism in allergic asthma susceptibility. Dr. Chapoval subsequently joined Jack Elias Laboratory at Yale University School of Medicine where one of her research projects was focused on lung DC biology. She developed a "Dendritic Cells" seminar for the Advanced Immunology course at UMSOM. Dr. Chapoval has authored and coauthored 40 papers, proceedings, and book chapters, including "Flow cytometry-based analysis and sorting of lung dendritic cells." She has served as an associate editor for the *Journal of Immunology* and as a reviewer for 28 peer-reviewed journals in the fields of immunology, molecular medicine, and drug discovery.

Contents

Preface

The identification of dendritic cells (DCs) as professional antigen-presenting cells, which initiate and shape the immune response, led to their further characterization in terms of their subsets, functions, and the development of new techniques for DC purification and cultures.

As the last book on DC biology and function was published in 2010 (Lorrain Welles, Nova Science Publishers), IntechOpen brought together an international group of researchers with the goal of providing an update on recent advances in DC research and clinical implications. This book contains six chapters that cover the following directions: DC subtypes and functions in different diseases, DC phenotyping, DC-NK cell interplay, roles of DCs in bone loss and repair, parasitic infections, and their therapeutic implications in cancer.

An introductory chapter provides a brief outline of recent reviews on DC subtypes, lymphoid and tissue-resident DCs, cell–cell interplay in immunity, and DCs in infectious diseases, allergy, autoimmunity, and cancer.

Chapter 2 is devoted to DC phenotyping and provides new insights into their molecular characteristics.

Chapter 3 deals with DC activation by NK cells, NK cell activation by DCs, and the importance of DC–NK cell cross-talk in cancer immunotherapy. It outlines the future perspectives in DC-based vaccination, which take into account NK cell-stimulating properties.

Chapter 4 studies DCs in parasitic infections with a specific focus on pattern-recognition receptors displayed by DCs such as Toll-like receptors, RIG-I-like receptors, NOD-like receptors, and C-type lectin receptors. It discusses in detail the role of DCs in adaptive immune responses against *Leishmania major, Leishmania mexicana, Toxoplasma gondii, Trichinella spiralis*, and protozoans of the *Plasmodium* genus. A better understanding of DC–parasite interaction will help in the development of new strategies and drugs to effectively treat parasitic diseases.

Chapter 5 studies the role of DCs in bone homeostasis and inflammation-related bone damage. It demonstrates the effects of different bone implant surfaces on DC maturation and function, and the resulting DC-mediated inflammatory responses. It establishes DCs as important cellular targets for controlling bone inflammation and destruction.

Chapter 6 defines the role of DCs in cancer and contains six major parts that cover a generation of DC-based vaccines based on the selection of a specific antigen; DC maturation by different stimuli; optimal delivery routes for DC-based vaccines; a combination of DC-based therapy with checkpoint inhibitors, chemotherapy, or other targeted therapies; DC mobilization and adjuvant-based activation; and in vivo DC targeting with nanoparticles.

This book is addressed to a broad audience, including academic researchers, clinical practitioners, and industry specialists interested in DC research and clinical application.

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Chapter 1

Introductory Chapter: Dendritic Cells

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Additional information is available at the end of the chapter

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1. Introduction

Dendritic cells (DC) are critical antigen-presenting cells (APC) of the immune system due to their unmatched ability to sample antigens and initiate T cell responses [1]. In addition to the induction of primary immune responses, DC are also important cells for the maintenance of immunological tolerance. DC were discovered by Ralph Steinman and Zanvil Cohn in 1973 [2, 3]. However, the peak of publications focusing on DC as main sentinel cells of the immune system happened 20–30 years after their discovery. This chapter discusses major advances in our understanding of DC biology, subtypes, phenotypes, cell-cell interplay, and roles in several pathologic conditions such as infectious, autoimmune, and tumorous diseases (**Table 1**). These new discoveries in DC biology and contemporary approaches in directed and tightly controlled DC manipulations will help in the development of improved therapeutic and vaccination strategies to fight many diseases.

2. DC subtypes, phenotypes, and functions

A recent comprehensive review of published research on DC migration in health and disease also shows the phenotypic characteristics of DC in different tissues, namely, in skin, intestines, lungs, and CNS [4]. In contrast to other organs, where several DC subtypes exist simultaneously, in skin epidermis, for example, only one type of DC is present, CD207+ Langerhans cells. In contrast to that, several DC subsets were found in dermis, such as conventional (c) DC1 and cDC2, distinguished by the presence or absence of XC-chemokine receptor 1 (XCR1) expression, correspondingly. There is a third cDC subpopulation in skin dermis, so called double-negative cDC (lacking XCR1 and CD11b expression). These three dermal DC subtypes have different origin/cellular progenitors, and perform distinct functions during skin inflammation.

In contrast, intestinal cDC1 is characterized by CD103 expression. At the same time, intestinal cDC2 expresses CD11b in addition to CD103. Surprisingly, lamina propria CD103+cDC induce regulatory T cells, as for other organs it is a selective function of plasmacytoid (p) DC [4]. The authors admit the presence of yet uncharacterized DC phenotypes in human gut.

Lung-resident cDC and pDC subpopulations have been characterized previously in mice [5] and humans [6]; however, more recent findings show the existence of two cellular subpopulations within lung cDC. In addition to cDC, the presence of monocyte-derived (mo) DC, which can be discriminated in the lungs based on the expression of cell surface markers such as CD103 (Integrin aE), CD207 (langerin, a C-type lectin with mannose binding specificity), DNGR1 (C-Type Lectin Domain Containing 9A, CLEC9A, a receptor for necrotic cells required by DC to cross-prime CTLs against dead cell antigens), Signal regulatory protein α (SIRPa), MAR-1 (Fc epsilon receptor I alpha), and Ly6C (a mid-stage cell development differentiation antigen, GPI-anchored glycoprotein) [4]. Moreover, CD64 and MAR-1 are considered to be the most selective markers for the effective separation moDC from cDC. In addition to that, the GenChip technology application to DC research has recently demonstrated that DC subsets display different transcriptional factor's requirement in their development and function [7, 8]. Several transcription factors including PU.1 (E26 transformation-specific family transcription factor), Bcl11a (C2H2 type zinc-finger protein), Irf4 and Irf8 (Interferon Regulatory Factors 4 and 8, correspondingly), E2-2 (basic helix-loop-helix transcription factor E protein), Id2 (Inhibitor of DNA

Topic	References
DC development	[1, 7, 9–11, 13, 14]
Antigen processing and presentation	[1, 9, 13, 14, 16–19]
Phenotypes, cell surface markers	[4, 9–15]
Transcriptional control and classification	[1, 7, 8, 10]
Functional characterization	[8–10, 13–15]
DC cytokine profile	[14, 15]
Immune tolerance	[14, 30, 31, 35]
Tissue- and organ-resident DC	[5, 13, 19]
DC migration	[4]
Cell-cell interaction	[16–23]
DC and allergy	[5, 12, 14, 24]
DC and cancer	[14, 24, 32]
DC and autoimmunity	[4, 14, 15, 24, 35]
DC and infectious diseases	[15, 24–28]
DC in osteoimmunology	[33, 34]
Novel techniques in DC research	[11, 20, 21]

Table 1. Main topics of research and discussion covered by cited manuscripts.

Binding Protein 2), Batf3 (basic leucine zipper transcription factor ATF-like 3), IFN regulatory factors (IRFs), zbtb46 (zinc finger transcription factor 46), Notch RBP-J (the main transcriptional mediator of Notch signaling), Icaros (DNA-binding zinc finger protein), and others have been found to be differentially regulated in different DC subsets [7, 9, 10]. The comprehensive review by Murphy and associates [10] discusses DC origins, heterogeneity, functions, phenotypic and functional homology between mouse and human DC subsets, and the requirement of several transcription factors for DC subtypes' development. According to a contemporary view on DC presented in this chapter, DC are divided into two main subpopulations, namely, interferon regulatory factor of transcription 8 (Irf8+) cDC (CD8a+) and Irf4+ cDC (CD11b+). Moreover, two subpopulations can be clearly distinguished even within Irf4+ cDC based on their developmental dependency on either Notch-2 or Klf4. The authors discuss the functional differences between different DC subsets based on the *in vitro* studies and the *in vivo* specific gene-knockout mouse evaluation. They also discuss the DC origins and transcription factors necessary for DC subtype's development from a precursor cell. As an example, Nfil3 (nuclear factor, interleukin 3 regulated; another abbreviation for it is E4BP4) is required for CD8α+ DC development, whereas Id-2 is required for Irf8+ cDC. Furthermore, a recently published international comprehensive study has used a combination of single-cell messenger RNA sequencing (scmRNAseq) and cell cytometry by time-of-flight (CyTOF) contemporary technologies to study individual human DC subsets and their precursors among blood CD135+HLA-DR+ cells [11]. The authors created a panel of 38 labeled Abs based on DC-specific markers including CD2, CX3CR1, CD11c, and HLA-DR. This panel also included Abs to cDC-associated markers such as CD11c, CX3CR1, CD2, CD33, CD141, and reported the existence of individual DC lineage-committed subpopulations, intermediate DC clusters, previously unrecognized human pDC heterogeneity. It definitely brought new insights into DC therapeutic potential in many diseases. Lineage commitment is directly regulated by several hematopoietic cytokines, where Flt3L, M-CSF, GM-CSF, Lymphotoxin β and TGFβ1 play the major roles in the individual DC subset's development [9].

In conclusion, the previous simplistic division of mouse and human DC on cDC and pDC and definition of their function as: the inductors of CD4+ T cell immunity (CD11b+ cDC), efficient Ag cross-presenters to CD8+ T cells (CD8α+ cDC), and rapid producers of type I IFN to fight viral infections (pDC), has been upgraded significantly [4, 8, 9, 12–15]. Human pDC differs from cDC as they are $CD11c^{low}$ and the expression of other lineage-associated markers such as CD3, CD14, CD16, and CD19 is not detected on their surface; however, they express BDCA-2 (blood dendritic cell Ag–c-type lectin, CLEC4C (CD303)), CD4, CD68, CD123 (IL-3R), and immunoglobulin-like transcript 3 (ILT3), or ILT-7. Similarly to human pDC, mouse pDC are also $CD11c^{low}$, but they express Ly-6C (a GPI-anchored glycoprotein - lymphocyte Ag 6 complex) and Siglec-H (sialic acid-binding immunoglobulin-like lectin H), which is not found on mouse cDC. The pDC ability to induce tolerance through IDO production and Treg cell induction explains, in part, their protective role in allergic diseases and transplant rejection [15]. However, pDC play tissue-damaging type I IFN-associated pro-inflammatory functions in autoimmune diseases [15]. The latter chapter has divided human DC into four subpopulations such as CD141+ DC, CD1c+ DC, pDC, and moDC, correspondingly to the mouse subsets such as CD8/CD103+ DC, CD11b+ DC, pDC, and moDC. Human moDCs have been shown to serve as an effective inducer of Th1 responses, which partially overlaps with CD141+ DC and CD1c+ DC functions [15]. Functional specializations of different DC subsets as well as the

clinical syndromes associated with DC deficiency are being discussed in details in a review by Merad and colleagues published in 2013 [9], whereas a review by Mildner and Jung [13] focuses on functional similarities and differences of organ-specific DC.

3. The role of cell-cell interplay in DC activation and function

DC uptake Ag, process it, and present it to T cells as Ag-derived peptide in the context of specific MHCI or MHCII molecules [1]. DC are subdivided on immature, Ag-sampling, and mature, Ag-presenting, cells. After Ag uptake, DC undergo maturation and migrate to the T cell areas of lymph nodes. In the lymph nodes, DC present Ag to T cells, which effective stimulation depends on two critical signals, MHC-TCR and costimulation. Several recent studies consider the necessity of a third, so called "polarization" signal, for an optimal T cell activation [16–18]. Such signal can be provided by certain cytokines [17, 18] or semaphorins [16]. A recent review by Federika Benvenuti [19] focuses on the structural and functional composition and significance of DC-T cell cross-talk, on the formation of an immunological synapse between these two immune cells and a synapse composition. The interesting current technical developments aimed to analyze DC-T cell interaction and resulting corresponding cell activation include two-photon intravital imaging technique [20] and Labelling Immune Partnerships by SorTagging Intercellular Contacts (LIPSTIC) [21]. Both techniques can be used to study cell activation *in vitro* and *in vivo* and beneficiary complement each other. Activation of T cells can be additionally analyzed by the use of dynamic *in situ* cytometry, Ca+ influx analysis, and/or transcription factor translocation [20].

Direct cell-cell contact between DC and other immune cells can significantly modulate DC themselves and the resulting DC-induced immune response to Ag. DC-macrophage interaction and its role in the immune response activation have been described for CD169+ (siloadhesin, Siglec-1) macrophages and BATF3-dependent CD8α+ DC [21]. Specific viral Ag targeting to CD169+ macrophages led to Ag transfer to cross-presenting CD8α+ DC and subsequent T cell activation. A review by Walzer and colleagues [22] discusses the activation of NK cells by DC after a direct cell-cell contact, DC maturation induced by such interaction, immature DC lysis by activated NK cells, and other effects of such interaction, which have all important consequences for antimicrobial response. A direct interaction of DC with neutrophils, critical cellular fighters of bacterial infection and regulators of immune response on the infection site, has been reported previously as the interaction between Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Nonintegrin (DC-SIGN or CD209) with Mac-1 or CEACAM1 on neutrophils [23]. These cell-cell interaction pathways play important connecting roles between innate and adaptive immunity. The communication of different DC subsets between themselves and the resulting changes/modifications in the immune response has never been addressed although such cellular cross-talk might significantly influence the outcome of immune response.

4. DC and infections

The main role of DC in infection is to induce an immune response leading to microbe elimination. DC are equipped with numerous Ag uptake receptors such as DEC-205, mannose

receptor CD206, CD209 (DC-SIGN), CD207 (langerin), CLEC4A (DCIR), which bind a whole array of microbes (reviewed in [24]). Following Ag uptake, CD8α+ DC cross-present Ag on MHCI to stimulate CD8+ T cells to kill infected cells. In addition to that, a protective Th1 response is generated over effective Ag presentation to CD4+ T cells. However, numerous microbial agents are capable of blocking DC maturation. Those include *Herpes simplex* virus, vaccinia virus, varicella zoster, CMV, measles, *Salmonella typhi*, *Plasmodia*, *Coxiella burnettii*, and others. Some microbial agents, such as *Yersinia pestis* or *Salmonella typhi*, can selectively destroy DC by injecting toxins into cells thus avoiding recognition by a host immune system. *Mycobacterium tuberculosis* blocks the expression of CCR7 on DC thus interfering with DC migration step. *Aspergilus fumigatus*, malaria and hepatitis C viruses modify DC function in such way that DC induce a Th2 type of immune response instead of a protective Th1 response. Furthermore, several microbes can multiply/replicate within DC cytoplasm, which leads to infection spreading. Those DC-disseminating microbes include, but not limited to, HIV-1, LCMV, *Toxoplasma gongii*, *Bacillus anthracis*, CMV, and Ebola virus. The *in vitro* pathogen invading study has been done in human myeloid DC cultures. As the numbers of pDC are significantly lowered in the blood of people with HIV, HTLV-1 and RSV infections, it is highly likely that these microbes can evade pDC as well. pDC produce a large amount of type I IFNs in response to either viral (HIV) [25] or nonviral (*Aspergillus fumigatus*) [26] pathogens. Besides pathogens, however, the healthy microbiota representatives, such as *Lactococcus lactis* [27] and *Bacteroides fragilis* [28], have the capacity to activate pDC. The latter studies suggest that pDC can function as the enhancers of corresponding probiotic's activity.

5. DC in allergy

DC in lung tissue continuously sample exogenous Ags and make sure the immune system does not generate a harmful response to such generally un-harmful agents as pollens, dust mites, cockroaches, and others. Basically, instead of T cell activation, lung-resident DC were shown to induce T cell tolerance to those innocuous proteins in steady-state conditions. One of the potential mechanisms of T cell silencing by DC is based on ICOSL molecule, which has been shown to co-stimulate Treg cell development [24]. Local lung cytokines, such as TSLP [29] or VEGF [5], play critical nonredundant roles in making lung cDC immunogenic toward inducing Th2 responses to subsequent allergen exposures. The main direction in DC-based immunotherapy of asthma is targeting a pro-allergic lung cDC function and making cDC tolerogenic, Treg cell-inducing cells [24]. Targeted activation of Treg cell-inducing pDC represents another direction in DC-based asthma immunotherapy [24]. Similarly to the mechanisms of lung-associated allergies, in the diseases associated with intestinal allergic inflammatory reactions, such as food allergy and inflammatory bowel disease, gut lamina propria CD103+ cDC sample Ag and migrate into local lymph nodes, stimulate Treg cells from gut-associated lymphoid tissues to induce and maintain tolerance to food antigens [30]. The main two directions in DC-based therapy for food allergy are based on the induction of oral tolerance to a specific food allergen by: (1) directly activating Treg cell-stimulatory mucosal CD103+ cDC, and (2) gastrointestinal pDC-mediated tolerance through *de novo* generation of iTreg cells [31].

6. DC and cancer

Tumor Ag-loaded DC present a basis for DC vaccine in cancer. Currently, three ways to obtain such DC have been used in pre-clinical practice and clinical trials, namely: (1) The *in vitro* PBMC stimulation with Ag and GM-CSF (PBMC contain other cell types besides DC); (2) DC direct isolation from blood (approximately 1% of total PBMC) and their *in vitro* direct stimulation; (3) the *in vitro* expansion and activation of DC precursor cells (monocytes or CD34+ bone marrow-derived hematopoietic progenitor cells) followed by Ag loading, and the use of them as a vaccine [32]. The immunogenicity of such vaccines is highly dependent on DC subtype and activation level. Therefore, additional DC stimuli are currently being evaluated in order to potentiate DC activity and ability to induce the desired Th1 and/or CTL anti-tumor response. These stimuli include: (1) TLR agonists; (2) a combination of poly-IC with TNFα, IL-1β, IFNγ, and IFNα; and (3) a genetic engineering of DC by mRNA electrocorporation (such as TriMix) [32]. The discussed chapter here also describes in detail the *in vitro* culture conditions for an effective DC anti-cancer vaccine formulation.

7. DC and bone biology

Normally, DC are absent in bone tissues (reviewed in [33, 34]). However, numerous DC were found in synovial and periodontal tissues surrounding bone tissue during inflammation. Both, immature CD1α+ cells expressing RANK (receptor activator of NF-kB) and RANKL, and mature RANK-expressing DC-LAMP+ DC were identified in synovial tissues of patients with rheumatoid arthritis. RANKL is an absolutely necessary factor for osteoclasts (OC) differentiation and for their role in bone-absorption. Moreover, the *in vitro* studies using different DC culture conditions have shown that DC can also serve as octeoclast's precursor cells. When immature CD11c+CD11b-DC were exposed to an Ag stimulation as a maturation factor and interacted with CD4+T cells using RANK-RANKL ligation, they develop into so called "dendritic cell-derived osteoclasts (DDOC)", which were phenotypically CD11c+MHC-II+TRACP+(tartrate-resistant acid phosphatase) CT-R+(G protein-coupled receptor that binds the peptide hormone calcitonin) cells. This is rather an alternative pathway in OC differentiation in addition to a classical OC developmental pathway from monocyte precursors. It is well established now that OC are multi-nucleated bone-eating cells, and the excessive production of such cells can lead to a bone loss. DC can also participate in bone homeostasis through their secretion of multiple osteoblast's inhibitory (IL-27) or activating (RANK, IL-1, IL-6, and IL-23) factors, or through activation of certain arms of T cell immunity, which, in their turn, secrete cytokines directly or indirectly involved in bone formation process [34].

8. DC in autoimmunity

Genetic alteration occurring in several types of immune cells, including DC, could lead to the appearance and persistence of self-Ag reacting T and B lymphocytes, which is a hallmark of autoimmune diseases. Most studies on the role of DC in autoimmunity were

done in mice. It has been shown that autoimmune diabetes type 1 prone NOD (nonobese diabetic) mice have lower number of CD8α+ DC, which are dysfunctional in Ag cross-presentation (reviewed in [35]) that reduces the cross-tolerance. The Batf3$^{-/-}$ mice, which lack these cross-presenting DEC205+CD8α+ DC, do not develop diabetes. The other tolerogenic type of DC in mice is characterized by CD11b and DCIR2 (Clec4A4/DC immunoreceptor 2) expression. DC are tightly involved in pathogenesis of another autoimmune disease, multiple sclerosis. The study in mice have demonstrated a reduced EAE severity in functionally pDC-depleted mice (pIII+IV−/− mice, which lack MHCII expression on pDC cell surface). Thus, it is believed that pDC induce autoimmune inflammation through type I IFN production and tolerance through Treg cell activation. Indeed, there is a strong connection of type I IFN levels (or "signature"—elevated expression of type I IFN-stimulated genes) (reviewed in [15]). PBMC obtained from patients with systemic lupus erythematosus (SLE) can be distinguished from those in healthy volunteers by an overexpression of 18 genes, 12 of which were IFN type I-regulated. Similarly, IFN "signature" was detected in skin of patients with psoriasis, in PBMC and sera of patients with Wiskott-Aldrich syndrome (WAS), and in atherosclerotic lesions. However, as it is mentioned above, pDC can also induce tolerance, and those two distinct functions of pDC in autoimmunity are particularly dependent on Ag nature and timing of exposure (an Ag priming phase or a chronic Ag exposure/inflammation phase).

9. Summary

Ralph Steinman was awarded the highest honor for an outstanding scientific discovery, a Nobel Prize in Physiology or Medicine, in 2011 in recognition of DC discovery as well as the characterization of DC main functions. More recently, several DC-modulating technologies were developed for their use as vaccines for infectious diseases, cancer, allergy, and autoimmune diseases [9, 24, 36]. New insights into molecular characteristics of DC and their roles in human diseases continue to be researched, which will lead to a development of novel therapeutic strategies aimed at targeting specific DC subsets and/or their products. These strategies include the manufacturing of DC with an optimal immunocompetence, capable in enhancing the strength of immune effector cells and making the disease-causing cells susceptible to immune attacks [32]. Therefore, DC, according to Steinman and Banchereau, present an "unavoidable target" in the design of effective treatments for many human diseases [24].

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Chapter 2

Dendritic Cell Subsets, Maturation and Function

Ghada Mohammad Zaki Al-Ashmawy

Additional information is available at the end of the chapter

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Abstract

Dendritic cells (DCs) are the most efficient and professional antigen-presenting cells of the immune system required for induction and dispersion of immune responses. DCs also have an important role in the induction and maintenance of tolerance. In response to infections, DCs drive the production of effector CD4+ T helper 1 (Th1) and CD8+ T cell-dominated immune responses. DCs can be designated to become tolerogenic and enhance regulatory T cells (Tregs) that regulate effector T cell responses, a process that is essential for the maintenance of immune homeostasis and control of autoimmune diseases and hypersensitivities. DCs can exist in three states: immature, semi-mature, and mature DCs. The difference between immature and mature DCs is distinctly based on variations occurring on a phenotypic level and functional level. Immature dendritic cells manifested characteristics of primitive cells, defined by expression of classical dendritic cell surface markers CD11c, CD11b and major histocompatibility complex class II (MHC-II). Phenotypic maturation is accomplished when DCs upregulate surface maturation markers such as CD80, CD83, and CD86.

Keywords: CD83, CD86, TLR, immunogenic, tolerogenic

1. Introduction

Dendritic cells (DCs) are rare, heterogeneous bone marrow (BM)-derived professional APCs that are disseminated ubiquitously in blood, lymphoid, and peripheral tissues, particularly at the gates of antigen entry. They originate from hematopoietic stem cells throughout specialized progenitor subsets and are essential in innate and adaptive immune capacity and in managing the balance between immunity and tolerance [1]. Under normal conditions, DCs are present throughout the body at low numbers representing ≈1–2% of white blood cells [2].

In the steady state, DCs reside in immature or semi-mature states in the periphery where they regularly take up and process self-Ags and maintain self-tolerance [3]. Immuno-stimulatory DCs have undergone maturation after recognition of exogenous and endogenous danger signals by Toll-like receptors (TLRs). These signals include pathogen-associated molecular patterns in the form of microbial products, such as products of damaged or dying cells [4].

DCs are matured by CD40 ligation and by pro-inflammatory cytokines that can produce DC maturation ex vivo, detached of CD40 ligation. Maturation is correlated with up-regulation of cell surface MHC gene products, co-stimulatory molecules (CD40, CD80, and CD86 and CD83), and relevant chemokine receptors that improve the ability of DCs to migrate to secondary lymphoid tissue, where they present Ag to Ag-specific T cells and induce T-cell activation and generation. Consequently, activated T cells drive DCs toward terminal maturation [5].

DCs produce from Hematopoietic stem cells (HSCs) in the BM and are originated from both myeloid and lymphoid progenitors, as illustrated in **Figure 1**. Both subsets, conventional DC (cDC) and plasmacytoid DC subsets (pDC), are derived from a common CD34+ progenitor [6]. The hematopoietic growth factor fms-like tyrosine kinase 3 ligand (Flt3L) represents a fundamental function in steady-state DC expansion; this is evidenced by the preponderance of DC precursors being Flt3+ (CD135+) and culture with Flt3L appearing in cDC and pDC

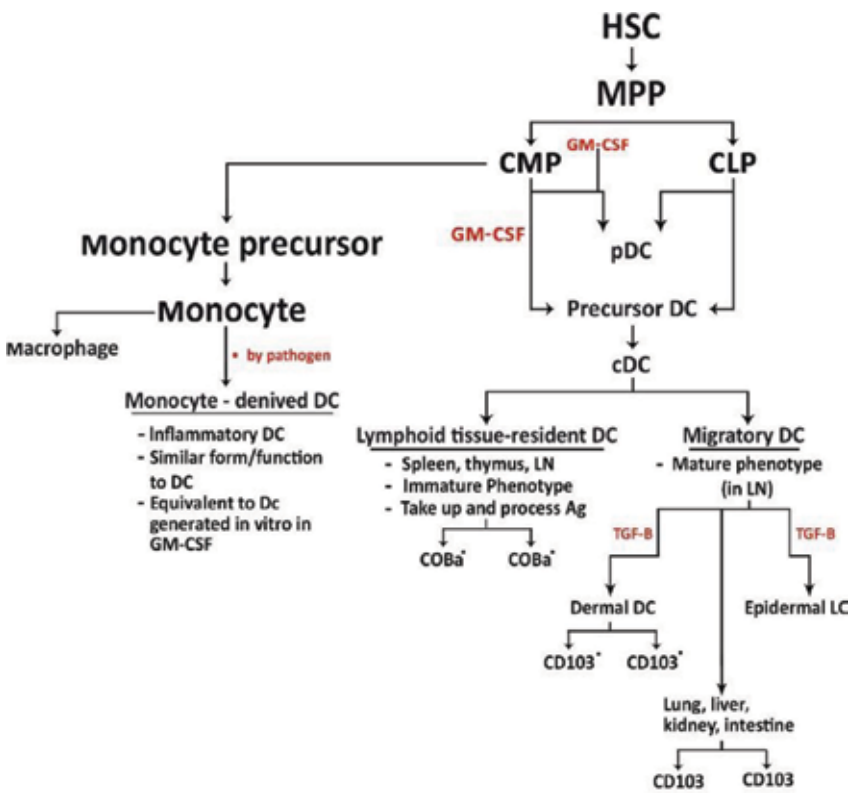

Figure 1. Dendritic cell hematopoiesis.

subsets. GM-CSF is also crucial in DC hematopoiesis, as it provides DCs from monocytes and immature progenitors in the deficiency of intact Flt3L signaling and provides DCs under inflammatory conditions [1].

DCs are divided into two principal cell populations, conventional DCs (cDCs) and plasmacytoid DCs (pDCs). In the steady state, cDCs present typical DC characteristics (e.g., cytoplasmic dendrites) and function (e.g., Ag uptake, processing, and exhibition). cDCs can be divided into migratory DCs, such as skin epidermal Langerhans cells (LCs), dermal DCs, which present Ag in lymph nodes following its uptake in peripheral tissue and resident DCs, which take up and process Ag within a lymphoid organ, such as splenic or thymic DCs [1]. Thymic DCs remove self-Ag-specific thymocytes and stimulate the expansion of immunoregulatory T cells (Treg). Thymic conventional DCs (cDC) readily received MHC class I and II from thymic epithelial cells (TEC), but plasmacytoid DCs (pDC) were less effective. Intercellular MHC shift was donor cell-specific; thymic DC readily gained MHC from TEC plus thymic or splenic DC, whereas thymic or splenic B cells were smaller donors [7].

Plasmacytoid DCs (pDCs) are a subset of precursor DCs which possess an immature phenotype in the steady-state and plasma cell morphology (e.g., lack dendrites). On activation, pDCs strictly match cDCs in form and function. Monocyte-derived DCs or inflammatory DCs are similar to cDCs in form and function and related to in vitro GM-CSF-generated DCs [3].

Under steady-state conditions, human pDCs display lower levels of MHC and costimulatory molecules compared with conventional myeloid DCs (mDCs). pDCs are less efficient in Ag processing and loading ability to excite T cells than mDCs. After their activation via TLR, pDCs produce high levels of type 1 interferon (IFN) and incite CD4+ and CD8+ T cells. This is in opposition to activated mDCs, which secrete IL-12 and enhance T-helper type-1 (Th1) cell differentiation and CD8+ cytotoxic T lymphocyte (CTL) responses [8]. Plasmacytoid DCs (pDCs) are a subset of precursor DCs which have an immature phenotype in the steady-state and plasma cell morphology (e.g., lack dendrites). On activation, pDCs closely resemble cDCs in form and function. Monocyte-derived DCs or inflammatory DCs are similar to cDCs in form and function and correlate with in vitro GM-CSF-generated DCs [3].

Under steady-state conditions, human pDCs display lower levels of MHC and costimulatory molecules compared with conventional myeloid DCs (mDCs). pDCs are less efficient in Ag processing and loading ability to stimulate T cells than mDCs. After their activation via TLR, pDCs secrete high levels of type 1 interferon (IFN) and stimulate CD4+ and CD8+ T cells. This is in contradiction to activated mDCs, which produce IL-12 and increase T-helper type-1 (Th1) cell differentiation and CD8+ cytotoxic T lymphocyte (CTL) responses [8].

pDCs have intrinsic tolerogenic features; in the steady position, human thymic pDCs provoke Treg, whereas liver and airway pDCs control oral and mucosal tolerance, respectively. pDCs have also been involved in the management of disease activity in experimental models of autoimmunity and revealed to exert disease-suppressing capacity [9].

It may be important after transplantation regarding donor engraftment (tolerance), which has clinical features that overlap with autoimmune disease. Epidermal LCs may be immunostimulatory or tolerogenic, depending on their state of maturity, inciting immunogen, and the cytokine environment [10].

DCs are characterized by high versatility, flexibility and multiple functional activities combined with their dual capacity to induce self-tolerance or trigger immune responses. The principal function of DCs is to scare the immune system toward heterogeneous and dangerous invasions and to defend self-tissues from destruction to keep self-tolerance [11]. The coordination of these supposedly multiple functions may open up new roads for stimulating or controlling immune responses and to promote defensive or therapeutic remedies for controlling inflammatory and autoimmune diseases or cancer, as well as designing unusual varieties of vaccines based on DCs biology [12].

A basic biological role of DCs relies on the constant sampling of their tissue environment, reacting to stress, risk signals and transducing the gathered molecular information to other cell classes of the immune system [13]. DCs are implemented with characteristics sets of pattern-recognition receptors, such as TLRs (Toll-like receptors), NLRs (NOD-like receptors), and RLRs (RIG-I-like receptors), which are specialized to recognize exogenous pathogen-associated molecular patterns (PAMPs) and endogenous danger signals, damage-associated molecular patterns (DAMPs) [14].

The response of DCs to MAMPs and DAMPs is achieved by the activation of pausing DCs by microbial components, noxious or toxic abuses. Activation of DCs sequences in the expression of costimulatory molecules, the generation of cytokines, chemokines and additional soluble mediators. Both are resting and stimulated DCs can switch their tissue position and transfer through peripheral and lymphoid tissues. Activation of DCs by MAMPs and DAMPS appears in the prompt, chemokine-mediated translocation of DCs to peripheral lymph nodes where they have the possibility to communicate naive T-lymphocytes to induct adaptive immune responses [15]. This process assures the transformation of molecular message obtained in the periphery toward other cell varieties of both innate and adaptive immunity such as neutrophils, granulocytes, NKs, killer T cells, T- and B-lymphocytes [16].

The response of DCs can be divided into the perception phase followed by phases of signal transduction pathways supported by adaptors and interfered by post-translational changes such as phosphorylation and ubiquitination reactions leading to the activation of transcription factors, and gene transcription followed by the secretions of soluble factors [17].

In this cascade, few receptor complexes ligated by their specific ligands allow substantial signal amplification. It has also been shown that the generation of fully active and stable DCs requires the parallel activation of multiple signaling pathways [18]. Signs through a particular receptor may produce partial stimulation only, which may be regressed by signals which promote the differentiation of regulative DCs. Signals produced by Toll-like receptors (TLRs), cytokines, chemokines, eicosanoids, free oxygen radicals, and several inflammatory mediators provide a signaling matrix and determine the phenotype and functional activities of DCs [19].

Five types of PRRs have been recognized: (i) transmembrane TLRs, which are combined to cell surface or endosomal membranes of different cell types, (ii) membrane C-type lectin receptors (CLRs) identified by the appearance of a carbohydrate-binding domain, (iii) three further classes of intracellular sensors, which are confined to the cytosol of multiple cell types and include NOD-like receptors (NLRs), RIG-like receptors (RLRs), and the latterly expressed AIM2-like receptors (ALRs), all with nucleotide recognition capacities [20].

Upon binding of their specific ligands, TLRs activate the NF-κB/AP-1 and the interferon-regulatory factor 7/3 (IRF-7/3) pathways to coordinate innate and initiate adaptive immunity [21].

RLRs are crucial viral sensors in the cytoplasm and contain retinoic acid-inducible gene-I (RIG-I), melanoma differentiation-associated gene-5 (MDA5), and laboratory of genetics and physiology 2 (LGP2), sequentially. RIG-I and MDA5 have been recognized as receptors toward double-stranded RNA [22].

Nucleotide-binding oligomerization domain (NOD)-like receptors mediate primarily antibacterial immunity through the activation of NF-κB or inflammasomes, whereas RIG-I-like helicases have a fundamental role in the induction of antiviral immune responses [23] (**Figure 2**).

The collaboration of PRRs and the resulting secretion of type I interferons and inflammatory cytokines can be extremely potent toward pathogens. Following infections, innate defense mechanisms are stimulated immediately and support the expansion of adaptive immune responses. DCs perform a crucial role in the orchestration of humoral and cellular immunity and the initiation and sustaining of long-term immunological memory [24]. Interaction of microbes with the innate immune system involves the induction of multiple PRR pathways triggered simultaneously by various PAMPs of the whole pathogen [25].

The possible interaction of two or more signaling pathways in biochemical systems can either be potentiating or hampering. For example, in moDCs and monocyte-derived Langerhans cells (moLCs), co-ligation of TLR3/TLR7 and TLR3/Dectin-1 lead to increased Th1/Th17 responses, in contrast to TLR3 and Langerin ligation, which had an opposite effect [26].

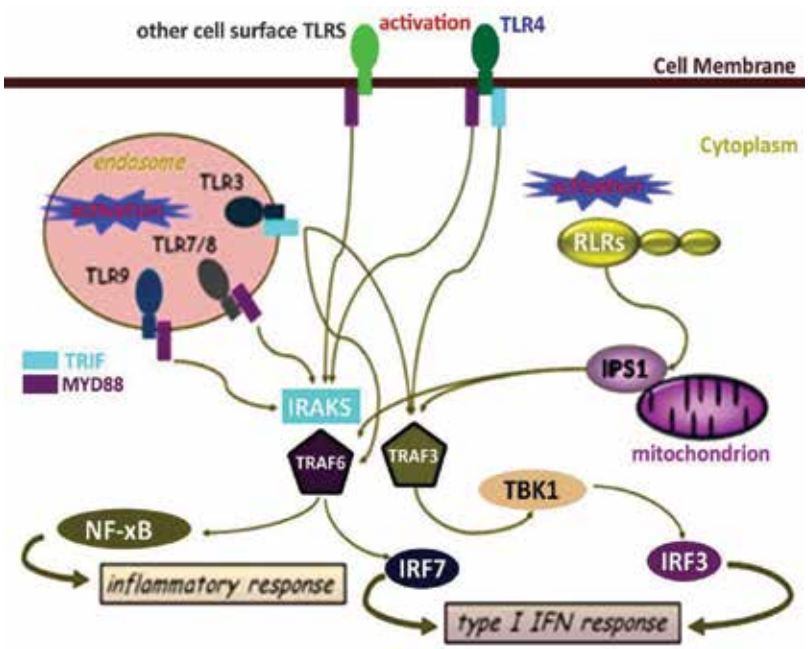

Figure 2. TLR and RLR signaling.

Similarly, another group found that RLR/TLR co-activation caused decreased Th1/Th17 responses upon bacterial infection. This cross-interference of RLR and TLR signaling might have significant implications in the design of future vaccination strategies, and the possible spectrum may be expanded to other non-immune cell types as well [27].

In vaccine construction, a primary purpose is to produce efficient, specific T-cell responses. This is accomplished by targeting antigen to cell surface molecules on DCs that efficiently direct the antigen into endocytic chambers for packing onto MHC molecules and stimulation of T-cell responses. Toll-like receptors (TLRs) expressed on DCs employed as intentions for antigen presentation for cancer and different disorders [28].

Depending on phenotypic and functional requirements, DCs may develop immunogenic or tolerogenic responses. Although several Toll-like receptors, such as TLR3, TLR4, TLR5, TLR7, and TLR8, provoke immune activation, others can quiet immune responses by tolerance initiation in DCs. Under certain conditions, TLR2 activation can lead to IL-10 production or Treg cell activation via repression of TLR7/TLR9 signaling and prevention of IFN-α and -β secretion from pDCs [29].

Despite the immunogenic capability of DCs in mounting immune responses, which has been assigned to the only target in the immune system, they have also been ascribed several roles in tolerance installation and silencing of immune responses. DCs express a fundamental role in the induction of several subsets of T cells, such as Th1, Th2, Th17 and regulatory T cells (Tregs). In the steady state, DCs play a critical role in the induction of tolerance against self-antigens. Complete ablation of DCs breaks self-tolerance of CD4+ T cells and results in fatal autoimmunity [30] (**Figure 3**).

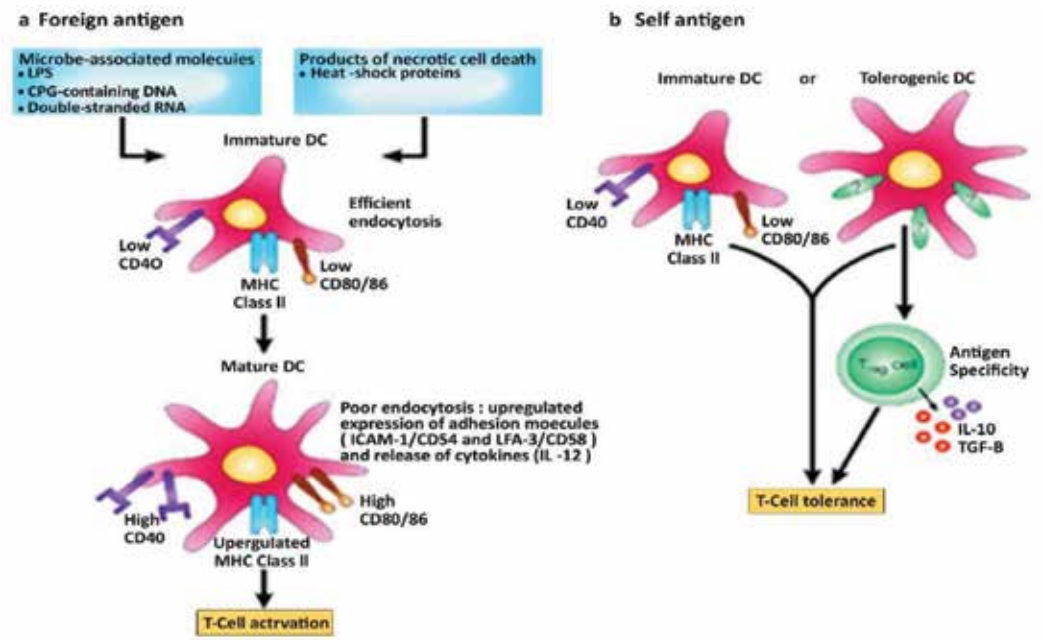

Figure 3. Dendritic cells in the choice between immunity and tolerance.

Although the general state of knowledge considers cDCs as inducers of immunity, while pDCs serve as the inducer of tolerance [31], their functions in the immune response to a diverse range of antigens are more complex.

pDCs are believed to be the critical effector cells in the early antiviral innate immune response by providing large quantities of type I interferons upon viral infection. pDCs increase immune responses by cross-talking with cDCs by the secretion of IFN-α, through performing a crucial role in active stimulation of adaptive immunity as well. In the interest to IFN-α secretion, it has been described that pDCs also express CD40L, which stimulates cDCs to secrete IL-12 [32] (**Figure 4**).

An association between the appearance and deficiency of multiple surface markers has been employed to identify DC subsets. These include the presence of significant expression of class II MHC antigens and the insufficiency of several progenitors' markers such as CD3 (T cell marker), CD14 (monocyte marker), CD19 (B cell marker), CD56 (natural killer cell marker) and CD66b (granulocyte marker). DCs further express a modification of adhesion molecules including CD11a (LFA-1), CD11c, CD50 (ICAM-2), CD54 (ICAM-1), CD58 (LFA-3), and CD102 (ICAM-3). DCs also represent costimulatory molecules including CD80 (B7.1), and CD86 (B7.2), which are upregulated through DC activation. CD86 designates to be a marker of primary DC maturation, while CD80 only increases in mature DC. Two additional markers of mature DC in humans are CD83 and CMRF-44. CD83 also is exposed by stimulated B cells, and CMRF-44 will also be exposed by macrophages and monocytes [33].

The identification of DCs by surface phenotyping may be accomplished by merely demonstrating a high level of MHC class II or a costimulatory molecule such as CD80 and the absence of lineage markers [34].

The conventional or myeloid DCs (cDCs) are characterized by a high exhibit of the phenotype LIN-CD11c and low HLA-DR + CD123, while plasmacytoid DCs (pDCs), derived from a lymphoid precursor, manifest low expression of the phenotype LIN-CD11c and high HLA-DR + CD123 [35]. The maturation state of DCs can categorize DCs. Immature DCs are located mainly in peripheral tissues, where they capture antigens, initiate their maturation and migrate to lymphoid organs, where they become mature to present antigen and stimulate naive T lymphocytes [36].

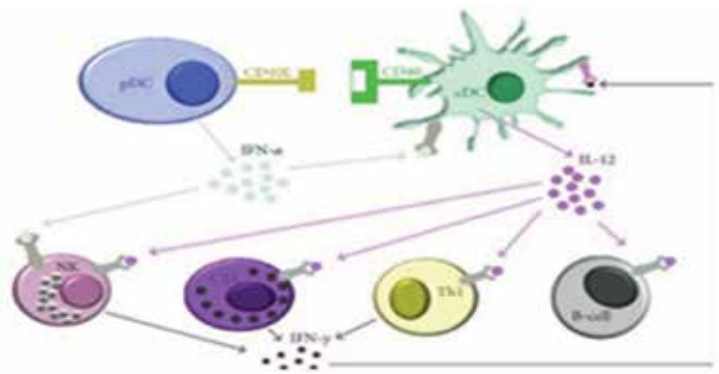

Figure 4. Cooperative action of different DC subsets to tackle both innate and adaptive immunity.

Buckley et al. [37] revealed that macrophages and DCs are positioned in the same splenic anatomical sections and yield monocyte-macrophage markers, proposing that both cell classes are relevant and probably originated from a familiar precursor. Vandenabeele et al. [38] illustrated in human thymus main classes of DCs, showing the low of the phenotype CD11b-CD11 + CD45RO, great CD83, CD86, HLA-DR and fewer DCs with high CD11b + CD11c, CD45RO population. They also recorded the appearance of pDCs with great CD123 in the thymic cortex. The role of DCs is tightly correlated to their anatomical location. In secondary lymphoid tissues, mature DCs present antigens, caught in the periphery, to naive T cells and produce immunity, while in the thymus DCs present self-antigens, produce negative determination of autoreactive T cells and improve the positive selection of regulatory T cells [39].

DCs can be generated via culturing CD34+ cells in the presence of several cytokines. One procedure which has been developed includes depleting the CD34+ cells of differentiated ancestors and next culture the cells in the presence of GM-CSF and IL-4 ± TNF-α. CD34+ cells can be collected from bone marrow or cord blood. Further procedure is to generate DC-like cells by culturing CD14+ monocyte-enriched peripheral blood mononuclear cells [40]. In the presence of GM-CSF and IL-4, these cultures lead to large numbers of DC like cells. These monocyte-derived DCs require additional conditioning *in vitro* with either TNF-α or lipopolysaccharides added to culture media to enable adequately function as a DC accomplished of preparing antigen-specific T cell responses [41].

Because of the established role of DCs in maintaining the balance between immunity and tolerance, tolerogenic (tol)DCs might be novel therapeutic targets to prevent undesirable (auto-) immune responses. The idea behind tolDC therapy is that it is a highly targeted, antigen-specific treatment that only affects the auto-reactive inflammatory response [42]. A tolerogenic state in DCs can be induced using several pharmacological agents, such as cyclosporine A, rapamycin, dexamethasone, vitamin A, vitamin D or other cytokines and growth factors [43].

Isolation and culture of leukocytes (buffy coats) obtained from heparinized human peripheral blood provide a valuable model for studies on DCs biology and may help uncover new means to manipulate DCs differentiation and function in therapeutic settings [44]. The buffy coat layer from human peripheral blood was cultured in the presence of GM-CSF and IL-4 to generate dendritic cell populations which were allowed to differentiate into mature DCs by TNF-α within 9 days [45] (**Figure 5**).

The *in vitro* effect of dexamethasone (DEX) on generation and differentiation of DCs through microscopic and phenotypic analysis was studied. The addition of DEX to the culture on day 0 prevented the differentiation of DCs to be tolerogenic. On the other hand, addition of DEX to the culture on day 7 or 8, either preceded or followed by addition of TNF-α, resulted in significant increase of CD83 expressing DCs; the greatest percent of tolerogenic DCs was obtained in the culture media to which DEX (1 μM) was added on day 8 and TNF-α (10 ng mLG1) was added on day 7. Although the addition of TNF-α to the culture 1 day prior to addition of DEX enhanced the differentiation of DCs (high percent of CD83 expressing DCs), TNF-α did not affect the morphological changes of DCs which became mature even in the absence of TNF-α. Opposite studies were reported that TNF-α is a maturation factor essential for the appearance of the morphological characteristics of DCs [46].

CD83 is an important marker for activated/mature DCs. It was recorded that both stimulated DCs and B cells secrete soluble form of CD83 and so low concentration of soluble CD83 are present in normal human sera [47]. The CD83 seems to possess regulatory roles for immune response. The soluble form of CD83 can repress immune responses, while being strongly up-regulated during DCs maturation and activation [48].

Fujimoto and Tedder (2006) revealed that CD83 has immunosuppressive roles such as the inhibition of surface molecules, such as MHC-II, reducing the dendritic cell-mediated T cell stimulation. The allogeneic stimulatory capacity of the DCs and immunosuppressant mechanisms of CD83 were illustrated significantly inhibiting anti-donor antibody responses [49]. The study of Ge et al. [50] reported that CD83 is capable of down-modulating expression of various DC [50]. The elevated CD83 expression suggests the possibility of DEX-generated cells to initiate a Th2-biased response where CD83 is able to inhibit DCs mediated T cells stimulation [51]. Furthermore, dexamethasone treated DCs possessed the capacity to convert CD4+ T cells into IL-10-secreting Treg potently suppressing the proliferation of responder T cells [52].

The CD83 is a surface marker that distinguishes immature and mature human dendritic cell populations. The CD83 is type 1 glycoprotein belonging to the immunoglobulin superfamily

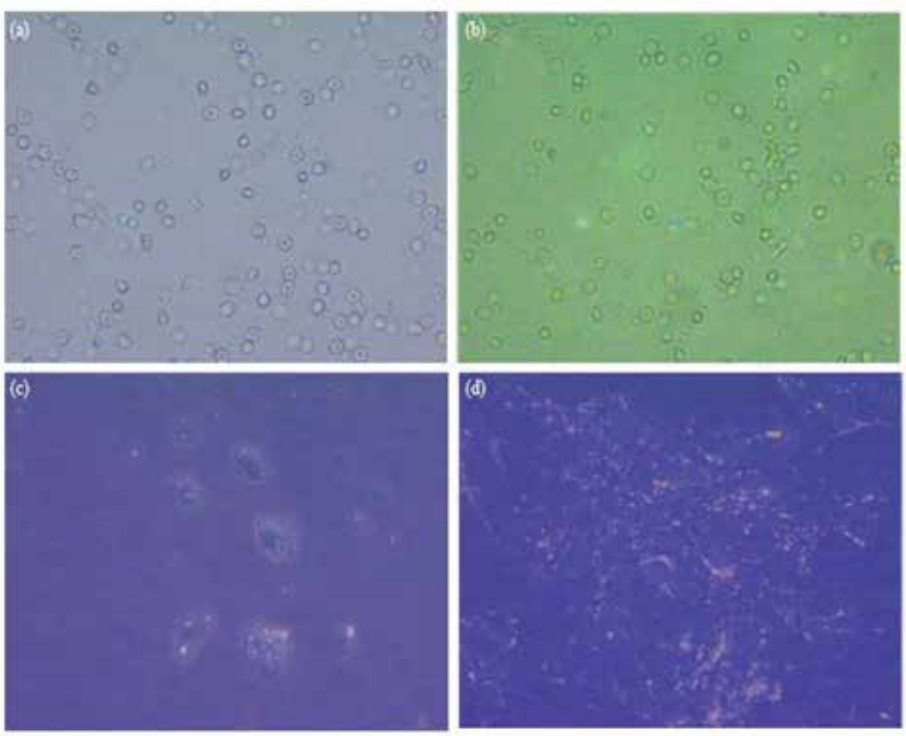

Figure 5. Morphological changes during generation and differentiation of DCs (40×), (a) adherent monocytes on day 0, (b) transforming monocytes on day 3, (c) generated DCs on day 7 and (d) mature DCs on day 9. The culture of buffy coat layer from human peripheral blood leads to generation of dendritic cell populations that in response to granulocyte-macrophage colony-stimulating factor (GM-CSF) plus interleukin-4 (IL-4) in 7 days and differentiate into mature DCs in response to maturation stimulus tumor necrosis factor-α (TNF-α). Morphological changes were examined under inverted microscope Carl Zeiss® using ZEN 2012® software, Germany.

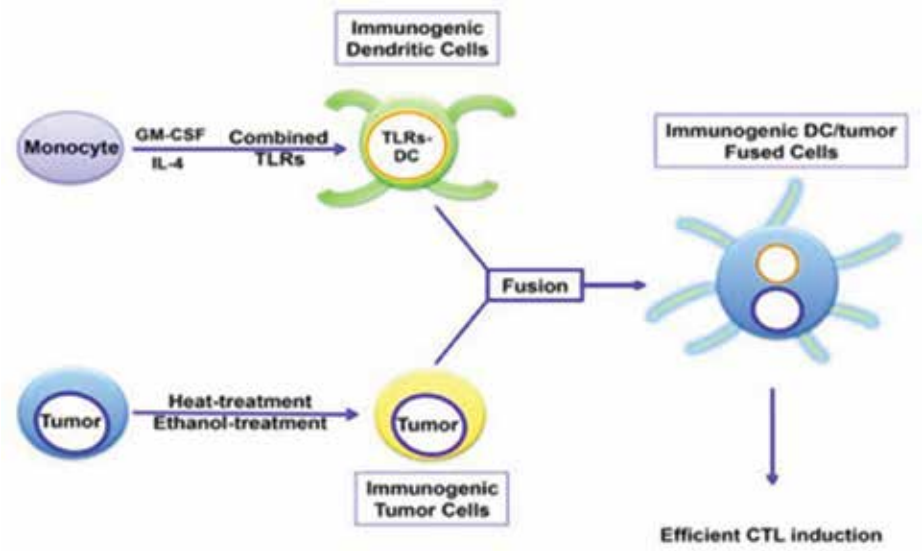

Figure 6. Generation of immunogenic cancer cells fused to activated dendritic cells.

and has been known to be one of the best markers. There is an outstanding deal of attention in how DCs might be developed as a manner of immunotherapy. DCs are being examined as adjuvants for vaccines or as a principal therapy to aggravate immunity against cancer. That DCs may show valuable in cancer has been most often studied in animal models. DCs burdened with tumor lysates, tumor antigen-derived peptides, MHC class I modified peptides, or whole protein have all been shown to yield anti-cancer immune responses and actions, including in some cases the ability to begin broad relapse of existing tumor [53] (**Figure 6**).

In conclusion, there is a pronounced hope to study these strategies and use tumor-antigen bearing DCs as a vaccine in humans. Human clinical investigations are continuing in numerous institutions to use DCs to initiate immunity to antigens against breast cancer, lung cancer, melanoma, prostate and renal cell cancers [54]. The study of immune-mediated mechanisms could be of value in avoiding and managing main immune disorders [45].

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Chapter 3

Highlighting the Role of DC-NK Cell Interplay in Immunobiology and Immunotherapy

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Additional information is available at the end of the chapter

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Abstract

Dendritic cells (DCs) and natural killer (NK) cells are both part of the innate immune system, also playing crucial functions in the regulation of adaptive immune responses. In recent years, numerous works have demonstrated that DCs and NK cells mutually influence each other with major consequences in the type and effectiveness of elicited immune responses. Among other effects, DC-NK crosstalk can result in NK cell activation and DC maturation or deletion, depending on its activation status. In this chapter and after a brief overview of DCs and NK immunobiology, we focus on the process of DC-NK crosstalk, highlighting the relevance of rationally exploring this interplay in the development of more effective cancer immunotherapies.

Keywords: dendritic cells, natural killer cells, DC-NK crosstalk, cancer, immunotherapy

1. Introduction

Dendritic cells (DCs) are a heterogeneous population of innate immune cells with unique capacity to process and present antigens to naïve T cells. They are, therefore, responsible for the orchestration of the adaptive immune responses, promoting either immunity or tolerance to self-antigens [1]. Given these unique characteristics, DCs have for long been used in clinical approaches, particularly to boost antitumor immune response during cancer treatment [2].

The results gathered from more than 20 years of experimentation and almost 350 clinical trials demonstrated that DC-based antitumor immunotherapy is safe and with relevant clinical outcomes [3].

One of the key observations from these experiments is that the success of DC-based vaccines relies not only on the capacity of these cells to polarize and activate T lymphocytes but also on their ability to bidirectionally interact with natural killer (NK) cells. In fact, it is known that an optimal antitumor immune response depends on a complex interplay between $CD8^+$ T cells, $CD4^+$ T cells and NK cells [4]. NK cells are large granular innate immune cells with cytotoxic functions. They are crucial for the initial defense against viral infections, destroying infected cells and also for the elimination of tumor cells or foreign and endogenous cells under stress [5]. The complex cell-to-cell crosstalk between DCs and NK cells has major consequences on the modulation of immune responses. Therefore, it is expected that the rational design of new DC-based immunotherapies encompasses this required interplay with NK cells in order to synergistically evoke a superior clinical outcome [6].

In this chapter, we focus on several key aspects: the DC and NK immunobiology, the mechanisms and consequences of DC-NK bidirectional crosstalk, and the potential and relevance of DC-NK crosstalk in cancer immunotherapy.

2. Dendritic cells

2.1. Dendritic cells at a glance

DCs were first described by Paul Langerhans in 1868, being erroneously characterized as part of peripheral nervous system. It took almost 100 years to reveal the real functions of these cells. In 1973 and subsequent years, Ralph Steinman and Zanvil Cohn demonstrated by a series of elegant experiments that DCs are crucial regulators of both innate and acquired arms of the immune system [7, 8]. These cells have the unparalleled ability to polarize naïve T lymphocytes into their different effector or regulatory subsets and are potent enhancers of NK cell cytotoxicity [9, 10] as well as fundamental accessory cells in the production of primary antibody responses [11]. They also take part in the preservation of tolerance to antigens, with thymus DCs helping in the shaping of the T cell repertoire through the deletion of autoreactive lymphocytes [12, 13].

Classically, DCs are found in an immature state at locations of possible antigen entry such as the skin and mucosal surfaces (e.g., genitourinary, gastrointestinal, and respiratory systems), as well as in the connective tissue of all solid organs and even in fat tissue, retina, and brain [8, 14–18]. Upon encountering a potential threat, DCs capture and process it, displaying the resultant antigens on major histocompatibility complex (MHC)-I or MHC-II molecules. During this process, DCs engage a program, termed maturation, characterized by several morphological, phenotypical and functional changes that strongly increase their immunogenic profile. Then, mature DCs enter draining lymphatic vessels and migrate to lymph nodes where they present processed antigens to naïve T lymphocytes, generating a specific immune response [19].

2.2. Dendritic cell subsets and characteristics

DCs are composed of a very heterogeneous family of innate immune cells with characteristics frequently overlapping between subpopulations, making hard to define an unambiguous classification. Hence, in 2014, a novel classification system was created, first centered on DC ontogeny and then on their location, function, and phenotype [20].

Concerning ontogeny, all DC subpopulations are derived from a common hematopoietic CD34+ stem cell precursor that originates multiple intermediate precursors, which differentiate into several DC subsets in a process that is highly dependent on hematopoietic cytokines and growth factors [8, 14, 21]. There are three major subpopulations of DCs: plasmacytoid DCs (pDCs) characterized by the expression of CD123, CD303, CD304, FCER1, ILT3, and ILT7; classical DCs 1 (cDC1) that express CD141, CLEC9A, XCR1, and CADM1; and classical DCs 2 expressing CD1c, CD11c, CD11b, CD2, FCER1, SIRPA, ILT1, and CLEC4A [2, 22, 23].

cDCs1 are 10 times less frequent than cDCs2 and can be found in the blood, lymph nodes, tonsil, spleen, bone marrow, and in non-lymphoid tissues such as skin, lung, intestine, and liver. Functionally, they are characterized by a high capacity to cross-present antigens via MHC class I to $CD8^+$T cells, promoting their activation to cytotoxic T lymphocytes (CTLs) [24]. Although they secrete low amounts of IL-12 when compared to cDCs2 [25, 26], they are highly effective in promoting Th1 cell polarization and NK cell activation. This is in part due to the expression of the chemokine receptor XCR1, which enables cDCs1 to closely interact with XCL-producing cells such as activated T lymphocytes and NK cells [25–28]. cDCs1 are particularly equipped for the recognition of viral and intracellular antigens: they express TLR3, TLR9, and TLR10, also being major producers of type III interferons IFN λ 1–3 [29].

Regarding myeloid cDCs2, they are the major DC population being found in blood, spleen, skin, lung, and intestine. They express a large panel of pattern recognition receptors (PRRs) namely TLRs 2, 4, 5, 6, and 8, NOD-like receptors (NOD2, NLRP1, NLRP3, and NAIP), and lectin receptors such as DEC205, CLEC4A, CLEC6A, CLEC7A, CLEC10A, CLEC12A, and also the asialoglycoprotein receptor. Once activated, cDCs2 produce high amounts of IL-12 and also secrete IL-1, IL-6, IL-8, IL-10, IL-23, and tumor necrosis factor-alpha (TNF-α) [25, 30]. The different blood and tissue resident cDCs2 orchestrate a wide range of immune responses against viral, bacterial, fungal, and helminthic infections as a consequence of their capacity to polarize naïve T cells toward Th1, Th2, Th17, Th22, and CTL effector populations [30–32].

pDCs present a further limited distribution, being mainly found in blood and T cell areas of lymphoid organs. This DC subset is specifically tailored to sense and respond to viral infections [2, 8, 14, 33]. Indeed, pDCs highly express TLR7 and TLR9, the sensors for single-stranded RNA and double-stranded DNA, respectively [34], and upon activation, they produce high quantities of type I and III interferons, TNF-α, IL-6, and granzyme B [35]. Due to their intrinsic capacity to cross-present antigens to $CD8^+$ T cells, they also play a relevant role in antitumor immunity [36].

Finally, Langerhans cells, the main DCs found in the skin epidermis, are CD45+, MHC-II+, positive for Langerin, and have a low expression of CD11c and a high expression of CD1a.

They are exclusively derived from embryonic precursors and are able to self-renew locally [37]. At a functional level, Langerhans cells can induce immunogenic or tolerogenic responses upon specific maturation stimuli and cellular microenvironment and are particularly effective in antigen cross-presentation [38].

The phenotypical and functional characterization of human DC subsets is an exciting area that remains in continuous evolution. It is conceivable that this increased knowledge in DC immunobiology will empower their use in the design of more rational and effective immunotherapies.

3. NK cells

3.1. NK cell immunobiology

NK cells represent 5–15% of the circulating lymphocytes and play a pivotal role in host defense against pathogens and cancer [5]. Although recognized for their spontaneous killing ability of virus-infected or transformed cells without prior immunization, these cells also play an important role in the modulation of immune responses through the secretion of multiple cytokines and chemokines [39]. Additionally, a growing body of evidence suggests that NK cells can mediate antigen-specific immunological memory, associated with adaptive immunity [40].

NK cell activity is tightly regulated by a complex array of germline-encoded activating and inhibitory receptors randomly generated during NK cell differentiation and maturation [41]. The integration of the signals transmitted by these receptors forms the basis of NK cell reactivity to their targets and determines the magnitude of NK cell–mediated cytotoxicity and cytokine production. The inhibitory receptors such as killer immunoglobulin-like receptors (KIRs) and the lectin-like CD94/NKG2A heterodimer recognize self-molecules of the major histocompatibility complex (MHC) class I expressed in almost all healthy cell types and protect themselves from NK cell-mediated killing [42]. Paradoxically, the engagement of MHC class I molecules maintains NK cells in a state of responsiveness to subsequent activation, a property referred to as NK cell licensing [43]. During the course of tumorigenesis or viral infection, cells often decrease or even lose the expression of MHC class I molecules and upregulate the expression of a wide spectrum of stress-induced surface ligands that are recognized by activating receptors in NK cells, including the natural cytotoxic receptors (NCRs: NKp46, NKp30, and NKp44), the C-type lectin receptors (CD94/NKG2C, NKG2D, NKG2E/H, and NKG2F), DNAM-1, and killer immunoglobulin-like receptors with a short cytoplasmic tail (KIRs, KIR-2DS, and KIR-3DS) [41, 44]. When multiple of these activating receptors are simultaneously engaged and reach a threshold that surpass the inhibitory signals, NK cells are shifted to an activated phenotype, exerting their cytolytic activity against target cells [41, 45]. Several cytokines have been described to activate and promote NK cells antitumor activity. The common gamma chain (γc) family of cytokines, like IL-2, IL-12, IL-15, IL-18 and IL-21, are the most well-recognized ILs to boost NK cell antitumor activities and have been used to improve the proliferation, differentiation and effector function of NK cells [46].

Regarding their cytolytic activity, the perforin/granzyme pathway is the main mechanism used by NK cells to kill target cells. Upon activation, NK cells polarize the lytic granules to

the immunological synapse formed with the target cell and release the membrane disrupting perforin that forms transient pores on the target cell membrane, allowing the entrance of granzymes, a family of serine proteases, that trigger an apoptotic-like cell death [47]. The death receptor pathways involving FasL and TRAIL are also employed by NK cells on target cell-induced apoptosis through a perforin-independent mechanism. Death receptor members of the TNF-α family such as FAS and the death receptor 5 (DR5) are usually upregulated in tumor cells and transduce apoptotic signals upon binding to their cognate ligands FasL and TRAIL on NK cells, resulting in a classical caspase-dependent apoptosis [48]. NK cells are also mediators of the antibody-dependent cellular cytotoxicity (ADCC), another type of granule-mediated cell death that occurs when the Fc receptor expressed by NK cells (FcγRIII or CD16) binds to the Fc portion of IgG1 antibodies-coated target cells [49]. This interaction results in a strong activation signal that overcomes the inhibitory signals, leading to a downstream cascade of activation events with the release of cytolytic granules and inflammatory mediators.

In addition to the direct cytotoxic mechanisms, NK cells also act as immunomodulatory cells engaged in reciprocal interactions with DCs, macrophages, and T cells through the release of various cytokines, chemokines and growth factors, which might augment or dampen immune responses [50].

4. DC-NK cell interplay

Currently, it is clear that DCs and NK cells have a crucial role in modulating innate and adaptive immune responses through a complex cell-to-cell crosstalk (**Figure 1**). Indeed, DC-mediated activation of NK cells contributes to the development of potent innate immunity, whereas, in turn, activated NK cells provide signals for DC activation, maturation, and cytokine production, promoting adaptive immunity [6]. This DC-NK crosstalk occurs, *in vivo*, in the lymph nodes [51, 52], at the sites of inflammation, in peripheral tissues such as the skin and mucosa [53] and in solid tumor microenvironments [54].

4.1. Activation of NK cells by DCs

Several studies have demonstrated the potential of DCs to influence the function of NK cells. Seminal works revealed a reduction of NK cell-dependent antitumor effects in mice depleted from CD8α DCs, suggesting a direct role of DCs in NK cells activation [55]. The triggering of NK cells by DCs seems to be dependent on both cell-to-cell contact and soluble factors. Accordingly, in human and animal studies, NK cells activation by DCs was significantly disrupted by transwell separation, reinforcing a major contribution of cell-to-cell contact to this close communication [55, 56]. Further studies have demonstrated that DC-produced IL-12 is also crucial for NK cells activation, namely for their production of IFN-γ [57]. This process comprises the formation of stimulatory synapses between DCs and NK cells, which promote the polarized secretion of pre-assembled stores of IL-12 by DCs toward NK cells [58]. Furthermore, Poly(I:C)-treated DCs and IFN-α-treated DCs also induce NK cells to secrete IFN-γ by the binding of activating NK cell receptor NKG2D to its specific ligands, such as MHC class I-related chains A and B (MICA/B) [59, 60]. Another relevant interaction that results in NK cell cytotoxicity and IFN-γ release occurs between CXC3CL1 expressed on DCs and CXC3CR1 on NK cells [61].

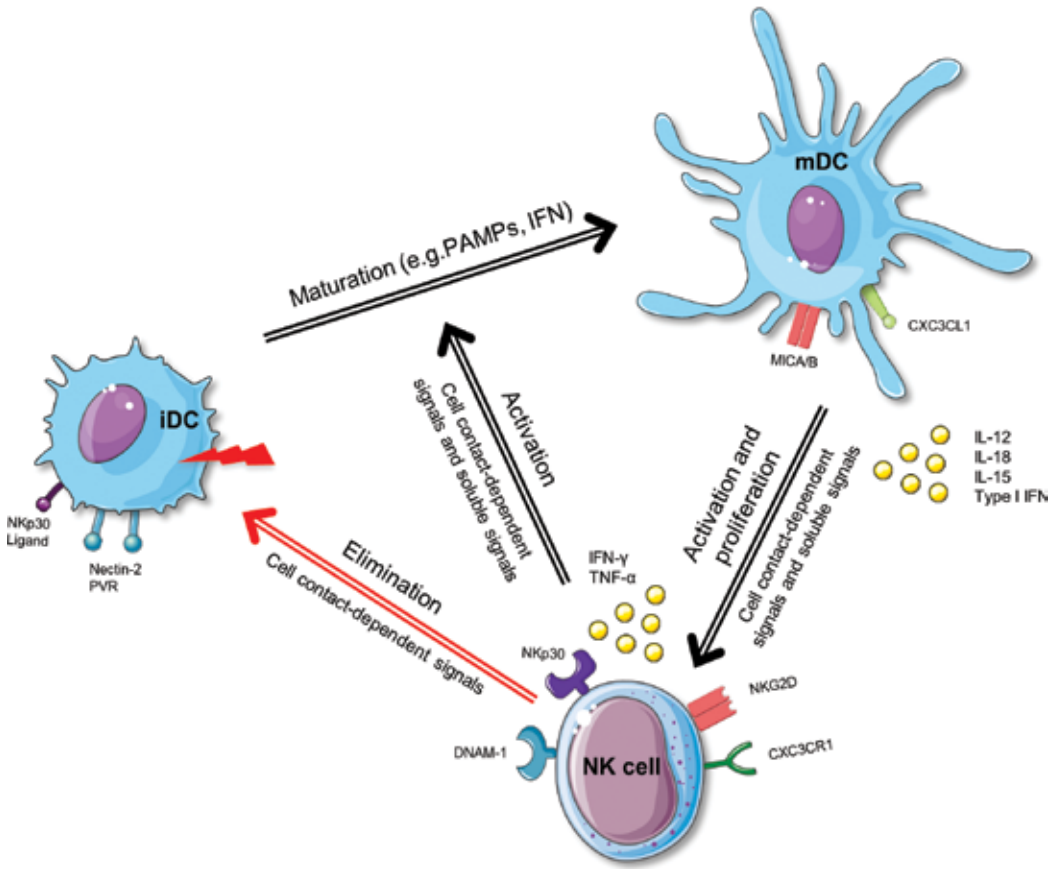

Figure 1. Dendritic cell-natural killer cell interplay. iDCs can undergo maturation by exposure to several stimuli, pathogen-associated molecular patterns (PAMPs), and IFN. The resulting mDCs secrete IL-12, IL-18, IL-15 and type I IFN, which in turn induce the proliferation and activation of NK cells, leading to further secretion of IFN-γ. NK-DC interaction by NKG2D with MICA/B and CXC3CR1 with CXC3CL1 can also lead to NK cell activation. Thus, activated NK cells are able to increase DC maturation, dependent on cytokines like IFN-γ and TNF-α, as well as the interaction of NKp30 receptor on NK cells with its ligand on DCs. On the other hand, activated NK cells can also eliminate MHC-I low-expressing iDCs by cell contact-dependent interactions of NKp30 receptor with NKp30 ligand and DNAM-1 with Nectin-2 or PVR. iDCs, immature dendritic cells; IFN, interferon; mDCs, mature dendritic cells.

IL-15 can be considered as an additional and important cytokine involved in the process of DC-mediated activation of NK cells. This cytokine can be found bounded to DCs membrane and is able to induce NK cell proliferation, survival, and to enhance their cytotoxic functions [57, 62]. Additionally, in DCs, transmembrane TNF, as well as membrane-bound IL-15, can enhance NK cells proliferation, CD69 expression, and IFN-γ secretion [63]. Both signaling mechanisms are mediated by cell-to-cell contact via simultaneous engagement of DCs transmembrane TNF and membrane-bound IL-15, with their respective NK cell receptors, leading to its activation. Furthermore, NK cells proliferation is also dependent on the interaction between CD40 and B7 molecules on DCs with CD40L and CD28 on NK cells, respectively [64].

IL-18 expressed by immature and mature DCs has also been implicated in NK cells activation. DC-derived IL-18, as well as IL-12, is involved in the upregulation of NK cell cytotoxicity [56].

Similar to IL-12, IL-18 seems to be delivered in secretory lysosomes at the NK/DC synaptic cleft, leading to NK cells activation [65]. In case of regulatory DCs, their insufficient production of IL-18 is involved in the restrained IFN-γ secretion by NK cells, downregulating NK cells activation [66]. In addition, studies performed in mice revealed that both IL-18 and IL-12 are also involved in the expansion of $Ly49H^+$ NK cells promoted by $CD8\alpha^+$ DCs [67].

The role of type I IFN (IFN-α/β) on NK cell activation has also been assessed during NK-DCs crosstalk. Type I IFN, secreted by plasmacytoid DCs [68], is required for NK cell cytotoxicity in response to virus infection [69, 70]. In the context of TLR stimulation, NK cells priming is dependent on the recognition of type I IFN signals by DCs and on the subsequent production and trans-presentation of IL-15 by DCs to resting NK cells [71]. Finally, IL-2 produced by bacterially activated myeloid DCs has also been shown to be required, both *in vitro* and *in vivo,* for NK cells activation and IFN-γ-efficient production [72].

4.2. DC modulation by activated NK cells

Over the last two decades, multiple studies have reported that NK cells play a relevant role in the DCs maturation process, either by killing DCs that did not properly acquire a mature phenotype or through direct DCs stimulation [73]. The process of immature DC lysis is dependent on NK-activating receptors as well as on the amount of MHC class I molecules on DCs [74, 75]. *In vitro* assays have demonstrated that activated NK cells can recognize and kill DCs via NKp30 natural cytotoxicity receptor, suggesting the expression of a still unknown NKp30 ligand on DCs surface. Mature DCs are susceptible to NK cell killing when NK inhibitory signal is blocked by MCH-I antibodies, confirming that mature DCs are naturally spared due to their high expression of MHC class I molecules [76]. In addition, DNAM-1-triggering receptor and its ligands, poliovirus receptor (PVR) and Nectin-2, have been demonstrated to be crucial in NK cell-mediated lysis of immature DCs. DNAM-1 receptor on NK cells cooperates with NKp30 receptor in the NK-mediated elimination of DCs. The degree of contribution of DNAM-1 appeared to correlate with the surface amount of its specific ligands PVR and Nectin-2 on DCs [77]. Other *in vivo* studies have also shown that NK cells efficiently kill injected immature bone marrow-derived DCs, via a pathway dependent on the TNF-related apoptosis-inducing ligand (TRAIL) [78]. Similarly, another study confirmed that NK cells can kill incompletely matured DCs in the context of a viral infection via TRAIL—Death Receptor 4 (DR4) pathway [79]. These findings led to the hypothesis that the killing of immature DCs by NK cells should promote the survival of the most immunogenic DCs, supporting and empowering efficient and protective immune responses. In fact, it has been demonstrated that the killing of immature DCs by autologous NK cells is particularly important for the expansion of cancer-specific CTLs [80].

On the other hand, in chronic viral infections, IL-10 produced by NK cells induces contrasting phenotypic changes in DCs; specifically, immature DCs exhibit aberrant resistance to NK cell-mediated elimination, whereas mature DCs had an increased susceptibility to NKG2D-dependent elimination. This process leads to the accumulation of poorly immunogenic DCs in lymph nodes, causing a progressive immune dysfunction [81]. Furthermore, DC lysis by NK cells can also negatively regulate the duration and effectiveness of virus-specific T cell responses *in vivo* by limiting the exposure of T cells to infected antigen-presenting cells, which

negatively impacts the quality of T cell responses and their ability to limit viral persistence [82]. Additionally, it has been shown that in solid organ transplantation, host NK cells kill allogeneic DCs via the perforin pathway. This will limit allogeneic antigen presentation to host lymphocytes, reducing T cell–mediated graft-versus-host disease [83].

Activated NK cells can also improve DCs maturation and activation, enhancing their ability to stimulate T cell responses. When NK cells are cultured with immature DCs in the presence of maturation stimuli, such as lipopolysaccharide (LPS), they strongly enhance DCs maturation, specifically by upregulating the DCs co-stimulatory molecule CD86 and IL-12 production. NK cells activated by IL-2 are also able to induce DCs maturation, improving their ability to stimulate allogeneic naïve $CD4^+$ T cells. These effects of NK cells on DCs maturation are cell contact dependent, although the secretion of IFN-γ and TNF-α is also relevant [84, 85]. These findings show that both effects of NK cells on DCs (DC killing and stimulation) are dependent on NKp30-triggering receptor.

Further studies have shown that NK cell-activated DCs produce higher levels of IL12p70 after subsequent CD40 ligands stimulation, leading to an increase in the induction of T cell responses [86, 87]. The effect of NK cells on DCs is also dependent on the type of NK cell activation; IL-2-primed "effector" NK cells can kill DCs, whereas IL-18-primed NK cells are just prone to enhance the ability of DCs to produce IL-12p70 dependent on CD40L stimulation [88]. In fact, NK cells are able to trigger immature DCs to secrete IL-18 through a $Ca2^+$-dependent and tubulin-mediated recruitment of IL-18-containing secretory lysosomes toward the adhering NK cell. Then, IL18-activated NK cells secrete the pro-inflammatory "danger signal" high-mobility group B1 (HMGB1), which induces DC maturation and protects DCs from lysis, thus favoring the development of adaptive immune responses [65]. Furthermore, human NK cells, exposed to different cytokines, are able to promote distinct pathways of Th1 priming. Specifically, IL-12- or IL-2-activated NK cells induce maturation of DCs capable of priming IFN-γ-producing Th1 cells, whereas IL-18-conditioned NK cells induce Th1 polarization only when co-cultured with both DCs and T cells, which release IL-12 and IL-2, respectively, promoting IFN-γ production. Thus, the local prevalence of IL-12, IL-2, and IL-18 at the inflammatory sites may differentially modulate the NK cell interaction with DCs, leading to different outcomes in naïve T cell polarization [89].

Recently, IL-23 was uncovered as an enhancer of NK cell ability to stimulate DCs. IL-23 induces NK cells activation and displays a synergistic effect with IL-18 for IFN-γ production by NK cells. This cytokine also potentiates the increase of CD86 expression and IL-12 secretion by LPS-treated DCs upon IL-18-stimulated NK cells contact [90].

5. DCs and NK cells in cancer immunotherapy

The main purpose of cancer immunotherapy is to change the balance from tumor escape or equilibrium to cancer cells elimination. Latest developments have been focused at increasing the activation status of the innate and adaptive immune systems, comprising cytokine administration, Car T cells, DCs and NK-based vaccines, checkpoint inhibitors and monoclonal

antibodies engineered to target high-yield elements in oncogenic-signaling pathways [91]. A crucial point for the development of such approaches was the definition of the optimal characteristics of an antitumor immune response. Specifically, it became evident that this response depends on a complex cells interplay involving DCs, $CD8^+$ T cells, $CD4^+$ T cells, and NK cells.

Given their positioning at the interface between innate and adaptive immunities and their unparalleled capacity to interact and modulate immune effector cells, DCs have been scrutinized and settled as highly desirable and full of translational and clinical potential. Since the 1990s, DCs have been used in more than 350 clinical trials as cellular antitumor vaccines [3]. Currently, there are three approaches exploring DCs in oncologic treatments: (1) non-targeted protein and nucleic acids-based vaccines captured by DCs *in vivo*; (2) direct targeting of antigens to DCs *in vivo*; and (3) vaccines composed of *ex vivo*-generated DCs matured and loaded with tumor antigens [19]. Notwithstanding the good safety profile of antitumor DC-based vaccines, the rate of success in inducing clear therapeutic outcomes is inconsistent [3]. Objective tumor responses are usually above 15% [92], and promising vaccines in early-phase studies [93, 94] often fail to present clinical benefits in pivotal phase III trials [95]. Differentiation of DCs *ex vivo* from blood monocytes followed by their injection back into the patient is by far the most common strategy [1, 8]. This approach suffers from some limitations: very few of the injected DCs migrate to the lymph nodes to present antigens to T cells, and it became evident that monocyte-derived DCs are functionally limited when compared to endogenous DCs subpopulations [96, 97]. *In vivo* targeting of antigens to specific DC subsets, tailoring of *ex vivo* differentiated DCs to particular phenotypes, and the combination of DCs-based vaccines with other antitumor therapies are critical steps for the effective success of new DCs immunotherapies [2–4, 92].

As referred in the earlier section, NK cells can directly eliminate tumor cells and indirectly enhance antitumor adaptive immunity by favoring DCs maturation and by killing immature DCs, thus enhancing immunogenic DC populations that will polarize antigen-specific CTLs [76, 98]. Importantly, the cell debris resultant from NK tumor cell destruction is also an important source of multiple tumor antigens for DCs cross-presentation to $CD8^+$ T cells [99]. Due to these characteristics, NK cells have been clinically explored in recent years in several immunotherapeutic strategies for cancer. There are mainly two therapeutic approaches based on NK cells: the use of NK cell stimulants/modulators to take advantage of endogenous responses and the adoptive cell transfers of fully differentiated and *ex vivo*-activated NK cells [100, 101]. The adoptive cell transfers of autologous NK cells have been tested and well tolerated in the treatment of several cancers, including glioma, lymphoma, and renal cell carcinoma, though clinical responses have not always been observed [102, 103].

6. Future perspectives

In parallel with the growing knowledge on immune cells and cancer biology, cell-based immunotherapies must be tailored to answer the new demands. Whereas initial research focused on generating mainly tumor CTLs responses, it becomes clear that the activation of multiple immune effector cells is the key to success for curative cancer vaccination.

Apart from CTL induction, DC-NK cell crosstalk is of major importance in antitumor immune responses [4]. Efficient DC-vaccine-mediated antitumor immunity has been shown to be strongly dependent on NK cell activity [6]. This was highlighted by experiments where NK cell depletion drastically impacted tumor elimination following DC vaccination [104, 105]. Moreover, data from several clinical trials on DC-based antitumor vaccines indicate that positive outcomes seem to correlate with high levels of activated NK cells in responder patients [106].

Taken together, these data underscore why future research efforts should also focus on optimizing the NK cell–interacting properties of DC vaccines, in addition to improving their T cell–stimulatory capacity. The NK cell–activating character of DC vaccine preparations can be imprinted at multiple levels, such as by (1) tailoring the phenotype of *ex vivo* differentiated DCs, (2) using specific DCs subsets, and (3) targeting endogenous DCs populations that are intrinsically prone to interact with NK cells. The former approach includes the use of DCs expressing high levels of

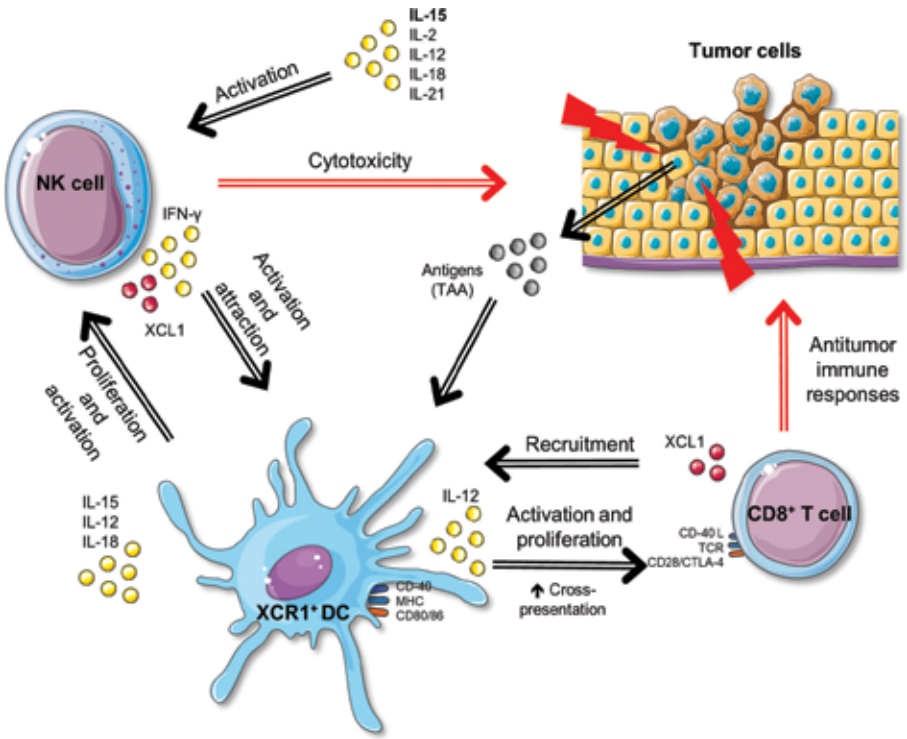

Figure 2. DC-NK cell crosstalk in cancer. NK cells can be activated by DCs, directly by cell contact and/or by DC-produced cytokines such as IL-12, IL-15 and IL18. Activated NK cells can then proliferate and secrete cytokines like IFN-γ, which will stimulate antitumor-acquired immune response. Specifically, IFN-γ induces CD8+ T cells to convert into antigen-specific CTLs and supports the creation of immunological memory against the tumor antigens. Additionally, IFN-γ can also stimulate the polarization of CD4+ T cells into Th1 subset that in turn stimulates CTL differentiation. Furthermore, activated NK cells, additionally to induce DCs maturation, will destroy tumor cells, fueling DCs with tumor antigens that are then cross-presented to CD8+ T cells. The cDCs1 population (CD141+ XCRI+) is particularly effective in this interplay: The expression of XCR1 receptor by this DC subset and of its ligand XCL1 by T and NK cells during infectious and inflammatory responses potentiates the interaction between these cells. Additionally, CD141+ XCRI+ DCs present an exceptional antigen cross-presentation capacity and are producers of high amounts of IL-12 following activation. CD-40 L, CD-40 ligand; IFN-γ, interferon γ; IL, interleukin; TAA, tumor-associated antigens; TCR, T cell receptor; XCL1, chemokine (C motif) ligand; XCR1, chemokine receptor for XCL1.

IL-12 and IL-15 [107] or manipulated to express the receptor XCR1. On the other hand, the other potential strategy is to target endogenous DCs subsets expressing XCR1. By using/targeting XCR1 expressing DCs, we potentiate their interaction with activated $CD8^+$T cells and NK cell, given that these are the main producers of the XCR1 ligand XCL1 (**Figure 2**) [26, 27, 108].

In conclusion, the design of new DC-based vaccination strategies should encompass NK cell-stimulating potency. Additionally, it would be of great value to systematically incorporate NK cells monitoring as an outcome in antitumor DC-based clinical trials.

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Conflict of interest

The authors declare no conflict of interest.

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Chapter 4

Role of Dendritic Cells in Parasitic Infections

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Abstract

Dendritic cells comprise a complex array of cell populations that play a leading role in immune defense. In an immature state, they have the capacity to sense and uptake different antigens. Upon capturing antigens, they become activated, mature, and migrate to lymph nodes where they present antigen-derived peptides to naïve T cells. Due to these excellent surveillance properties, dendritic cells play an important role against parasitic infections. Also, dendritic cells are an important source of IL-12, which is a fundamental proinflammatory cytokine in the control of intracellular parasites. The aim of this chapter is to review the most important characteristics and functions of dendritic cells and their role in the control of infection by parasites.

Keywords: dendritic cells, pattern recognition receptors, pathogen-associated molecular patterns, protozoan parasites

1. Introduction

1.1. Generalities of dendritic cells (DC)

Dendritic cells (DC) were discovered by Paul Langerhans in 1868 when he described dendritic, nonpigmentary cells in the epidermis that he considered intraepidermal receptors for extracutaneous signals of the nervous system [1]. Afterwards, Langerhans discovery fell into oblivion and almost 100 years elapsed until, in 1973, Steinman and Cohn described a cell population in the spleen of mice similar to the one described by Langerhans that had appearance and behavior different from monocytes and macrophages and were named as dendritic

cells [2]. They observed that this new cell population had a great capacity to initiate and modulate the immune response [3, 4] and expressed high levels of MHC-II and CD11c [5, 6].

Currently, DC are recognized as a heterogenous cell population whose members differ in ontogeny, anatomic localization, migration, and due to the great repertoire of functions they perform, make them key participants in the immune response. DC are localized in lymphoid and nonlymphoid tissues including blood. At the periphery, they capture antigens through the recognition of pathogen-associated molecular patterns (PAMPs) by PAMPs recognition receptors (PRRs), migrate, and transport them to lymphoid organs where they are specialized in antigen processing and efficiently present endogenous and exogenous antigen-derived peptides in both MHCI and MHCII contexts. DC also have the unique capacity of presenting exogenous noncytosolic antigen-derived peptides in the context of MHCI by cross presentation [7], a critical mechanism for the immune response against viruses and intracellular bacteria [8]. In addition to the primordial role of DC in antigen processing and presentation, their participation has been broadly documented, along with other immune cells, in the production of cytokines that modulate the immune response toward a Th1 or Th2 response, in the regulation of cytotoxic T lymphocytes, and in immunologic tolerance [9–15]. The crucial role of DCs in the initiation and regulation of adaptive immunity has led to their use in dendritic cell-targeted vaccination [16]. It has been documented that following loading with pathogenic antigens and adoptive transfer, DC mediate protection against a wide spectrum of infectious diseases [17–19]. However, it has been shown that the employment of *ex vivo* antigen-loaded DC for first-line prophylactic vaccination is not adequate. Targeting dendritic cells *in situ* through antigen-DC receptors has circumvented this obstacle. Indeed, this new strategy has proven to be effective against different infections, including parasitic infections [20] and can be explained by the facility of exposing antigens to DC and their regulated presentation pathways. The outcome of these studies emphasizes that targeted delivery of antigens to DC surface endocytosis receptors such as C-type lectins increases antibody and cell-mediated immunity [21].

Due to their complexity, the establishment of the origin of DC and their classification has encountered some difficulties; nevertheless, researchers have reached a consensus on these topics, which is discussed next.

1.2. Origin and subpopulations of DC

The origin of DC has been more precisely deciphered in the murine model as compared to humans. It has been established that during their differentiation, hematopoietic precursors $CD34^+$ in the bone marrow give rise to common myeloid progenitors (CMP) characterized for the expression of Lin^- $CXCR1^+$ $CD11b^-$ $cKit^+$ $CD135^+$ [22]. These CMP give rise to the common progenitor of monocytes and dendritic cells (MDP) [9, 15, 23, 24], which in turn originate the precursors of plasmacytoid DC (pre-pDC) and conventional DC (pre-cDC). These cells abandon the bone marrow to the circulation to later colonize the tissues as immature DC where they develop and differentiate to DC [23, 25–29]. It has been shown that the growth factor FMS-like tyrosine kinase 3 ligand (Flt3L) [30–32] is essential for the process of differentiation of mouse DC, while M-CSF and GM-CSF are indispensable for the development of progenitors, but not for their maturation [33].

In relation to the origin of human DC, for years it has been difficult to establish their ontogenic pattern. Nevertheless, the culturing of human hematopoietic stem cells $CD34^+$ performed by Lee and colleagues [34] shed important information about their origin. They showed that these precursors give rise to the human progenitor of granulocytes, monocytes, and DC (hGMDP), which in turn originate the progenitor of human DC and monocytes (hMDP). The hMDP gives rise to monocytes and the common progenitor of DC (hCDP). Differently from GMP (granulocytes and macrophages progenitor), hCDP are found not only in cord blood and bone marrow, but also in peripheral blood and lymphoid tissues and originate the different types of DC [34]. These cells are characterized for a high expression of MHCII, but lack the typical markers of CD3 lineage (T lymphocytes), CD19/20 (B lymphocytes), and CD56 (NK cells), reason why DC have been traditionally named as $HLA\text{-}DR^+$ $lineage^-$-cells. There are two principal subtypes of DC: conventional DC (cDC), also called myeloid DC (mDC), and plasmacytoid DC (pDC) [15].

1.2.1. Conventional (cDC) or myeloid DC (mDC)

cDC or mDC share an ontogenical origin with monocytes and macrophages, and thus, GM-CSF is essential for their differentiation *in vitro*. These cells are characterized for the expression of typical myeloid antigens such as CD11c, CD13, CD33, and CD11b. CD11c is also expressed in human cDC and monocytes, but cDC lack CD14 or CD16 and may be subdivided into cDC $CD1c^+$ and cDC $CD141^+$ fractions. These two fractions share homology with mouse classical DC expressing either CD11b ($CD1c^+$ DC) or CD8/CD103 ($CD141^+$ DCs) [35]. cDC are also characterized for the expression of different Toll-like receptors (TLR) such as TLR1, TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, and TLR10 [36]. Additionally, human mDC have been subdivided according to their anatomical localization into three groups: (1) peripheral tissue-resident, (2) secondary lymphoid organ-resident, and (3) circulating blood mDC [35]. In particular, peripheral tissue resident DC encompass different mDC subsets localized in human skin. These include Langerhans cells (LC) and dermal interstitial DC (intDC). The origin of these two cell populations is still controversial, since some authors suggest that the precursor is of myeloid origin and one of the intermediaries is the monocyte [37]. Contrarily, other authors have proposed that the origin of LC and intDC proceeds from a fetal progenitor that also originates the glia in the central nervous system [38]. LC are localized in the epidermis and express CD1a, langerin, and E-cadherin. intDCs are localized in the dermis and express DC-SIGN, CD11b, XIIIa factor, and CD14. Besides, these cell populations differ in the response to certain stimuli and production of cytokines and chemokines. For example, the stimulation with CD40L induces the production of IL-10 by intDCs, but not by LC. On the other hand, intDCs produce IL-6 and IL-12 that induce the differentiation of B cells toward IgM-producing plasma cells and stimulate Th cells for the production of Th1 cytokines, favoring this type of response. LC stimulate Th cells for the secretion of IL-4, IL-5, and IL-13, resulting in a Th2 response [35].

1.2.2. Plasmacytoid dendritic cells (pDC)

These cells owe their name to their similar appearance to plasma cells and to the fact that they proceed from a lymphoid progenitor, reason why they require IL-3 instead of GM-CSF for

their differentiation *in vitro*. pDC are characterized for the production of type I IFN in response to viral recognition through TLR7 and TLR9. Apart from these TLR, they also express TLR1 and TLR10 [36]. In mice, pDC are localized in bone marrow, blood, and lymphoid organs and are characterized for the expression of CD11c, CD45RA, CD317, and CD172 with a low expression of MHCII and negative for CD11b. They also show a significant expression of Ly6C, Ly49Q, and Siglec-H [39–41]. It has been shown that mouse pDC are not as efficient in antigen presentation and T cell priming as cDC, even when activated. Instead, murine pDC participate in peripheral tolerance through the induction of regulatory T cells (T regs), which has been proposed to occur by two mechanisms. In one case, it has been suggested that T regs are generated via the production of IDO and subsequent T cell tryptophan starvation upon CD200R engagement. On the other hand, the suggestion is that T regs generation is dependent on IL-10 and TGF-β production by pDC. Other studies have also demonstrated that pDC induce T cell anergy or deletion [42–44]. In relation to human pDC, it has been established that they are strong activators of T cells and share the capacity of their murine counterparts to induce tolerance. Some characteristic markers of human pDC are BDCA-2 (CD303), BDCA-4 (CD304), $CD123^{hi}$, and $CD1c^{low}$ (BDAC-1) [41], and they have been divided into two populations based on CD2 expression [45]. Both pDC subsets demonstrate strong activation and cytokine production in response to viruses, but CD2hi pDC express IL-12p40 during influenza infection, are better stimulators of naïve T cells, and have a better survival rate in response to stress and glucocorticoid treatments [46–48].

1.2.3. Dendritic cells that respond to specific microorganisms: tip-DC

It has been shown that some populations of DC develop in response to specific microorganisms. Such is the case of Tip-DC ($CD11b^{int}$, $CD11c^{int}$, $Gr\text{-}1^{+}$, $DEC\text{-}205^{-}$, $CD14^{-}$, $F4/80^{-}$) that produce TNF and iNOS/NO upon the infection with some microorganisms such as *Listeria monocytogenes* and *Brucella melitensis*, which results in an effective mechanism against infection. Nevertheless, in some cases, this response has been associated with tissue damage [49–51].

It has also been demonstrated that during infection with the intracellular parasite *Trypanosoma brucei*, tip-DC represent the major pathogenic M1 liver subpopulation. $CD11b^{+}$ $Ly6C^{+}$ monocytic cells migrate from bone marrow to the liver of infected mice through CCR2 interactions, then differentiate to immature inflammatory DC ($CD11c^{+}$ CD80/CD86/$MHC\text{-}II^{low}$) in an IFN-γ and MyD88 signaling-independent, and finally mature to functional Tip-DC, whose signaling depends on IFN-γ and MyD88. Interestingly, IL-10 dampens Tip-DC function during *T. brucei* infection by limiting their differentiation and maturation and CCL2 expression [52].

1.2.4. DC $CD14^{+}$

DC $CD14^{+}$ are a group of myeloid DC $CD11c^{+}$ localized in diverse nonlymphoid tissues as well as in lymph nodes. They were originally described as interstitial DC and are characterized by the presence of CD14, which suggests that they probably originate from monocytes with which they share more features than with $CD11c^{+}$ and $CD141^{+}$ DC [35].

1.2.5. Monocyte-derived dendritic cells (moDC)

As already mentioned, DC originate from precursors present in the bone marrow; nevertheless, some of them can differentiate from other cells, as is the case of moDC. In humans, there are three types of monocytes: classic ($CD14^+$, $CD16^-$), intermediate ($CD14^+$, $CD16^+$), and non-classic ($CD14^{low}$, $CD16^+$). Currently, it has not been accurately defined from which monocyte subtype moDC derive *in vivo* [53]. According to transcriptomic analysis, it has been suggested that in humans, skin DC $CD14^+$ as well as DC $CD103^-$ $CD172a^+$ from intestine are related to monocytes [54, 55], reason why they are considered authentic moDC. On the other hand, in inflamed tissues, inflammatory DC express $CD11c^+$, $CD1a^+$, and $CD14^+$ that are most probably derived from monocytes and therefore are also considered moDC [56, 57].

DC are one of the most important effectors in the immune response due to the multiple functions they play such as recognition of PAMPs and activation to produce proinflammatory and regulatory cytokines, phagocytosis of pathogenic organisms, migration to spots where danger and pathogenic signals exist, and processing and presentation of antigens through with MHCII and CD1 to T lymphocytes. In the next section, we will discuss the receptors present in dendritic cells that interact with pathogenic organisms [58].

2. Pattern recognition receptors (PRRs) present in dendritic cells

As already mentioned, DC are the surveillance cells that need to distinguish between self and nonself. They are able to recognize different molecules such as proteins, lipids, carbohydrates, and nucleic acids of bacterial, viral, fungal, or protozoan origin known as PAMPs. To achieve this surveillance task, DC possess distinct types of receptors among, which are: Toll-like receptors (TLR), RIG-I-like receptors (RLR), NOD-like receptors (NLR), and C-type lectin receptors (CLR) [59].

2.1. Toll-like receptors (TLRs)

Innate immunity is the first line of host defense against pathogen infection, and infected hosts need to detect the invasion of pathogens to prevent their spread. TLRs are transmembrane proteins that present two principal domains, leucine-rich repeats (LRR) and a Toll/IL-1R (TIR), which recognize PAMPs and initiate signaling pathways, respectively. Currently, 15 mammalian Toll-like receptors are found (TLR1-15), of which 10 are in humans. TLR3, 7, 8, and 9 are intracellular receptors and the other ones are extracellular [59–61]. The binding partners of the recently discovered TLR10, TLR12, TLR13, and TLR15 are unknown. TLR11 is only expressed in mice, and recent studies suggest that it associates with molecules originating from uropathogenic bacteria and *Toxoplasma gondii* [62, 63]. TLRs exert their functions through the formation of homo- or heterodimers. To date, five dimers have been described that are: TLR1/TLR2, TLR2/TLR6, TLR4/TLR4, TLR5/TLR5, and TLR10/TLR10 [64]. The TLR1/2 heterodimer recognizes bacterial triacyl lipopeptides, and TLR2/6 recognizes bacterial diacyl lipopeptides. In addition, each TLR in individual

form recognizes different ligands and induces the production of various cytokines and chemokines [65–67]. TLR1 and TLR2 recognize triacyl lipopeptides, TLR2 and TLR6 sense diacyl lipopeptides, lipoteichoic acid, and zymosan. Also, TLR2 recognizes peptidoglycans, lipoarabinomannan, porins, glycosylphosphatidylinositol-anchored mucin-like, and hemagglutinin. TLR3 recognizes double-stranded RNA, TLR4 senses LPS and envelope proteins. TLR5 recognizes flagellin, TLR7 and 8 can recognize single-stranded RNA and finally TLR9 recognizes DNA CpG and malaria hemozoin [68]. DCs show different expression levels of TLRs and respond dissimilarly to TLR ligands. The expression of TLRs varies with species, DC subtype, and maturation stage. All human TLRs are present in immune cells, specifically in DC, TLR1, 2, 3, 4, 7 and 9 have been shown to be present [69]. TLR10 is not present in human dendritic cells; nevertheless, it has been recently demonstrated that this receptor could be found intracellularly in endosomes and can recognize dsRNA [70]. When TLRs bind their ligands (PAMPs), this (among another variables) can trigger changes in the maturation, migration, and actions of DC. This phenomenon initiates an inflammatory response characterized by the production of cytokines, cellular migration, and directly or indirectly the activation and generation of Th1, Th2, Th17, Treg, and even B lymphocyte responses [71].

2.2. RIG-I-like receptors (RLRs)

Retinoic acid-inducible gene-I-like receptors (RLRs) are intracellular receptors and, to this date, are a family of three members, which are RIG-I, melanoma-differentiation-associated gene 5 (MDA5), and laboratory of genetics and physiology 2 (LGP2). The ligand for RIG-I is RNA (5′ -PPP single-stranded RNA and short double-stranded RNA), MDA5 recognizes RNA (poly IC and long double-stranded RNA) and LGP2 recognizes RNA [72]. All three receptors are composed of a central DExD/H box helicase domain, which coordinates RNA binding and a C-terminal regulatory domain that is implicated in RNA binding. In addition, RIG-I and MDA5 contain two CARD domains (caspase activation and recruitment) that have critical signal activity and are involved in the innate immune and inflammatory responses upon RNA detection [73]. The activation of these receptors induces the production of type I IFN characterized by its antiviral and antibacterial activity; thus, RLRs are an important complement of the immune response, particularly, with intracellular pathogens, and could be important in the response against antigenic RNA from intracellular protozoa. It has been showed that RLRs responses are independent of TLRs responses [74].

2.3. NOD-like receptors (NLRs)

One important family of receptors found in DC is the nucleotide-binding oligomerization domain-containing receptors (NLRs), which are a family of 22 protein members in humans that can be classified into five subfamilies according to their structure: NLRA (CIITA), NLRB (NAIPs), NLRC (NOD1, NOD2 and NLRC3-5), NLRP (NLRP 1-14), and NLRX [74]. Their basic structure consists of a central nucleotide-binding domain (NBD), a carboxy-terminal leucine-rich repeats (LRR), and an amino-terminal effector domain. Some of the ligands that bind to these receptors are iE-DAP, MDP, RNA, ATP, bacterial toxins, uric acid, CPPD, amyloid-b, and anthrax lethal toxin [68]. NLRs are linked to different functions, as is the case of

NOD1, NOD2, CIITA, and NLRP10 that are signaling receptors and are associated to adaptive immunity. NLRC4 and NLRP3 are linked to the formation of the inflammasome and finally NLRP1, NLRP6, NLRP12, and NLRC5 participate both in signaling as well as in the formation of the inflammasome [75]. In relation to the formation of the inflammasome, NLRP 1, 2, 3, 6, 7, 12, NLRC4/N, and AIP 2, 5 have been found to participate when those receptors sense PAMPs or DAMPs (damage-associated molecular patterns). Also, it has been documented that CIITA and NLRC5 can be activated by cytokines that lead to the transcription of MHC I and II, and NOD1-2 have TLR-like activity with the recognition of PAMPs and triggering of inflammatory signaling cascades. Other members such as NLRP 2, 4, 6, 11, 12, NLRX1, NLRC3, etc. participate in the inhibition of the NF-κB pathways [76, 77]. Finally, NLRP 10 and 12 participate in cellular migration. In particular, NLRP10 plays a critical role in antigen-primed DC migration in draining lymph nodes [78], and NLRP-12 regulates migration of DC to a CCR7 chemokine gradient, thus resulting in a reduced T cell response to antigen [79].

In conclusion, this large family of intracellular receptors can recognize viral, bacterial, protozoan, and fungal antigens that trigger cellular innate and adaptive immune responses.

2.4. C-type lectin receptors (CLRs)

Myeloid cells, including DC and macrophages, express a large number of C-type lectin receptors that are one of the most important PRRs that recognize carbohydrates [80]. They can have a transmembrane or soluble localization and can trigger phagocytic and inflammatory responses through the recognition of PAMPs, DAMPs, and tumor-associated molecular patterns (TAMPs) [81]. The common structure in these receptors is the carbohydrate recognition domain (CRD), which can be found in different numbers and arrangements. They also present an ITAM (immunoreceptor tyrosine-based activation) like motif or an ITIM (immunoreceptor tyrosine-based inhibition) motif [82]. A wide array of carbohydrates are recognized by CLRs, among those that can be found are high mannose glycans, mannose, mannosyl fatty acids, fucose glycans, Lewis antigens, glucans, among others. The recognition of the ligand by a CLR can activate or inhibit responses dependent or independent of the Syk signaling pathway [83]. This family of receptors has been classified according to their structure in 17 groups that comprise almost 50 proteins. The groups that comprise the soluble receptors are: I (proteoglycans), III (collectins), VII (free CTLD), IX (tetranectin), and XII (CTLD/acidic neck). On the other hand, transmembrane receptors are grouped as follows: II (type 2 receptors), IV (selectins), V (NK cell receptors), VI (MMR), X (polycystin), XI (attractin), and XIV (endosialin) [82]. Several CLRs are present in DC and the best characterized are DCIR, DCSIGN (CD209), Dectin1, Langerin, and MMR (CD206). Langerin (CD207) on LC in the epithelial layer of the skin has unique carbohydrate specificity for high mannose and LeY carbohydrates and is involved in the recognition of various viruses [84]. DEC 205 has extensively been employed for target delivery of antigens to DC in murine and human studies [16]. It has been shown that targeting antigen to the DEC205 receptor improves humoral and cellular immune responses when DC are stimulated with activating agents or adjuvants such as poly I:C [85]. In mice, this receptor is expressed on cortical thymic epithelium, thymic medullary DC ($CD11c^+$, $CD8\alpha^+$), and subsets of peripheral DC (splenic, lymph node DC, dermal, interstitial DC, and Langerhans cells) [86]. In contrast, DC-SIGN (CD209) is merely expressed on moDCs and

on CD14+ dDCs in dermal layers of the skin and has specificity for mannose and all Lewis type carbohydrates (Lewis A, B, X, and Y) [87]. This receptor is often endosialin with the MR (CD206) and shares mannose specificity [88]. DCIR (for DC immunoreceptor) (CLEC4a) has broad carbohydrate specificity for mannose and fucose [89, 90] and has been shown to participate in T cell responses. Different from other CLRs, DCIR contains an ITIM motif and, upon triggering with Abs, inhibits the production of inflammatory cytokines and, thus, has been associated with homeostatic control and control of inflammation [91–93]. Also, it has been shown that DCIR can participate in the capture of HIV-1 and promote infection in trans and in cis of autologous $CD4^+$ T cells from human immature monocyte-derived DCs [94].

Finally, these receptors can recognize (and opsonize) pathogens and the responses to these events are phagocytosis for antigen uptake, cell migration, cell adhesion, inhibition of cytokine production, and interaction between DC and T lymphocytes [95]. In conclusion, CLRs are key participants in the immune response that spot important signals of PAMPs, DAMPs, and TAMPs with which immune cells (e. g., dendritic cells) sense and recognize the environment. Its interactions and intersections trigger, adapt, and regulate immune responses [96].

In addition to the PRRs just mentioned, it is important to note that some orphan receptors have been described for which a ligand has not been found, as well as the family of ALR intracellular receptors (AIM2-like receptors) and another family of lectins (I-lectins) Siglecs (sialic acid binding Ig-like lectins) [97].

3. Role of dendritic cells in the infection with parasites

Due to a wide variety of functions that dendritic cells display both in the innate immune response as well as in the adaptive immune response, they are key participants in the defense against parasites. DC have the capacity to recognize different molecules in the surface of parasites and are efficient phagocytes; thus, several intracellular parasites reside inside DC. Once DC phagocytose intracellular parasites, they can exert their microbicidal capacities, although it has been shown that they are not as efficient in the destruction of microorganisms as other phagocytes such as macrophages and neutrophils. Once internalized, DC process antigens for presentation to T cells. DC have the unique property to migrate to regional lymph nodes where they activate naïve T cells, as well as produce cytokines and participate in the modulation of the immune response, the amplification of the innate immune response, and can also participate in immunological tolerance.

Protozoan infections that persist in urban environments including leishmaniasis, Chagas disease, malaria, and zoonotic diseases such as toxoplasmosis are a matter of great concern due to their prevalence, morbidity, and mortality [98]. Our best hope to counteract them is the development of new and innovative technologies. For this development, the better understanding of the biology of these parasites and their interaction with their host is of outmost importance. We chose the above-mentioned diseases and analyzed their interaction with DC that are one of the most important participants of the immune response.

3.1. Interaction of dendritic cells with *Leishmania*

3.1.1. Generalities of Leishmania

Leishmania is an obligate intracellular parasite that presents two morphological stages: the flagellated promastigote that is found in the salivary glands of the insect vector and the aflagellated amastigote that is the intracellular form found in the vertebrate host. This genus of parasites is constituted by diverse species that are morphologically indistinguishable and are grouped in three subgenera: *Leishmania*, *Viannia*, and *Sauroleishmania*. The species that cause infection in humans and other mammals are found in the subgenus *Leishmania* and *Viannia*. *Sauroleishmania* has been only found to infect some reptiles. Species belonging to *Leishmania* are characterized for having a suprapyloric development and among these are: *donovani, chagasi (infantum), major, tropica, aethiopica, mexicana (pifanoi), amazonensis,* and *venezuelensis*. On the other hand, the species of the subgenus *Viannia* have a peripyloric development and are *braziliensis, guayanensis, peruviana,* and *panamensis. Leishmania* is transmitted by Diptera belonging to the family Psychodidae, specifically, by females of the genus *Lutzomyia* and *Phlebotomus* [99, 100]. *Lutzomyia* is the transmitter of leishmaniasis in America, and *Phlebotomus* transmits this pathology in Africa, Asia, and Europe [101, 102].

3.1.2. Leishmania life cycle

Leishmania life cycle starts when the insect vector feeds blood from the vertebrate host and inoculates promastigotes in the superior dermis [103]. Then, promastigotes are recognized through different receptors such as CR3 [104], C-type lectin receptors, and FcγR and are phagocytosed by dermal macrophages, where they transform to amastigotes [105] and also infect neutrophils. Thanks to a series of events such as the inhibition of phagocytosis, resistance to microbicidal mechanisms, and inhibition of host cells apoptosis, amastigotes manage to survive inside macrophages and duplicate until they lyse them and infect new surrounding cells, such as dendritic cells. If the parasite is not able to inhibit the different microbicidal mechanisms of macrophages, they will be able to destroy them mainly through the production of nitric oxide and induce the activation and recruitment of proinflammatory cells such as cutaneous mast cells, neutrophils, and inflammatory monocytes that lead to the development of a focus of chronic inflammation in the site of infection, which will be evident in the patient [105, 106]. The female sand fly when ingests blood from an infected host draws infected cells as well as free amastigotes and in this form the life cycle continues.

3.1.3. Interaction of Leishmania major with macrophages and DC

One of the most studied interactions of DC with a parasite is the interaction with the intracellular parasite *Leishmania.* In the murine model of infection and using *Leishmania major*, it has been shown, as already mentioned, that promastigotes infect macrophages and neutrophils that are localized near the site of inoculation. Promastigotes are phagocytosed by macrophages mainly through CR3 [104], which permits parasites to enter to this host cell without activating it [107]. Interestingly, the infection with *Leishmania* downregulates the capacity of macrophages to produce IL-12. Even the stimulation of macrophages with IFN-γ/LPS does not

elicit the production of IL-12 when cells are infected with *L. major* [108]. Once promastigotes enter to macrophages without activating them, they differentiate into amastigotes and start dividing in the parasitophorous vacuoles. Then, amastigotes are released to the extracellular milieu where they are phagocytosed by neighboring cells such as DC. DC phagocytose amastigotes mainly through FcγRI and FcγRIII, inducing DC maturation, migration to lymph nodes, and IL-12 production [109]. It has also been shown that *Leishmania*-infected DC upregulate the levels of costimulatory molecules such as CD40, CD54, CD80, and CD86, as well as of MHCII; that is, the maturation of DC enables them to initiate the activation of T lymphocytes [110]. Other authors have demonstrated that during the course of chronic infection of C57BL/6 mice with *L. major,* the main producers of iNOS are inflammatory DC, which are recruited in a CCR2-dependent manner and the induction of iNOS depends on the development of a local Th1 microenvironment [111]. In addition to the analysis of the infection of murine DC with *L. major*, other authors have shown that in the case of human DC infected with *L. major* metacyclic promastigotes, the production of high amounts of IL-12 needs the interaction of CD40-CD40L, although infected DC are able to produce some IL-12. Also, the infection of human DC with *L. major* promastigotes does not inhibit the process of maturation [112].

3.1.4. Role of DC in the adaptive immune response against Leishmania major

Once DC capture *Leishmania* parasites, they migrate to the lymph nodes were they activate naïve CD4 and CD8 T lymphocytes in order for them to respond specifically against the parasite with an immune response dominated by the presence of IFN-γ and cytotoxic T lymphocytes species specific in what has been called a type Th1/Tc1 immune response. This type of response permits mice to control infection and eliminate the parasite [113]. IL-12-producing DC have been observed until week 4 postinfection with a peak in week 6 just before the Th1/Tc1 IFN-γ-producing response develops completely [114]. Nevertheless, the reason for the delay in DC maturation still remains to be clarified. Iborra and colleagues have described a route that attempts to understand this delay in the maturation of DC after *Leishmania* infection. They found that *L. major* parasites secrete a soluble factor that binds to the soluble macrophage c-type lectin receptor (Mincle) of DC, which inhibits its maturation. They showed that Mincle deficiency favored stronger DC activation represented by a higher expression of costimulatory molecules, migration to dLNs and priming of a Th1 response. Thus, mice deficient in Mincle receptor are capable of controlling parasite replication and indeed had smaller lesions [115]. It has been shown that in the infection of mice with *L. major* metacyclic promastigotes, Langerhans cells induce the activation of regulatory T lymphocytes [116]. Murine $CD103^{+}$ dermal DC have been shown to be responsible of inducing a protective immune response against *L. major* since mice lacking this DC subtype develop an immune response dominated by regulatory and Th2 lymphocytes infection [117].

3.1.5. Interaction of DC with L. mexicana

While *L. major* is an etiologic agent of cutaneous leishmaniasis (CL) in the Mediterranean region, *L. mexicana* is in Mexico and Central America. Although both species cause CL, the case of *L. mexicana* is of particular interest due to the fact that this species can cause localized cutaneous leishmaniasis (LCL) and diffuse cutaneous leishmaniasis (DCL). Different studies

have shown that the interaction of *L. mexicana* and its principal host cells, macrophages and DC, are substantially different as compared to what has been observed with the *L. major* model. It has been observed that in DC infected with *L. mexicana,* the parasite manages to be internalized without the initiation of cell maturation or the production of IL-12. Although the infection inhibits IL-12 production, *L. mexicana* does not eliminate the capacity of DC to produce it and, indeed, when an external stimulus such as LPS is used, infected DC are capable of producing and secreting IL-12. In addition it was observed that the internalization of the parasite is independent of opsonization [118]. In relation to the recognition of *L. mexicana* by DC, the information about the receptors involved is scarce. On one hand, it has been reported that DC-SIGN is capable of binding *L. mexicana* LPG [119]. Recently, our group showed that DC-SIGN participates in an important manner in the internalization of *L. mexicana* promastigotes since the blockade of the receptor with a specific antibody diminished significantly the interaction of monocyte-derived dendritic cells with *L. mexicana* promastigotes [120]. Using the same strategy of blocking DC-SIGN with an antibody, its role in the infection of DC with *L. pifanoi* amastigotes was shown to be relevant since it dramatically diminished the interaction of DC with amastigotes [121].

3.1.6. Interaction of DC with other species of Leishmania

It has been observed that DC can interact with both *L. infantum* y *L. pifanoi* amastigotes and promastigotes through DC-SIGN and that it binds more avidly the infective metacyclic forms. Interestingly, the interaction of DC with *L. major* metacyclic promastigotes does not depend on DC-SIGN [122]. Also, it has been shown that plasmacytoid DC do not internalize *L. infantum* promastigotes, although contact of the parasites with these cells induces the secretion of IFNα/β, but not of IL-12 [123]. Also, it has been described that in the early stages of *Leishmania* infection, the inflammatory milieu that is produced is ideal for the induction of monocytes toward Tip-DC.

3.1.7. Molecular mechanisms involved in the modulation of DC by Leishmania

The molecular mechanisms involved in the modulation of DC during the infection with *Leishmania* have not been fully analyzed. In the murine model of infection with *L. major*, it has been clearly established that susceptible mice (Balb/c) mount a Th2-type response that enables them to eliminate the parasite while resistant mice (C57/BL6) mount a Th1 response that permits the elimination of the parasite. In contrast, C57/BL6 mice infected with *L. mexicana* metacyclic promastigotes do not resolve infection and it becomes chronic, what has been associated with a decrease in the recruitment of monocytes, a reduction in inducible nitric oxide synthase (NOS2) synthesis in moDC along with a reduction in the migration to the lymph node, which results in an insufficient activation of a Th1 response [124]. *Leishmania* parasites possess an extraordinary capacity to manipulate host cells. In particular, it has been shown that the infection of murine DC with *L. mexicana* promastigotes rapidly affects DC molecular mechanisms necessary for the development of a protective immune response, among them, an increase in tyrosine phosphatases, which translates in an inactivation of mitogen-activated protein kinases (MAPK), and decrease in the translocation of transcription factors such as

NF-κB and AP-1. In addition, parasites also modulate DC maturation markers decreasing the surface expression of antigen-presenting and costimulatory molecules [125]. Our group has worked for several years in the modulation of DC by *L. mexicana*. We have shown that LPG purified from *L. mexicana* promastigotes induces a major secretion of IL-12 and NF-κB translocation in human DC as compared with monocytes [126]. We also showed that the infection of murine DC with *L. mexicana* amastigotes downregulates NOS2 and thus diminishes NO production [127]. On the other hand, we have also shown that the infection of human DC with *L. mexicana* amastigotes and promastigotes inhibits the activation of MAPK JNK and p38, and the infection of human DC with *L. mexicana* amastigotes activates AKT during camptothecin-induced apoptosis [128, 129].

In addition to the modulation of DC biology exerted by *Leishmania* that has just been described, another intracellular parasite whose interaction with DC has been deeply studied is *Toxoplasma gondii.*

3.2. Dendritic cells in the infection with *Toxoplasma gondii*

3.2.1. Generalities of T. gondii

Toxoplasma gondii is an intracellular parasite that causes toxoplasmosis, and it can be hosted by diverse warm-blooded animals and is present in two interconvertible stages: the lytic, invasive, and active tachyzoites and the slow-growing, encysted bradyzoites. The oocysts present in the definitive host, a feline, are highly infective and long-lived and are shed in the feces for a limited time [130]. The infection initiates with direct contact with oocysts or by consumption of undercooked meat containing bradyzoite cysts. Bradyzoite cysts convert to tachyzoites in the small intestine of the intermediate host and can infect almost all nucleated cells. Here, they replicate within a parasitophorous vacuole (PV), egress by lysing the cell, and infect neighboring cells. Tachyzoites elicit a potent immune response that eliminates most parasites. However, some tachyzoites can evade this response, convert back to bradyzoites, and persist mostly in nonreplicative cells such as those in the brain or heart of their intermediate host [131].

3.2.2. Immune response to T. gondii

Toxoplasma orchestrates a carefully balanced string of events between various cell types including neutrophils, DCs, and macrophages upon first encountering the host innate immune defense. A complex network of molecular signaling pathways leads to the activation and regulation of cytokines and ultimately to the production of effector molecules [132]. Acquired resistance to *T. gondii* infection is mediated by a mucosal and systemic Th1 cellular immunity [133]. The deviation to a Th1 response depends enormously on the production of IL-12 by different cells such as conventional DCs, macrophages, and pDCs. Parasite infection causes damage to the intestinal epithelium resulting in the translocation of microflora and subsequent MyD88-dependent signaling and IL-12 production. IL-12 triggers the proliferation of NK cells, CD4 T cells, and CD8 T cells, which mediate cytotoxicity and the production

of high amounts of IFN-γ [134, 135]. It has been previously showed that DC pulsed with *T. gondii* antigens elicit protective immunity against chronic toxoplasmosis in mice [136, 137].

3.3. Interaction of DC with *T. gondii*

3.3.1. Recognition of T. gondii by DC

DC are crucial participants in the immune response against *T. gondii* and one of the leading roles that they play in the production of IL-12, which, as previously mentioned, promotes the production of IFN-γ and thus deviates the immune response toward a Th1. DC recognize diverse *T. gondii* molecules; in particular, it has been shown that a soluble parasite extract (STAg) has a major capacity of eliciting IL-12 from splenic DC as compared to other PAMPs such as LPS and CpG oligonucleotides [138]. The production of IL-12 induced by *T. gondii* in DC is dependent on MyD88, an adaptor molecule in TLR signaling pathways, and the chemokine receptor CCR5, since the production of IL-12 decreases dramatically in mice lacking MyD88 or CCR5 [139]. Interestingly, CCR5 in DC is induced with cyclophilin-18 from *T. gondii* [139]. The participation of MyD88 in the induction of IL-12 by *T. gondii* presupposes the recognition by TLRs. Indeed, it has been shown a profilin-like protein, which is not required for the intracellular growth of *T. gondii*, but is indispensable for host cell invasion and active egress from cells [139], was identified as a ligand of TLR11 and the profilin-like protein is also recognized by TLR-12 [62], and is critical for IL-12 production, especially in plasmacytoid DCs (pDCs) [140]. TLR11 has been localized intracellularly associated with the nucleic acid-sensing TLR trafficking protein UNC93B1.52. A mutation in this protein impedes TLR intracellular trafficking, which has been shown to cause a reduction in IL-12 production in mice infected intraperitoneally with *Toxoplasma* bradyzoites and increases susceptibility to infection [141, 142].

Both cyclophilin-18 and the profilin-like protein stimulated IL-12 production in $CD8\alpha^+$ DC and $CD8\alpha^-$ DC. Although humans do not express either TLR11 or TLR12, human monocytes produce proinflammatory cytokines in response to *T. gondii* infection, suggesting that other TLRs in humans recognize different compartments of *T. gondii* to produce IL-12 in antigen-presenting cells. Additionally, DC also produce chemokines such as CCL2 and CXCL2 upon recognition of parasite components including virulence factors [143]. These chemokines induce the migration of $Ly6C^{high}CCR2^+$ monocytes and neutrophils to the infection site [144]. Albeit not demonstrated specifically in DC, other TLRs, such as TLR2, can also be activated in response to *Toxoplasma* [145]. TLR2 and TLR4 both signal after binding *Toxoplasma* glycosylphosphatidylinositol (GPI) anchors [146]. However, single absence of either TLR2 or TLR4 in DC did not reduce the production of IL-12 in response to STAg [147].

3.3.2. Effector functions of DC against T. gondii

It is possible that DCs can directly act as effector cells to eliminate *Toxoplasma* as suggested by their ability to display oxygen-dependent microbicidal activity after IFN-γ activation [148]. Moreover, plasmacytoid DCs (pDCs) have been shown to be efficient at autophagy

[149], a process known to eliminate *Toxoplasma* in primed macrophages and to involve the family of p47 GTPases [150]. The various subsets of DC possibly recognize either direct infection with *Toxoplasma* or sense parasite products differently, and are thus important mediators of parasitic elimination and facilitators for the development of an efficient adaptive immune response. Conventional $CD11c^+$ DC have been shown to play key roles in host resistance to *Toxoplasma* bradyzoite cysts administered i.p. [151, 152].

3.4. Role of DC in the infection with *Plasmodium*

3.4.1. Plasmodium life cycle

Malaria is a disease caused by the blood protozoan of the genus *Plasmodium* about which there have been described five species capable of infecting humans that are *P. falciparum, P. vivax, P. malariae, P. knowlesi,* and *P. ovale* [153]. The life cycle of this parasite starts when a hematophagous female mosquito of the genus *Anopheles* introduces its proboscis, containing infective sporozoites into the mammalian host. There is evidence that once the sporozoites have been inoculated, they can remain on the skin for hours and slowly release into the blood [154, 155]. Once sporozoites reach the bloodstream, they migrate to the liver where they infect hepatocytes and transform into merozoites that infect red blood cells. Some of these merozoites transform into male and female gametocytes to finally complete their cycle within the mosquito's digestive tract [156, 157].

3.4.2. Interaction of DC with Plasmodium

Several studies have revealed that DC play an important role during *Plasmodium* life cycle, as well as in the pathophysiology and outcome of the disease. Although *Plasmodium* does not infect DC, it has been shown that the interaction of DC with infected erythrocytes has an effect on DC functions. In particular, it has been shown that erythrocytes infected with *P. falciparum* and co-cultured with human DC manage to induce in them an arrest in the process of maturation, thus inhibiting their ability to stimulate and activate T lymphocytes; however, the effect is dependent on the parasite: DC ratio [158, 159]. Also, during the acute phase of infection with *Plasmodium*, there is an increase in $BDCA3^+$ DC, but not in $CD1c^+$ DC, which correlates with a severe presentation of the disease [160, 161]. Other DC populations that change during *Plasmodium* infection are pDC ($HLA\text{-}DR^+CD123^+$): mDC ($HLA\text{-}DR^+CD11c^+$). It has been reported that the ratio of pDC:mDC diminishes in patients suffering from the acute phase of *P. vivax* infection. In addition to this decrease, there also exists an arrest in DC maturation demonstrated by a reduction in CD86 in patients infected with *P. vivax* and a decrease in CD83 and HLA-DR in individuals in the acute phase of infection with *P. vivax* and *P. falciparum*. This diminution of CD functions in these infections is accompanied by an increase in the number of cells that die by apoptosis, as well as by the decrease in their ability to capture, mature, and present antigens to T cells [162–164].

Other research groups have addressed the role of DC in the induction of the response of T and B cells in *Plasmodium* infection. It has been shown that $TCD8^+$ cells, previously activated by DC, exert their cytotoxic effect by inducing death of *P. vivax* sporozoites housed in

hepatocytes, thus, reducing parasite loads in hepatocytes. There is evidence that suggests $CD8^+CD11b$-DC located in the peripheral lymph nodes near the mosquito inoculation site are the same subtype of DC responsible for the activity of $TCD8^+$ cells [165–168]. The protective response of $TCD8^+$ cells is associated with the production of IFN-γ, which is induced by two subtypes of mature ($CD40^+$) spleen DC and ($CD8\alpha^+CD11b^-$ and $CD8\alpha^-CD11b^+$) D C) [169, 170]. There is evidence that, of these two subtypes, only the $CD8\alpha^-CD11b^+$ DC are responsible for the activation of $TCD4^+$ cells, in the acute phase of the infection, while the $CD8\alpha^+CD11b^-$DC participate in the process of antigen cross presentation throughout the infection [171, 172]. In relation to the activation of $TCD4^+$ cells, in different study models, it has been shown that the presentation of *Plasmodium* antigens by DC to $TCD4^+$ cells expresses MHC-II, CD40, and CD80 and produces IL-12, IL-6, and TNF-α, thus inducing $TCD4^+$ cells to express IL-2, IFN-γ, and TNF-α. Finally, the stimulation of DC with *Plasmodium* antigens also induces their migration of DC to lymphoid organs [173–177].

Apart from the interaction of DC with protozoan parasites that has been discussed, we chose *Trichinella spiralis* as an example of the interaction of a helminth with DC.

3.5. Interaction of *Trichinella spiralis* with DC

Trichinella spiralis is a parasitic helminth that belongs to the group of nematodes. Its biological cycle begins when a mammal (usually mouse, rat, pig, and human) ingests raw or undercooked meat containing cysts (encysted larvae). As it passes through gastric acid and pepsin, the larva is released from the cyst and invades the mucosa of the small intestine, where it matures to the sexually differentiated adult state that initiates reproduction [178]. After 1 week, females release larvae, which migrate to skeletal striated muscle where they encyst and the cycle closes when cysts are ingested by another mammal [178]. In order for *T. spiralis* to remain for long periods in the muscle, the parasite must have mechanisms to evade the immune response and inhibit tissue inflammation. It has been described that *T. spiralis* muscle larvae have structural carbohydrates on their surface that contribute to the activation of the immune response that results in Th2/anti-inflammatory response [179]. It has been shown that *T. spiralis* glycans affect the anti-inflammatory environment and can interfere with the development of inflammatory diseases [180]. On the other hand, *T. spiralis* muscle larvae excrete a molecular complex called excretory-secretory antigen (ES L1) that has been shown to alter DC maturation [180]. In addition, immature DC resulting from the exposure to ES L1 induce a Th2 and regulatory response with the production of IL-10 and TGF-β, but without increasing $CD4^+CD25^+Foxp3^+$ effector T cell population [179]. Thanks to these two mechanisms, the induction of an anti-inflammatory response and the inhibition of DC maturation, *T. spiralis* survives as long as it is the muscular larva without inducing inflammation or an adequate immune response.

4. Conclusion

Dendritic cells represent a wide constellation of cells that perform crucial roles in the immune response covering from recognition and phagocytosis, to antigen processing and presentation

to naïve T cells and immune tolerance. Due to this wide array of functions, they constitute a bridge that connects the innate immune response with the adaptive and are very important against parasites. DC are able to recognize diverse PAMPs present in parasites through different PRRs such TLRs, CLRs, NLRs, and RLRs, some of which, upon binding their respective ligand, induce phagocytosis and/or signal for the production of different molecules. Parasitic infections cause great morbidity and mortality. For the majority of them, there are no vaccines and the treatments that are not always effective. The better understanding and gaining of knowledge on the biology of parasites and their interaction with the immune system, in particular with DC due to the important role that play in the immune responses, will permit the development of new strategies and drugs to effectively treat the pertaining diseases mentioned in this work.

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Chapter 5

The Role of Dendritic Cells in Bone Loss and Repair

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Additional information is available at the end of the chapter

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Abstract

The cells of innate immunity, such as neutrophils, macrophages, and dendritic cells (DCs), stuck to the bone implant walls release reactive radicals, enzymes, and chemokines, which induced subsequent bone loss. DCs do not play a big role in bone homeostasis in steady-state conditions, but could act as osteoclasts precursors in inflammation foci of bone. The potent antigen-presenting cells responsible for activation of native T cells and modulation of T cell activity through RANK/RANKL pathway and other cytokines associated with osteoclastogenesis determine critically situated at the osteoimmune interface. The titanium (Ti) and magnesium (Mg), the metallic candidate in implant, including calcium-phosphate coating formation on them by method plasma electrolytic oxidation were used to evaluate the immune-modulatory effects of DCs. The calcium-phosphate coating on metals induced mature DC (mDC) phenotype, while Ti and Mg promoted a noninflammatory environment by supporting an immature DC (iDC) phenotype based on surface marker expression, cytokine production profiles, and cell morphology. These findings have numerous therapeutic implications in addition to DC's important role in regulating innate and adaptive immunity. A direct contribution of these cells to inflammation-induced bone loss establishes DC as a promising therapeutic target, not only for controlling inflammation but also for modulating bone destruction.

Keywords: osteoimmunology, dendritic cells, bone, titanium, magnesium, biocompatibility

1. Introduction

There are two forms of the immune response of organism: innate and adaptive ones and dendritic cells (DC) serve as a bridge between them. The role of innate immunity cells

in inflammation-induced osteoclastogenesis and subsequent bone loss is critical, thereby establishing a new paradigm of osteoimmunology [1–3]. According to the long-standing definition, septic and aseptic total joint and bone replacement loosening are two distinct conditions with little in common. Septic bone replacement loosening is driven by bacterial infection whereas aseptic loosening is caused by biomaterial wear debris released from the bearing surfaces. The initial injury to the tissue surrounding a bone implant induces an inflammatory response mediated by the cells of innate immunity, such as neutrophils, macrophages, and DCs. The cells, which are stuck to the implant walls, release reactive radicals, enzymes, and chemokines, which cause a cascade of inflammatory responses [4, 5]. The mobilization of functional activity of innate immunity cells is directed by chemokines, cytokines, and integrins. Recently, it has been recognized that the mechanisms that drive macrophage activation in septic and aseptic total joint replacement loosening resemble each other [6]. Accumulating evidence indicates that in addition to mediating bacterial recognition and the subsequent inflammatory reaction, toll-like receptors (TLRs) and their ligands, pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPS), play a key role in wear debris-induced inflammation and cellular activation. Furthermore, metal ions released from some total joint replacements can activate TLR signaling similar to bacterial derived PAMPs [7]. Likewise, metal ions can function as haptens activating the adaptive immune system similar to bacterial derived antigens [8]. Thus, it appears that aseptic and septic joint and bone replacement loosening share similar underlying pathomechanisms. DCs derive from the mononuclear cell pool and are characterized by high expression levels of CD11c/major histocompatibility complex (MHC) II. Conventional DCs are capable of antigen presentation and reverse migration, i.e., are able to migrate to lymph nodes via afferent lymphatic vessels. Immature progenitor conventional DCs are unable to prime T cells, but are equipped with high phagocytic activity [9]. It was shown that, DCs can influence the type of immune response through the induction of regulatory mechanisms. Immature DCs express chemokine receptors (CCR-1, −2, −5, −6 and CXCR-1) and are able to migrate actively to the inflammatory focus in response to the appearance of chemokines [10]. The process of maturation of DC is initiated by various factors, including biomolecules of microorganisms, pro-inflammatory cytokines and products of necrosis of cells and tissues. Maturation of the DC is a continuous process, in which there is a decrease in the endocytic potential, the expression of antigen-recognizing receptors, while, on the opposite side, the expression of adhesion molecules CD54 and CD58 increases. This changes the structural organization of cellular organelles, increases the activity of lysosomal enzymes, there are immunoproteasomes which process the intracellular protein antigens. Fully differentiated DCs actively produce pro-inflammatory cytokines, such as IL-12alpha (IL-12p35), through which T-lymphocytes are activated, including regulatory lymphocytes [11].

Researchers recently began to investigate a possible direct role of DC in inflammation related bone damage. DCs are known for their role of antigen presenting cells (APCs) and do not appear to play a role in bone homeostasis in nonpathological conditions, but some data suggest that DC could act as OC precursors in an inflammatory milieu, transforming into

DC-derived-OC according to phenotypic and functional characterization studies. Moreover, DCs modulate T cell activity through RANK/RANKL and osteoclastogenesis-associated cytokines [12–14]. The role of DC, as the key components of the defensive response of the organism in the pathology of bone, was demonstrated in the field of osteoimmunology research. It is indicated, that normally the localization of the DC in the stroma proper or adjacent to the bone tissue, is rare and DC do not take part in the restoration of its defects [15, 16]. On the other hand, the presence of DC in patient's synovial periodontal fluid during periodontitis and in joints of patients with rheumatoid arthritis has been documented [17, 18]. With these diseases, localized in the bone stroma the DC can form aggregates with T- cells, forming inflammatory foci, where migrate through chemotaxis and adhesion molecules RANK-RANKL. It is shown that, during the inflammation of bone tissues, the expression of these receptors on the surface of the DC induces indirectly through regulation of T-cell activity and through the process of differentiation and survival of osteoclasts bone degradation [15, 16, 19]. Rivollier et al. have shown that myeloid DCs of human peripheral blood can be transformed into osteoclasts in the presence of macrophage colony-stimulating factor M-CSF and the soluble form of receptor RANKL, suggesting direct participation of DC in osteoclastogenesis [16, 20, 21]. Further, co-cultivation of $CD11c^+$, $CD11b^-$ DC, similar to the classical precursors of osteoclasts, under the influence of granulocyte-macrophage colony-stimulating factor GM-CSF and interleukin 4 IL-4, their transformation into osteoclasts was demonstrated. This suggests that transformed into functional osteoclasts $CD11c^+$ DC, under the condition of their immune interaction with $CD4^+$ T cells and other factors in the surrounding bone tissue environment, can induce the bone resorption process in vivo. Also installed an important protein currently considered as a master regulator of osteoclastogenesis—dendritic cell-specific transmembrane protein (DC-STAMP). It is assumed that DC-STAMP plays an imperative role in bone homeostasis by regulating the differentiation of both osteoclasts and osteoblasts [22]. In general, these data point to a critical effect of DC on the process of osteoclastogenesis in inflammatory bone diseases, where they act not only as powerful antigen-presenting cells, that activate and regulate the cells of immune system, but also influence directly to the destruction of bone tissue. There is a lack of definitive evidence about the physiological relevance of this phenomenon in vivo but DCs could act as an osteoimmune interface, contributing to bone loss in inflammatory diseases [12, 16, 21].

At the moment, in the field of endoprosthetics, there is a tendency in studies aimed at creating biomaterials that can replace damaged tissue sites of the human organism. Most successfully, these studies are made while treatment of the pathology of the musculoskeletal system, including in the endoprosthetics of large joints. At the moment, stainless steel and titanium alloys are the main materials used for the manufacture of immersion implants. Nevertheless, the use of fixatives from bioinert metals in osteosynthesis requires repeated surgical interventions aimed at removing the metal implants that have performed their role, and this is often no less traumatic, than osteosynthesis itself. Therefore, it remains relevant to search for bioresorbable materials that are suitable for creating implants used in osteosynthesis, that could be completely metabolized by the organism without exerting a pathological effect on surrounding tissues and the organism as a whole [23, 24]. Such materials include magnesium

alloys, which, due to the strength properties, are suitable for the production of various types of implants. This material has good biocompatibility, sufficient corrosion resistance and shows a positive effect of magnesium biodegradation products on osteogenesis, but the mechanism of their action is not fully studied [25]. Both bioinert and bioresorbable materials, when introduced into the organism, are contacted with antigen-presenting cells and their properties, such as topography of the surface, chemical composition, play an important role in initiating a pro- or anti-inflammatory immune response.

Thus, DCs are suitable cells for evaluating of their response to biomaterials because they can transform into osteoclasts under bone inflammation and also initiate and modulate the immune response to the implants materials. This way, the determination of the ability of biomaterial to influence on the phenotype of DC is quite applicable for determining their compatibility properties with the organism. Only several metal-based nanoparticles were reported to activate T cell responses or homeostasis. For example, TiO_2 nanoparticles provoke inflammatory cytokines and increase DC maturation, expression of co-stimulatory molecules, and prime native T cell activation and proliferation [26]. Most importantly, pattern recognition receptors signaling activations also can enhance antigen presentation via upregulating the expression of MHC and co-stimulatory molecules (CD80 and CD86) on DC leading to adaptive immunity activations [1, 7]. Thus, the study of the phenotype and functional activity of the DC after exposure to biomaterials corrected for properties suggests a direction in the development of the immune response induced by their introduction into the organism and makes it possible to compose an immunomodulating design of such biomaterial.

From all the listed above, the aim of the work is to reveal the immunomodulating properties of bioinert (titanium) and bioresorbable (magnesium) metal implants according to the degree of their influence on DC markers.

2. Materials and methods

2.1. Materials

Bacterial lipopolysaccharide (LPS, Abcam, USA) and the disks of implants were prepared from 1-mm thick sheets of commercially pure Ti (wt. %: Fe 0.25; Si 0.12; C 0.07; O 0.12; N 0.04; H 0.01, Ti—the remaining part balance) and Mg alloy MA8 (1.5–2.5 wt. % Mn; 0.15–0.35 wt. % Ce; Mg—balance) were used. The samples of a size of 15 mm × 20 mm × 2 mm have been undergone preliminary mechanical treatment until the roughness parameter of Ra = 0.12 μm. After mechanical treatment, samples were thoroughly washed with deionized water and ethanol and dried in the airflow. The samples appearance after volumetric tests was observed using a Stemi 2000CS stereo-microscope (Zeiss, Germany).

The electrolyte was prepared in 2 liters of deionized water by adding the following components: 30 g/l of calcium glycerophosphate dihydrate ($C_3H_7O_6P$), $Ca{\cdot}2H_2O$ and 40 g/l of calcium acetate monohydrate ($Ca(CH_3COOO)_2{\cdot}H_2O$). The electrolyte pH was adjusted to 10.9–11.3 by adding 20% NaOH solution [27]. Plasma electrolytic oxidation was carried out using a reversible thyristor rectifier, as power supply, equipped with an automated control system with appropriate software. All the samples were treated in the unipolar PEO-mode at

a current density of 0.67 A/cm^2. The treatment time was 300 s and the final voltage equaled to 540 V. Experimental series were carried out with the sample coatings, which included calcium and phosphorus (Ca and P) on Ti and Mg alloy substrate. The samples were denoted in the text as: uncoated titanium—Ti 1; titanium with calcium-phosphate coating—Ti 2, and uncoated Mg alloy—Mg 1; with coating—Mg 1. The samples were punched to be 15 mm in diameter for snug fit in the wells of 6-well tissue culture polystyrene (TCPS) plates (Thermo Scientific, Germany). The samples were sterilized in a laboratory oven (Thermo Scientific, Denmark) at 180°C for 15 min (with controlling of surface properties), in accordance with the rules for sterilization of medical devices.

2.2. Animals

Study approval from the local Ethical Committee of the Pacific State Medical University (Vladivostok, Russia) was received under No. 2015–0102. For the experiments, adult, three-month old, 250 g of weight male rat was used. Animals were euthanized using carbon dioxide asphyxiation as approved by the MIT committee on animal care (National Institute of Health Guide for the Care and Use of Laboratory Animals, NIH Publications No. 80023, 1996).

2.3. Dendritic cell (DC) culture

The two primary cell types were used in this study are human peripheral blood mononuclear cell (PBMC) and rat bone marrow derived DCs (RMDC). Human PBMC were obtained from donor blood (Border station of blood transfusion, Primorye, Vladivostok, RU). All donors were in good health and were negative for blood-borne pathogens as detected by standard blood bank assays. The aphaeresis product was processed to enrich the PBMC fraction by using ficoll-hypaque (BioLegend, CA, USA) density gradient separation according to standard protocols as previously described [28]. RMDC were generated, as previously described, by Onai et al. [29]. Briefly, BM cells were removed from a male of rats and cultured in 24-well-culture plates, at a concentration of 5×10^6 cells per well, in 800 μl of RPMI-1640 (Lonza, Belgium) supplemented with heat-inactivated 10% fetal calf serum (FCS), 100 μg/ml of penicillin, 100 μg/ml of streptomycin, 5×10^{-5} m 2-mercaptoethanol (Lonza, Belgium) plus GM-CSF (50 ng/ml) and IL-4 (10 ng/ml). On days 3, 6, and 9 the supernatant was gently removed and replaced with the same volume of the supplemented medium. On day 9 of culture, ≈80% of the cells were CD11c$^+$ DC.

2.4. Cell viability assay

To determine toxicity levels of samples the cellular cultures RMDC were prepared at approximately 2000 and 20,000 cells per well, respectively, in 96-well flat bottom tissue culture plates. A mitochondrial colorimetric assay (MTT assay) by the percent of total succinate dehydrogenase (SDH) released was used [30]. In the each well with cellular monolayer, leaving 200 μL, to which 40 μL of MTT 1.2 mM solution (3-(4.5-dimethylthiazol 2-yl)-2.5-diphenyltetrazoliumbromide, Sigma-Aldrich, USA) was added. The cells were incubated at 37°C and 5% CO^2 for 4 h. The upper medium was removed carefully, and the intracellular formazan was solubilized by adding 200 μL of dimethyl sulfoxide to each well (Sigma-Aldrich, USA). Then, the contents of the wells were mixed thoroughly using a pipette. Two hundred microliters from

each well were transferred into a separate well on a 96-well ELISA plate (Corning Costar, Lowell, MA, USA). The absorbance was measured at 570 nm. The results expressed as optical density (OD) were obtained for three different experiments from each surface modification.

2.5. Dendritic cell phenotyping

Phycoerythrin (PE), allophycocyanin (APC), peridinin chlorophyll protein (PerCP-Cy5.5)—conjugated monoclonal antibodies (mAbs) specific for human CD14 (M5E2), CD34 (MEC14.7), CD38 (90), CD86 (2331), CD83 (HB15e), and HLA-DR (L243) (all from BioLegend, CA, USA) were used to determine the cell surface receptors expressed on the reverse transmigratory human PBMC DCs. Isotype control antibodies included MIgG2a (G155–178) and MIgG1 (MOPC-21) were also purchased from BioLegend. DCs were collected and labeled with the abovementioned specific antibodies for 45 min at 4°C in PBS containing 2% bovine serum albumin (BSA) and 0.05% sodium azide. Flow cytometry was used to determine the abundance of each cell type (subpopulation) expressing markers using MACSQuant™ Analyzer 10 (Miltenyi Biotec GmbH, Germany) and Kaluza 1.5 analysis software (Beckman Coulter, USA).

The mean fluorescence intensity (MFI) value for the expressing CD14, CD34, CD83 on RMDC (polyclonal antibody with species reactivity human, mouse, rat, 1:200, MyBioSource, Inc., USA) was analyzed using a confocal scanning laser microscope (Zeiss, Germany) connected to an Evolution MP Color Camera (Media Cybernetics Inc., Bethesda, MD, USA). The camera used Image-Pro Plus 7.0 software (Media Cybernetics Inc.), and the acquired digital images were processed using ImageJ software (National Institutes of Health, Bethesda, MD, USA) for qualitative analysis.

2.6. Methods of determination of the cells functional activity

RMDC incubated with samples of implants at 37°C and separate supernatant were frozen and stored at −20°C. The disrupted cells were mixed with 100 μL of Griess reagent, which consisted of equal volumes of 0.1% N (1 naphthyl) ethylenediamine dihydrochloride and 1% *n*-aminobenzene sulfanilamide (ICN, USA) in 2.5% phosphoric acid solution. After incubation for 10 min, the absorbances were measured at 540 nm using a Multiskan Titertek Plus spectrophotometer (Flow lab, Finland). Determination of the ATPase was determined by adding to the cellular monolayer 20 μl of substrate for ATPase (8 mg ATP on 1 ml of Tris HCl buffer (pH 7.8), which contained 87 mg of NaCl, 28.7 mg of KCl, and 52 mg of $MgCl_2$ 6 H_2O, ICN, USA) and the samples were left for 30 and 60 min. The reaction was stopped by adding 100 μl mixture of ascorbic and molybdenum acids at a ratio of 1:1. After 20 min, the optical density of the substrates was measured on a spectrophotometer at a wavelength of 620 nm. For the determination of the activity of lactate dehydrogenases (LDH), the Lloyd method in his own modification was used. 100 μl of substrate was added into wells of plate with adherent cells (2 mg/ml iodine nitro tetrazolium on phosphate buffer pH 7.2 with 0.4% $MnCl_2$, Sigma-Aldrich, USA) and incubated at 37°C for 30 min. Diformazan pellets were dissolved by adding 100 μl of isopropyl alcohol and acidified with 0.04 M HCl for 20 min. The optical density of the substrates was determined on a spectrophotometer at a wavelength of 650 nm. The activity of cytochrome oxidase was determined by adding to the cellular monolayer 100 μl of 0.1 M acetate buffer, pH 5.5, containing 10 mg/ml of $MnCl_2$, 0.33% hydrogen peroxide, and 2 mg/ml of diaminobenzidine. After 10 min incubation at room temperature, the reaction was stopped

by the addition of 10% sulfuric acid (100 μl per well). The quantity of formed product was determined by measuring the absorption at 492 nm. Samples containing the substrate solutions and 10% sulfuric acid were used as the control.

The results were obtained for three different experiments from each surface modification. The spectrophotometric data of optical density were evaluated as the simulation index (T), which was calculated as the ratio of the difference between the mean values of the optical density of the solutions containing reaction products of control and experimental cells, versus the mean value of optical density of intact cells (and expressed as per cents).

2.7. Cytokine assays

Measurements of RMDC cytokines RANTES, TNF α, IL-1, IL-6, IL-10 and IL-12 in the supernatants were performed by using specific solid-phase sandwich enzyme-linked immunosorbent assay (ELISA). Capture and detection cytokines used were purchased from Mouse ELISA Kit (Abcam, USA), using the procedure recommended by the manufacturer. The absorbances were measured at 450 nm by use of a microplate ELISA reader.

2.8. Scanning electron microscopy (SEM) of adherent RMDC

The qualitative analysis of cell adhesion was determined at 1, 3, 6, and 9 days. The disks were washed three times with warm D-PBS to remove the non- or loosely adherent cells. The cells samples were fixed with 1 ml 0.2 M cacodylate buffer (pH = 7.4) included 2% glutaraldehyde, 3% paraformaldehyde and 0.02% (w/v) picric acid (Sigma) overnight. The cells were washed three times with 0.2 M cacodylate buffer and were post-fixed with 0.5 ml 1% osmium tetroxide (OsO_4, Sigma) for 1 h. The cells were then dehydrated in a sequential series of increasing concentrations of acetone: 15, 30, 45, 75, 90, and 100% acetone for 30 min at each concentration. Subsequently, the samples were dried in an E3000 Critical Point Dryer (Quorum Technologies, Canada) and sputter coated with a thin layer (~5 nm) of carbon (JEE-420, JEOL, Japan). The micrographs were collected using scanning electron microscopy ULTRA PLUS-40-50 (Zeiss, Germany) in accelerating voltage 5 kV.

2.9. Statistical analysis

Data for the differentiation assay were analyzed by analysis of variance (ANOVA) and the Mann–Whitney method for comparisons between groups. The levels of cytokines and cellular proliferation, as well as the fluorescence intensity of the mature DC, were also analyzed by "ANOVA" followed by the Newman-Keuls test to determine multiple comparisons. Values were considered significant when different at $P < 0.05$.

3. Results

3.1. The morphology and activity of dendritic cells

Innate immunity is nonspecific and the first line of organism defense is carried out with the help of pattern-recognition receptors, which plays a significant role in the early reaction and the

subsequent pro-inflammatory response. The physical and chemical properties of the implants initiate various cellular reactions, such as absorption and intracellular biodistribution, which lead to a certain form of immune response [10, 31]. SEM micrographs showed that RMDC after contact with Ti 1 and Mg 1 had a spherical shape with an estimated size of 20 μm in diameter (**Figure 1B**, **E**). DCs treated with calcium phosphate coated exhibited more dendritic processes associated with mature DCs, the cells were larger with a folded surface; the formation of numerous dendritic appendages was also observed (**Figure 1D**, **F**). While in contact with Mg 1, the diameter of the cells was within the limits, the architectonics of the surface had folding, which indicated their activation (**Figure 1E**). In contact with coating Mg 2 the morphology of DC changes were similar with the morphology of cells contacted with Ti 2 (**Figure 1F**).

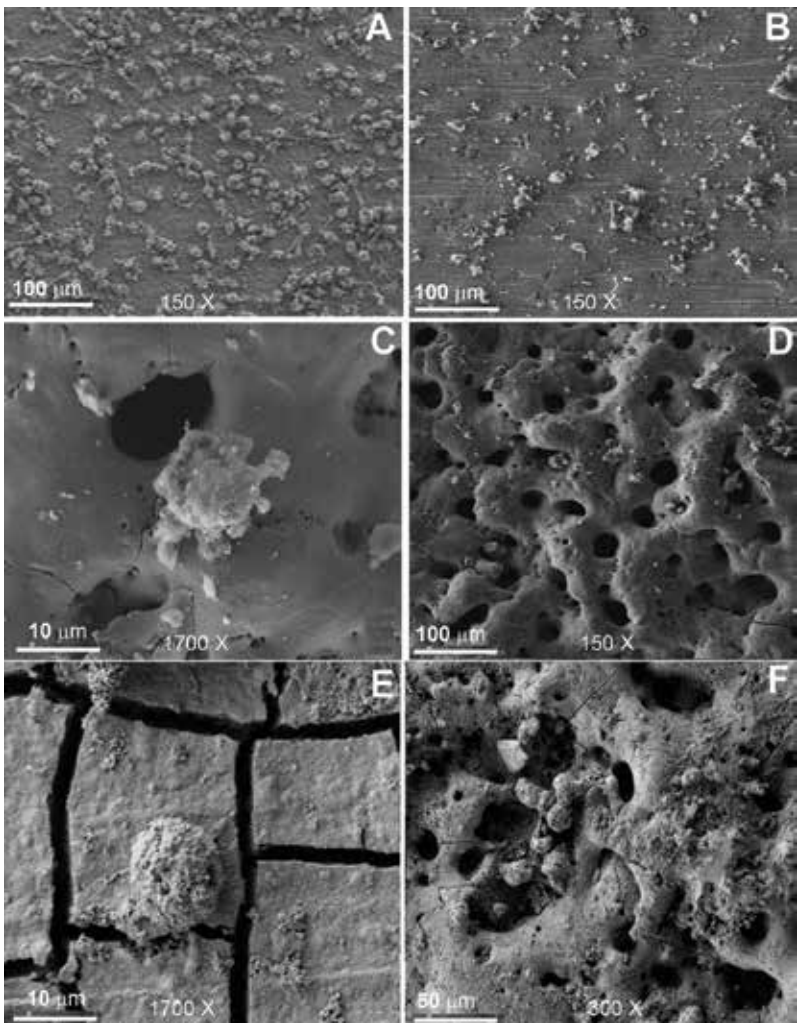

Figure 1. SEM of RMDC on the surface of collagen-coated glass (A) and samples. A typical architectonica of surface cell adhered to titanium Ti 1 (B) and Mg 1 (E); DC has a flattened shape without the presence of dendrites which is associated with immature DCs. In contact with calcium phosphate PEO-coated Ti 2 (C, D) and Mg 2 (F), the cells exhibited dendritic morphology which is associated with mature DCs; there have been numerous folds, and this surface was round and detected numerous pseudopodia. Data are from one of the two separate experiments, both with comparable results.

3.2. Cell viability

It is known that the MTT assay provides an estimate of total enzyme succinate dehydrogenase (SDH) released. SDH is a flavoprotein dehydrogenase and belongs to the succinate oxidase enzyme complex that forms the membrane respiratory chain of mitochondrion. The flavin group of this enzyme contains four iron atoms and is bound covalently to the protein [32]. We found no decrease of SDH activity of RMDC in the initial observation period (2 days) in DC contacting with Ti 1, Ti 2 and Mg 1, which indicates to a lack of cytotoxic effect of the studied samples (**Figure 2A**). A significant decrease of intracellular content after 3 days in cells in contact with Mg 1 and Mg 2 indicated a stimulated effect of these samples. It should also be noted that the dynamics of the cellular response on the samples with coatings was similar despite the different materials.

3.3. Enzymes activity of cells

Nitrite levels, an indirect measure of nitric oxide production, were assessed by the Griess Assay. Indicators for RMDC stimulated by Ti 1 was 14.5 uM, which was below than that stimulated by Ti 2, Mg 1 and Mg 2 (22.8; 21.9 and 19.03 uM), but the difference was not significant (P = 0.07). The level of cellular membrane released enzymes (ATPase) was significantly lower in the titanium Ti 1 and Mg 1 (T = −3.75 ± 0.5 and T = −6.25 ± 0.6%, respectively, 3 days contact) than in the coated samples (P = 0.024, **Figure 2B**). ATPase of membrane participates in

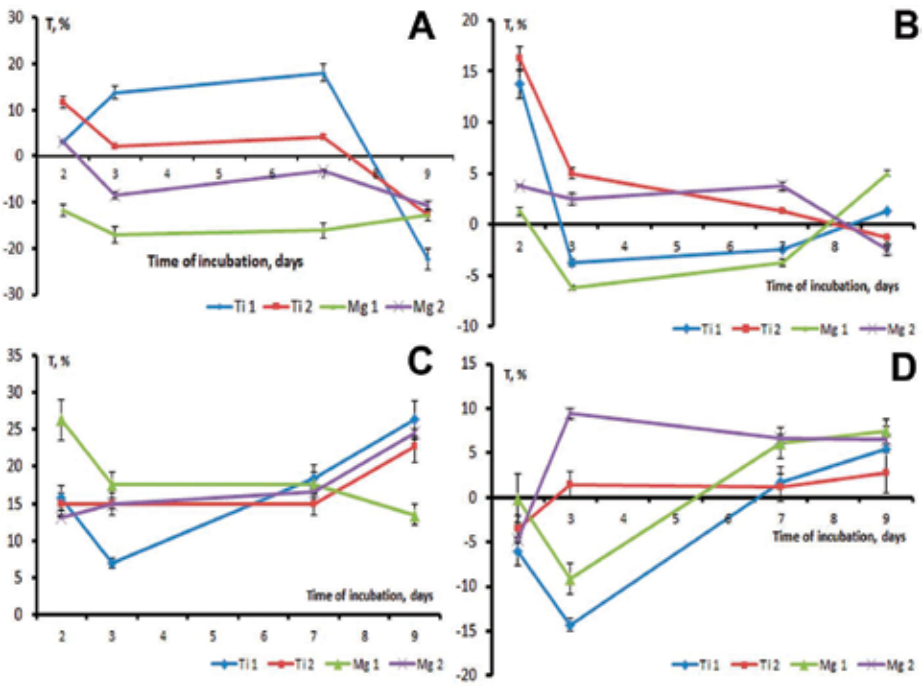

Figure 2. Enzyme activity of RMDC after contact with samples: A—succinate dehydrogenase; B—ATPase; C—lactate dehydrogenases; and D—cytochrome oxidase. Data presented as mean ± standard deviation of three independent experiments of time-course of cell contact. Ti 1—titanium without coating; Ti 2—calcium phosphate PEO-coating; Mg 1—Mg alloy without coating; Mg 2—calcium phosphate PEO-coating.

the hydrolysis of phosphate bonds and is indicators of stimulation of the cellular metabolism with a decrease in intracellular content. These data shows maximum stimulation of cells associated with the adhesion on samples surface within the first days and a difference ($p < 0.05$) of indices for coated on the titanium and magnesium (Ti 2, Mg 2) with less stimulating effects as compared with pure metals (Ti 1, Mg 1).

The LDH is a coenzyme dependent dehydrogenase and catalyzes the transfer of a reduced equivalent (hydrogen) from lactate to NAD^+ or from NADPH to pyruvate. LDH acts on the last step of hydrolysis that occurs under anaerobic conditions and results in the reduction of pyruvate yielding lactate and NAD^+. Most of the enzyme in the cell is weakly bound to the cell structure and localized in the cytoplasm, a smaller part being attached firmly to mitochondrial membranes [33]. We found no decrease LDH activity in the observation period, which indicates to a lack of cytotoxic effect of the studied samples (**Figure 4C**). A significant increase of intracellular content of this period reflected the increase of metabolic activity cells after contact with the samples at higher index stimulation for cells with Mg 1 ($p = 0.035$).

Cytochrome oxidase and SDH are the main components of the normal aerobic oxidative system of the tissue cells that are also known as the succinate dehydrogenase complex, where SDH is the first component and cytochrome oxidase is the second. Cytochromes are subdivided into three groups according to their chemical structure and spectrum: cytochromes a, b, and c. Oxidized cytochrome oxidase is reduced by cytochrome c catalyzing the transfer of four electrons to the oxygen molecule. Thus, cytochrome oxidase is a representative of the third group of oxidases. The difference was observed between indicators of enzymes depending on the sample type: the highest one was detected in cells contacted with Ti 2 and Mg 2 (2 and 3 days, **Figure 2D**). Thereafter, these parameters de-creased, thus showing the cells stabilization. Such a change in cell metabolism was associated with the components contained in coatings on the samples.

3.4. Effect of samples on DC maturation

Influenced of various stimulus DCs are undergoing a process of maturation that allows them to become more potent inducers of the adaptive arm of the immune response. In the absence of stimuli, the vast majority of DCs are immature. It is unclear whether or not treatment with metals implants can trigger DC maturation, but the ability of calcium phosphate PEO-coating to induce ROS in phagocytes suggests the possibility that these materials might activate this potent antigen-presenting cell (APC) population. DC maturation can be connected to the increased expression of the activation markers, CD1a and CD83, similar to what is observed in vivo [10, 11]. The RMDC treated with RPMI 1640 showed the typical expression of cell surface molecules as immature DC. In contrast the RMDC treated with PEO coated showed a clear change of the expression levels of CD14, CD34, and CD83 (**Figure 3**).

The $CD34^+$ cells in bone marrow are precursors of both the DC and granulocytes, and such cells are of the "intermediate" type on the 6th day of culture under the influence of an inducer are able to differentiate in DC or in leukocytes. In order to study the role of implants as maturation inducers, the receptor phenotype of human PBMC was analyzed. The primary culture of human PBMC was placed in vials with samples and cultured in the presence of GMCS and IL-4. As a control, cells adhered to the surface of specialized plastic coated with lectin

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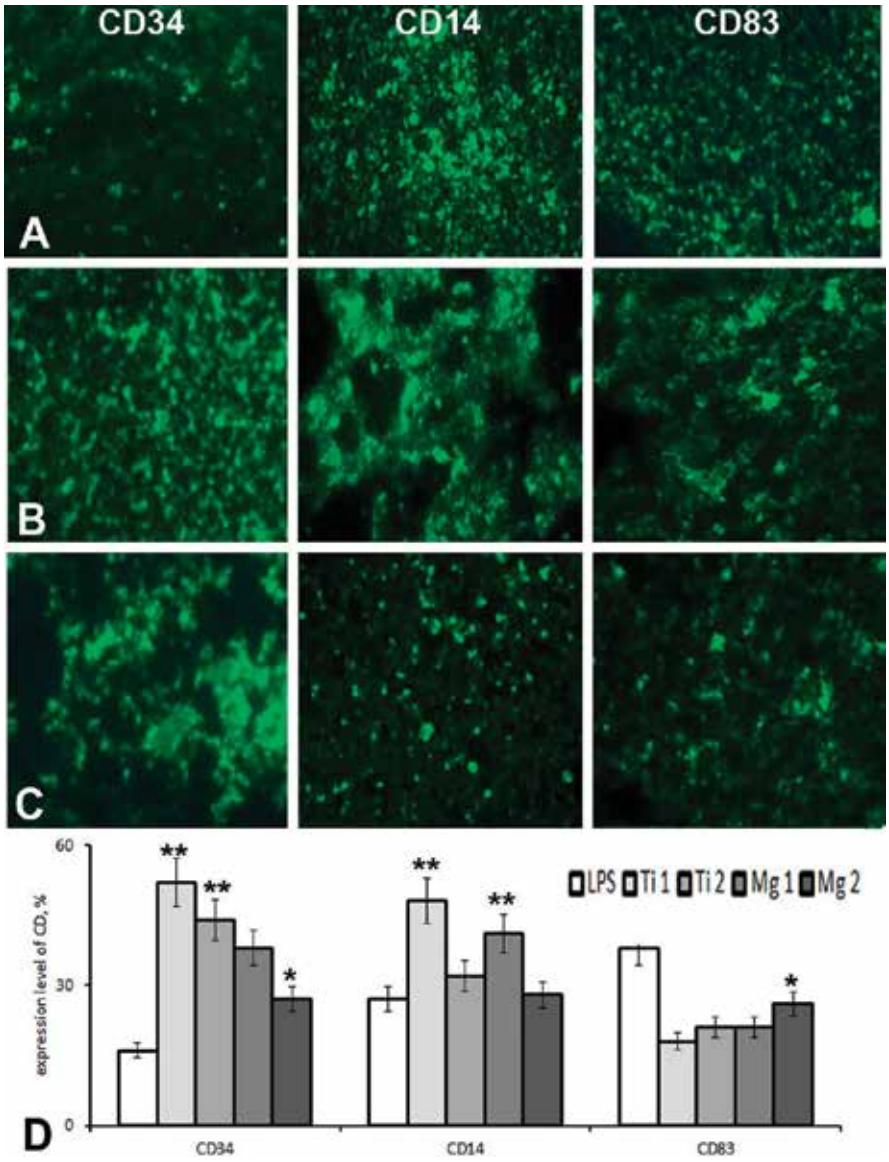

Figure 3. RMDC were cultured 9 days with LPS (A), magnesium Mg 1 (B), and calcium phosphate PEO-coating Mg 2 (C) and isolated. Representative photographs of immunofluorescent staining. DCs for FITC-conjugated anti-rat CD34, CD14, and CD 83 were analyzed by confocal scanning laser microscope, magnification 400×. Data are representative of three independent experiments (D); error bars represent mean ± SD (N = 20) with *$p \leq 0.05$ and **$p < 0.01$ as compared to LPS-stimulated DC.

were used, and lipopolysaccharide *Escherichia coli* (LPS) was added to obtain a population of mature DCs. We already know that culturing of DC in the presence of GM-CSF and IL-4 supplemented with 2.5 ng/ml of LPS stimulates maturation of DC and reduces the number of macrophages in culture. It was determined that the maximum expression of CD34 on the DK surface was observed on the 1st day of joint incubation with LPS, and the cell content was 72 ± 5.8%. Later, their number decreased, reaching the minimum figures by the end of

the observation period (1.6 ± 0.08%). Under the influence of implants, the number of $CD34^+$ cells compared to the control was lower. This way for samples with titanium after 1 day the index was 56 ± 4.8% and for samples with magnesium 48 ± 4.6%. The minimum number of these cells was noted at the end of the observation period (21 s) and amounted to 1.8 ± 0.2% and 2.4 ± 0.6%, respectively. Thus, the data obtained by us indicate an identical effect of the implants on the expression of the adhesion receptor, and the percentage of the content of these cells, reduced relative to the control, on their expressed effect as inducers of cell maturation.

As an indicator reflecting the direction of hematopoietic pool cell differentiation under the influence of implants, the degree of expression of membrane glycosylphosphatidylinositol-bound CD14 protein was determined. This component is an element of the CD14/TLR4/MD2 receptor complex, which recognizes the LPS, and is expressed on the surface of myeloid cells, especially on macrophages. We found that, in the control sample under the conditions indicated above, the minimum number of cells with a high degree of CD14 expression was determined on the ninth day, while the content of cells positive for detecting the DC CD83 terminal differentiation marker at these times the observation was maximum. The indicators were 14 ± 1.8 and 67 ± 5.8%, respectively. These data indicate that the introduction of LPS has a pronounced effect on the maturation of the DC population. During studying the degree of expression of these receptors in cell populations that contacted the implants, it was found that magnesium had the most activating effect on differentiation in the direction of the DC. Thus, on the 9th day of a joint incubation with magnesium, the $CD14^+$ cell count was 26 ± 2.8% and CD83 + 58 ± 4.6%, while on contact with titanium 32 ± 3.1 and 48 ± 3.6%. In subsequent observation periods, the number of $CD14^+$ cells was at the specified level, and when in contact with the implants, it decreased slightly. The data cited indicate a pronounced effect of the magnesium implant on the directionality of differentiation of hematopoietic pool cells mainly toward the DC.

The great interest is in the data obtained by us on the degree of expression of costimulatory molecules CD83 and CD86 on the surface of the DC during their interaction with implants, depending on the incubation time. These receptors of intercellular adhesion interact with the corresponding ligands with high avidity, under condition of their expression on cell membranes by clusters. Under effect of LPS on the degrees of expression receptors on day 9, the indicator of $CD14^+$ DC was minimal against the maximum of $CD83^+$ (2.4% ± 0.2 and 62.4 ± 0.6%, **Figure 4C**). Despite the fact that, at the initial time of observation, LPS activated maturation of DC more than implants, over time the level of expression of costimulatory molecules on the cell surface under the influence of titanium and magnesium increased (**Figure 4C**). Moreover, with relatively low values of $CD83^+$ and $CD86^+$ DC, the intensity of their luminescence increased (**Figure 4**) and expression of CD83 molecules on DC, incubated with implants remained elevated (23.8 ± 2.6 and 21.2 ± 3.4%, respectively) in comparison with DC treated with LPS (0.6 ± 0.2%) until the end of the observation period. In contact with titanium Ti 1, the number of $CD14^+$ cells in this period was 32 ± 2.1% and $CD83^+$ − 48 ± 2.6%, with coated titanium Ti 2–26.4 ± 2.1% and 52.6 ± 4.6% (**Figure 4**). These data indicate to the effect of coated on titanium as inductors of DC differentiation.

The leukocyte antigen CD38 is a bifunctional enzyme that combines ADP-ribosyl cyclase and cADP-ribosyl hydrolase activity, is expressed on hematopoietic cells, respectively, by their

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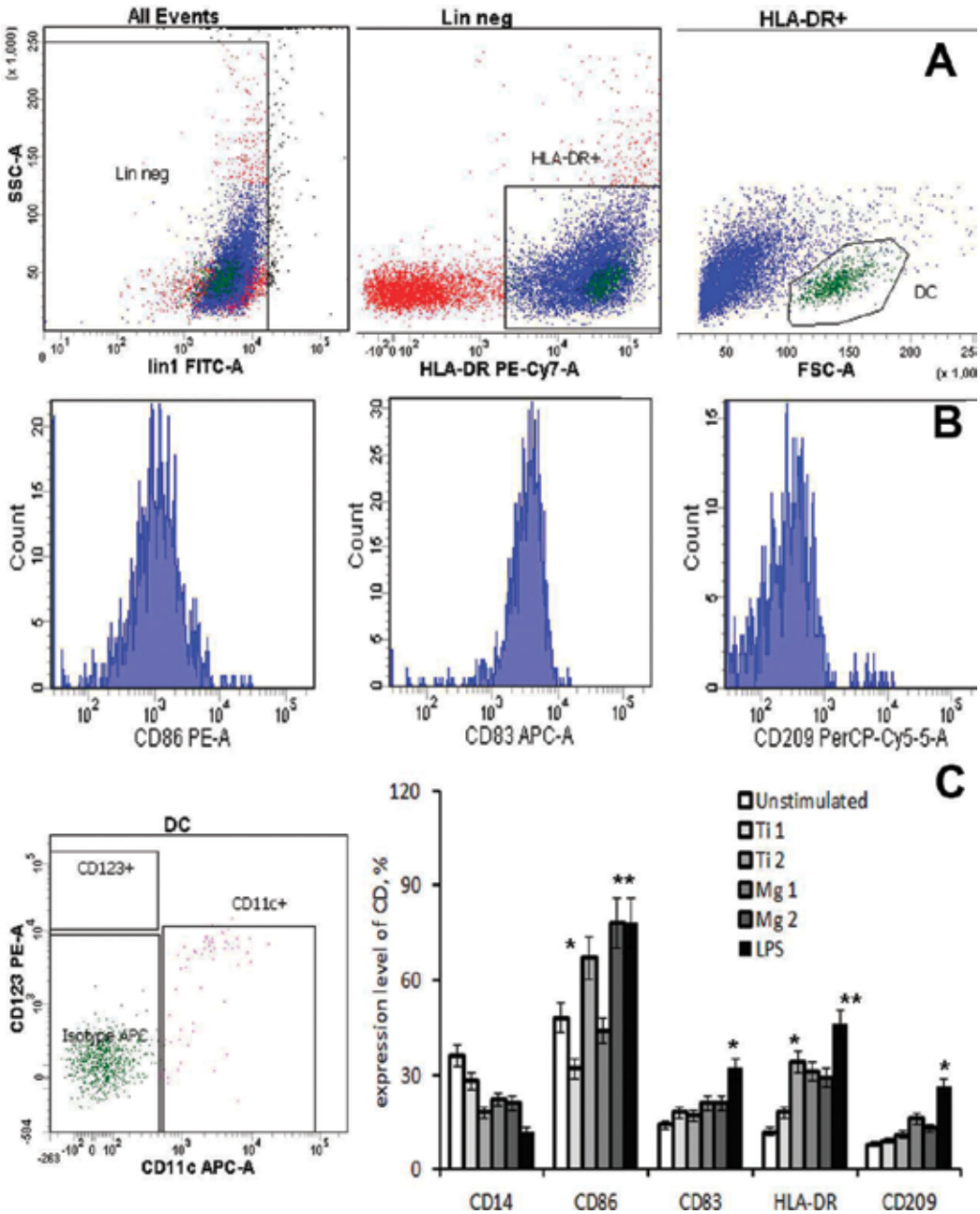

Figure 4. Phenotype profiles of DCs from human PBMC. The cells were cultured for 9 days in the presence of GM-CSF and IL-4 immature DCs (unstimulated), maturate LPS-stimulated DC (LPS) controls, and samples were isolated. (A) DCs were analyzed by flow cytometry for HLA-DR surface marker. (B) DC were stained with PE-conjugated CD86, APC-A-conjugated CD83, PerCP-Cys5–5-A conjugated CD209 and then examined by flow cytometry. (C) DCs were examined for CD11c, CD14, CD 83, CD 86, CD123 and CD 209 expression by flow cytometry. Data are representative of three independent experiments; error bars represent mean ± SD (N = 20) with *$p \leq 0.05$ and **$p < 0.01$ as compared to unstimulated DC.

degree of differentiation or proliferation. The product of the enzymatic activity of CD38-cyclic ADP-ribose is a universal catalyst of calcium from the internal depot. Moreover, the main function of the CD38 receptor is to regulate the activity of bone marrow cells, lymphoid tissue and peripheral blood, stimulating their production of cytokines, and also participates in the migration of the DC. Increased regulation of CD38 serves as a marker of cell activation, in particular, the process of differentiation of B-lymphocytes into plasmocytes. When studying the amount of $CD38^+$ phenotype in a pool of undifferentiated cells of the myeloid series before contact with samples and LPS, their content was determined to be 19.29 ± 1.74%. In the dynamics of interaction of these cells with LPS, the amount of $CD38^+$ increased, with a maximum value of 3 seconds after incubation, the indicator was 98.2 ± 9.7%. Then, in the samples incubated with magnesium, an increase in the number of $CD38^+$ cells on day 2 (91 ± 8.92%) was revealed with a subsequent decrease to the end of the observation period (28 ± 2.4%). Upon contact with titanium, the maximum $CD38^+$ cell content was observed only for 9 s of incubation and their value was 82 ± 7.8%. The above data indicate the presence of the inducing effect of implants on the maturation of DC, depending on the contact time, and more magnesium than titanium.

Treatment of pathogenic associated molecules generated a population shift from a precursor DC phenotype, traditionally $CD14^+$ and $HLA\text{-}DR^+$, to an increased number of immature and mature DC phenotype $CD14^-/HLA\text{-}DR^{+low}$, $CD14^-/HLA\text{-}DR^{+high}$, respectively. $CD14^+$ DCs express C-type lectin DC-specific intercellular adhesion molecule (ICAM)-3-grabbing non-integrin (DC-SIGN, CD209), which may also be found on monocyte-derived DCs, especially those generated under tolerogenic conditions such as IL-10. Analysis of the stimulated $CD14^-/HLA\text{-}DR^+$ population demonstrated significantly enhanced expression of mature DC-specific marker CD83, CD209 and the costimulatory molecule CD86 after contact with calcium phosphate PEO-coating implants compared to unstimulated controls and was similar to cultures stimulated with LPS (**Figure 4C**). There is an approximate 3-fold increase in DC-specific maturation marker CD83 expression on DCs treated with Ti 2 and Mg 2 over those given for cells on titanium ($p < 0.05$). This shift can be explained by stimulation of the resident cells population exposure properties of calcium-phosphate coating and the presence of molecular point-like effects on receptors. The increase in CD86 and CD123 indicate the DCs maturing ability for the costimulation of lymphocytes, triggering their subsequent activation and proliferation, further suggesting the potential to drive such an adaptive response.

3.5. Cytokine production of cells

DCs are a unique antigen-presenting cell that can both participate in inflammatory reactions, by producing a variety of inflammatory mediators, and directly respond to the product of these innate pathways. The study of titanium Ti and its coating effect on cellular production of cytokines showed that the level of pro-inflammatory cytokines, much difference between the indices for intact cells and after their contact with the samples, was found for the two cytokines TNF α and regulated on activation of cellular expressed and secreted RANTES (**Figure 5**). The greatest number of cytokine producing was in contact with titanium Ti 1 and a lower number in contact with calcium phosphate-coated Ti 2. This dependence was established in relation to anti-inflammatory cytokines production by cells—interleukin 6, 10, and 12. These data indicates that, in comparison with other studied samples, the smallest immune stimulatory effect applies to the calcium phosphate coated Ti 2.

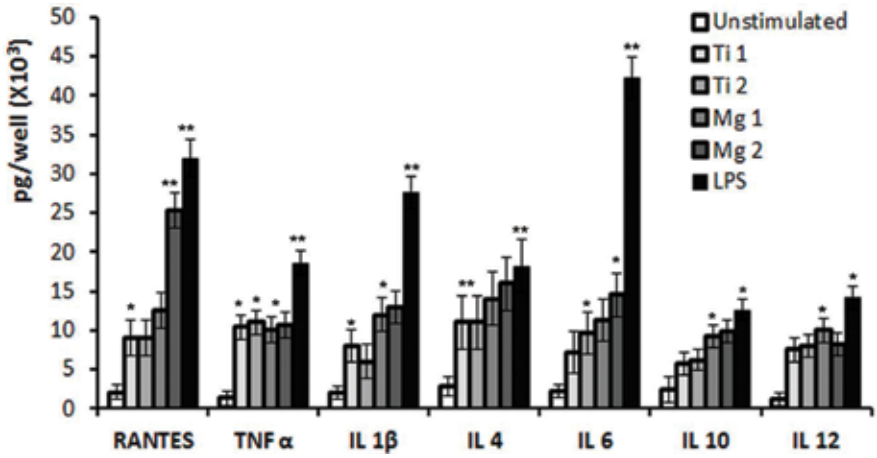

Figure 5. Cytokine release of RMDC treated with samples (Ti 1, Ti 2, Mg 1 or Mg 2) as compared to the immature DC (unstimulated) and maturate DC (LPS-stimulated DC) controls. Supernatant was harvested from each well and examined by cells ELISA. The cytokine amount was normalized to the total cell number in the well. Error bars represent mean ± SD (N = 120) with *$p \leq 0.05$ and **$p < 0.01$ as compared to unstimulated DC.

4. Discussion

During the maturation of the DC, the endocytic potential, the degree of expression of antigen-recognizing receptors decrease, and, on the opposite, the expression of adhesion molecules, which are bound to the plasma membrane, increases. The tolerant properties of highly differentiated DCs in triggering an immune response are manifested not only by their ability to present antigens to lymphocytes, but also by unique migration properties, that allow them to attach antigens to various tissues of the organism and transport them to regional lymphoid organs. In addition to the changes in cell organelles morphology, the activity of lysosomal enzymes and immunoproteas, which process the intracellularly synthesized protein antigens, is increased, the production of pro-inflammatory cytokines and various growth factors, through which T-lymphocytes and connective-tissue cells are activated [1, 10, 34]. These unique properties allow DC to be important participants in the process of bone tissue regeneration as initiators of osteolysis, by activating the differentiation and maturation of osteoclasts. From this point of view, the property of bioresorbable magnesium implant, revealed in our study, has a more pronounced effect, in comparison with bioinert titanium, on the process of directed differentiation and maturation of the DC, which is of a particular interest to us.

The data obtained by us indicate the identical effect of the implants on the expression of the CD34 adhesion receptor of hemopoietic cells, and the percentage of these cells' content, reduced relative to control factor, to their expressed effect as inducers of cell maturation. The cited data indicate a pronounced effect of the magnesium implant and calcium phosphate coated on the directionality of hematopoietic pool cell differentiation, mainly toward the DC, in comparison with titanium. Despite the fact that at the initial time of observation, LPS activated DC maturation more than implants, over time the level of expression of co-stimulatory molecules on the cell surface under the influence of titanium and magnesium has increased. The CD38 receptor

appears on $CD34^+$ committed stem cells and specific progenitor cells of lymphoid, erythroid and myeloid cells. It is considered that CD38 expression persists only in lymphoid progenitor cells during the early stages. The above data indicate the presence of the inducing effect of implants on the maturation of DC, depending on the contact time, and more magnesium than titanium.

The phenotype and morphology of DCs was differentially modulated by metal and calcium phosphate coated surfaces. Specifically, although the expression levels of DC maturation marker, CD83 and HLA-DR were not altered significantly, calcium phosphate coated treatment of DCs induced higher co-stimulatory molecule, CD86, expression relative of iDCs (Unstimulated). DC treatment with Ti 1 did not affect CD86 expression as compared to iDCs, presumably promoting a noninflammatory environment. Was showed that CD86 is the most sensitive marker for DC response to biomaterial treatments and is a valid variable for determining DC maturation levels [31]. Furthermore, DCs contacted with calcium phosphate coated exhibited much more extensive dendritic processes, a morphology associated with maturated. Consistent with the CD86 expression results, DCs incubated with Ti 1 and Mg 1 possessed a rounded morphology that is associated with immatureted. Despite the non-stimulating nature of metal implants cells were able to fully mature upon LPS challenge (data not shown).

The bio-anodized surface contains Ca and P ions incorporated from the electrolytic solution improves biological properties of metal implants [35, 36]. The developed unique electrolyte composition and the formation method helped the creation of biologically active PEO coating on the surface of titanium and magnesium, which might affect the occurrence of aseptic inflammation. The results presented herein that calcium phosphate coated contacted DCs were non-stimulating indicated the importance of surface porosity as a material property that modulates DC phenotype and enzymes activity. Whereas maximum stimulation of cellular enzymes associated with the adhesion on surface within the first hour and a difference between indices for coated titanium with less stimulating effects as compared with titanium. Most expressed stimulation of the cytochrome oxidase in DCs in contact with a hydroxyapatite coated was established. DC in the inflammatory response by regulating cytokines such as nitric oxide and pro- and anti-inflammatory cytokines including the development of inflammation in the tissue surrounding the implant [37]. Cytochrome oxidase and SDH are the main components of the normal aerobic oxidative system of the tissue cells that are also known as the succinate dehydrogenase complex, where SDH is the first component and cytochrome oxidase is the second. Oxidized cytochrome oxidase is reduced by cytochrome c catalyzing the transfer of four electrons to the oxygen molecule. The cytochrome oxidase activity in the cells reflects the level of oxidative metabolism. This enzyme contains cytogemmin with which molecule of NO communicates. In this case, at interaction of super oxygen anion with NO is formed peroxynitrites—the powerful oxidizer capable inhibits activity of mitochondrial enzymes cell. Definition of activity this enzyme allows indirectly estimating ability of cells to production NO on nitrite reductase ways [38]. Reduced cell response upon contact with the coating indicates to better properties with respect to biocompatibility as compared to metal implants. In general, these data indicate to ambiguous reaction of cell in contact with coatings and property of metal.

In addition, DCs contacted with samples surfaces produced differential cytokine profiles. Contrary to the high expression level of CD86 and dendritic morphology, calcium phosphate coated and Mg 1 contacted DCs released higher amounts of anti-inflammatory cytokine,

RANTES, IL 1β, and IL 6, compared to immature DCs or Ti 1 treated DCs. Although some trends in the release of TNF-α and IL-12 were observed, the differences were not statistically significant. A wider array of cytokines and chemokines were subsequently analyzed in order to better delineate the cytokine responses upon DC treatment with Ti surfaces. Treatment of DCs with calcium phosphate coated on Mg promoted enhanced production of the chemokine RANTES of the mediator acute and chronic inflammation, compared to immature DCs, and to a level similar to LPS treated mature DCs.

Unlike titanium the magnesium after a certain time interval in the insertion into the injury site, is resorbed by osteoclasts and other professional phagocytes and the highly-differentiated DCs, already presented at the site of aseptic inflammation, are of a great importance. These cells, due to the fact that they have already acquired the properties of highly specialized antigen-presenting participants in the process of elimination of undesirable components of inflammation, can also have an indirect effect on the process of osteosynthesis by producing a variety of factors, including cytokines, into interstitial space to attract connective tissue cell elements. The relatively low degree of activating effect of the titanium implant on the DC confirms its property of bioinertness in relation to the immune system, which indicates its positive qualities as a material that continuously stays in the organism. DC participates in the inflammatory response by regulating cytokines such as nitric oxide and pro- and anti-inflammatory cytokines. The materials of coating exhibited better biological compatibility than metal implants. The immunomodulatory properties of currently available implant coatings need to be improved to develop personalized therapeutic solutions. DCs exposed to the implantable materials ex vivo can be used to predict the individual's reactions and allow selection of an optimal coating composition, that take prospects for use of this cells for diagnostic and therapeutic approaches to personalized implant therapy.

5. Conclusions

Calcium phosphate-coated surfaces have a very similar chemical composition, but differs a kind of metal substrate with different properties; titanium is bio inert and magnesium is bioresorbable. The comparable levels of CD86 expression for DCs contacted with Ti 2 or Mg 2 surfaces suggested that kind of metal substrate is not crucial in modulating DC phenotype. The calcium phosphate coated surfaces have the same roughness, were prepared to retain their high surface energy by plasma electrolytic oxidation and were treated in the unipolar PEO-mode.

In this study, different of surfaces metal implants and coated were shown to induce differential DC phenotype upon treatment. DCs treated with calcium phosphate surfaces exhibited a more mature phenotype, whereas DCs treated with Ti 1 and Mg 1 surfaces maintained an immature phenotype. These results indicate another benefit of metal surfaces for promoting bone formation and integration by providing a local noninflammatory environment. Furthermore, calcium phosphate surfaces indicated possible material property–DC phenotype relationships for implant design. There is mounting evidence to suggest the involvement of the immune system by means of activation by metal ions released via biocorrosion, in the pathophysiologic mechanisms of aseptic loosening of orthopedic implants.

However, the detailed mechanisms of how metal ions become antigenic and are presented to T-lymphocytes, in addition to how the local inflammatory response is driven, remain to be investigated.

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Conflict of interest

The authors state that there is no conflict of interest.

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Chapter 6

Dendritic Cells: The Tools for Cancer Treatment

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Additional information is available at the end of the chapter

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Abstract

During cancer immune editing, the immune system shapes tumor fate in three phases through the activation of innate and adaptive immune mechanisms. After the elimination and equilibrium phase, the escape phase represents the final phase in which immunologically sculpted tumors begin to grow progressively. In this chapter, we will discuss which efforts are made to restore the balance in favor of the immune system making use of dendritic cells (DCs). The first approach is adoptive cell transfer, in which autologous DCs are generated and activated ex vivo. Secondly, we will discuss attempts in which pro-inflammatory or pro-migratory factors are delivered to attract and activate DCs in situ. Both strategies have the general goal to activate and mature DCs able to induce a robust tumor-specific T cell response. In addition, this chapter will discuss the clinical impact of DC-based therapies in cancer treatment focusing on the safety, feasibility, immunological responses, and clinical outcome.

Keywords: dendritic cells, immunotherapy, cancer, cell therapy, in situ vaccination

1. Introduction

The concept of a key role for the immune system in the protection against tumor development is longstanding and was formulated for the first time by Paul Ehrlich. He postulated that the immune system has the ability to suppress the majority of carcinomas [1]. However, the immune system can also promote tumor progression through chronic inflammation, selection of poorly immunogenic variants, and suppression of antitumor immunity. Together, the dual host-protective and tumor-promoting actions of immunity are referred to as cancer immune editing. According to this hypothesis, there are three phases in an immune response against tumors, the elimination, the equilibrium, and the escape phase, referred to as the three Es of cancer immune editing. During

the elimination, phase cells belonging to the innate and adaptive immune system can recognize and kill the tumor cells. When the tumor cells cannot be completely eradicated, the tumor and the immune system achieve a balance. During this equilibrium phase, the immune system controls the tumor cells but cannot eliminate the tumor. In the escape phase, the tumor cells can evade the immune control and develop further toward a tumor with clinical manifestations [2, 3].

DCs play an important role in the interface between innate and adaptive immunities. DCs have the unique capacity to take up antigens and present them in the context of major histocompatibility complex (MHC) I and MHC II in order to activate $CD8^+$ T cells and $CD4^+$ T cells, respectively. In order to be fully activated, T cells must be confronted with different activation signals. The initial interaction between the DC and the T cell, through the MHC and the T cell receptor (TCR), provides the first signal. In addition to MHC–peptide complexes, a costimulatory signal, the so-called signal 2, is required for T cell activation. $CD8^+$ T cells also require cytokine signals (signal 3), produced by macrophages and DCs, at different stages of their activation for the optimal generation of effector and memory populations and for their survival [4, 5]. The absence of signal 2 and/or signal 3 or the presence of immunosuppressive cytokines could either activate T helper 2 cells or elicit immune suppression through the induction and activation of regulatory T cells (Tregs), myeloid-derived suppressor cells (MDSCs), or dysfunctional DCs [6]. Tumors can disrupt these signals by increasing the production of immunosuppressive cytokines, by reducing levels of MHC I molecules, and by losing tumor antigens, thereby evading immune responses and eventually escaping immune control.

The development of DC vaccines is based on these properties. A successful therapeutic vaccine is mostly dependent on antigen-specific $CD8^+$ T cells educated to generate cytotoxic T lymphocytes (CTLs) that can directly kill cancer cells, but also a T helper response is important. Antigen-specific helper T cells are needed to sustain the CD8 response and induce longtime memory. The desired properties of vaccine-elicited $CD8^+$ T cells associated with elimination of cancer cells comprise (i) high TCR affinity and T cell avidity, (ii) production of high levels of granzymes and perforin, (iii) trafficking of T cells into the tumor and persistence in the tumor site, and (iv) high proliferation and renewal potential [7]. The components of the immune system necessary for the induction of such effector cells include (i) the presentation of antigen by appropriate antigen-presenting cells (APCs) and (ii) the generation of $CD4^+$ T cells producing cytokines which can help $CD8^+$ T cell proliferation and differentiation [6, 7]. Since DCs play a key role in the recognition and processing of antigens present on the tumor cells and in the induction of T cells capable of eliminating tumor cells, their use in the treatment of cancer is heavily investigated.

In this chapter we will describe different strategies that are explored to strengthen the immune system in the fight against cancer, focusing on the key role for DCs in this process. This chapter can only be a summary due to the constraints of time and space. We refer also to excellent reviews that have recently been published elsewhere [8–10].

2. Ex vivo DC vaccination

In order to generate a DC-based vaccine, autologous DCs (the so-called natural, circulating DC) or monocytes, that are induced to differentiate towards the so-called monocyte-derived

DCs, are isolated from patients by a leukapheresis. In order to fulfill the need for signal 1, these DCs are subsequently loaded ex vivo with tumor-associated antigens (TAAs). Afterward, the DCs are activated with different maturation stimuli, required to give these cells the capacity to provide signals 2 and 3 to the T cells. This ex vivo generated DC vaccine is then reinjected into the patient, in order to activate preexisting tumor-specific T cells and induce de novo antitumor immune responses leading to tumor regression.

Each step in the generation of a DC vaccine (isolation of specific DC subset from the peripheral blood or tumor of the patient, choice of TAA and loading of the DCs, maturation stimuli, and injection route) has an impact on the phenotype of the DCs and their capacity to activate T cells, thereby influencing the efficacy of the therapy. These factors will be discussed in detail below.

2.1. DC subset and source of the DCs

Two main subsets of natural DCs in the blood and lymphoid tissue can be distinguished: the myeloid DCs (mDCs) and the plasmacytoid DCs (pDCs). These DC populations can be distinguished based on the differential expression of phenotypical markers, their localization, and specific function. mDCs, also called conventional DCs (cDCs), are characterized by a high expression of MHC II and are efficient in inducing T cell proliferation. This subtype can be further divided into a $CD141^+$/$BDCA3^+$ population, called the cDC1 subset, and the $CD1c^+$/$BDCA1^+$ population, also called the cDC2 subset. Some studies have shown the superior ability of the cDC1 subtype to cross-present exogenous antigens on MHC I molecules, resulting in a robust $CD8^+$ T cell response [11, 12]. The cDC2 subpopulation is the predominant subset present in the blood and has been shown to be a potent $CD4^+$ T cell activator. Due to high expression of TLR3 and TLR8, these cells are able to secrete IL-12, resulting in the polarization of naïve $CD4^+$ T cells to IFN-γ-secreting Th1 cells, a critical step for tumor rejection in many models [13]. IL-12p70 has also been shown to promote natural killer (NK) cell activation, thus contributing to antitumor responses [14]. In contrast, pDCs are characterized by a low MHC II expression and lack of CD11c expression. These cells are important effector cells in immune responses due to their ability to produce high levels of type I interferon. pDCs also express high levels of TLR7 and TLR9 in endosomal compartments enabling them to respond to viral infections [15]. These subtype-specific functions may be harnessed in the design of a DC vaccine in order to elicit potent antitumor responses. Although natural DCs only constitute 1% of peripheral blood mononuclear cells (PBMCs), clinical trials have shown the potential of this approach and the promising clinical efficacy. A study in metastatic melanoma patients used the cDC2 subset or pDC subset isolated from the peripheral blood of the patients and loaded these cells ex vivo with tyrosinase and gp100 peptides. The vaccination approach using the cDC2 subset has been shown to be safe, technically feasible and resulted in a prolonged progression-free survival (12–35 months) in 4 out of 14 patients [16] (NCT01690377, NCT number, or ClinicalTrials.gov identifier). The pDC vaccine has also been shown to be safe and feasible and resulted in the induction of antigen-specific $CD4^+$ and $CD8^+$ T cell responses and a systemic type I IFN signature [17]. The use of natural DCs in the clinic is thus a promising, although challenging, avenue. Currently, several trials are ongoing in different cancer types aiming to compare the effect of using cDCs or pDCs as starting material to generate a DC-based vaccine, including melanoma (NCT02574377) and prostate cancer (PCa) (NCT02692976). Moreover, it has been shown that mDCs and pDCs can work together and

act synergistically, resulting in better antitumor efficacy of the vaccine. For example, a murine in vivo assay demonstrated the superior ability of a vaccine composed of both the mDC and pDC subsets to activate $CD8^+$ T cells and subsequently enhance antitumor responses compared to treatment with only one of the subsets [18]. Also, human mDCs and pDCs are able to cross activate each other. Co-application of DC subsets induced plasma cell differentiation from B cells and secretion of high levels of IFN-γ by peripheral blood lymphocytes and NK cells [19]. Further research is needed to confirm that the combination of different subsets of DCs included in a DC-based vaccine could result in better clinical results in different types of cancer patients compared to vaccines composed of only one subset.

Besides the blood and lymphoid DC subsets, there are two main subsets that have been characterized in the human skin: dermal DCs and epidermal Langerhans cells (LCs). A subpopulation inside the dermal DC population, characterized by the expression of CD14, seems to specialize in developing humoral responses, mainly by inducing naïve $CD4^+$ T cell differentiation into follicular helper T cells. LCs are more efficient in cross-presenting antigens and inducing naïve $CD8^+$ T cell differentiation into effector cytotoxic T lymphocytes [20]. LCs derived from $CD34^+$ hematopoietic stem cells are currently being tested in two phase I clinical trials in melanoma patients and patients with multiple myeloma (NCT01456104, NCT01995708).

The main obstacle to use these naturally occurring DCs to generate a DC-based vaccine is the difficulty to obtain large amounts of cells needed for the vaccine generation. Moreover, it has been shown that DCs isolated from cancer patients are often dysfunctional and impaired in their capacity to process and present TAA and subsequently activate T cells, resulting in the development of anergic T cells. In this regard, the discovery that DCs can be generated starting from PBMCs or $CD34^+$ hematopoietic progenitors allowed the production of clinical grade DCs ex vivo and opened new opportunities in the field of DC-based vaccination in cancer patients. PBMCs can be differentiated toward immature DCs by culturing these cells with GM-CSF and IL-4, while $CD34^+$ progenitors differentiate into DCs in the presence of TNF-α, GM-CSF, and Flt3L. The advantages of these approaches are the ability to overcome DC dysfunction in cancer patients and to obtain large numbers of DCs allowing multiple rounds of vaccination. Although time-consuming and costly, this approach is used in the majority of clinical trials.

2.2. Antigen selection and loading

2.2.1. Types of antigen

TAAs can be divided into overexpressed self-antigens, mutated self-antigens, and tumor-specific antigens. Although they are preferentially expressed by tumor cells, TAAs are oftentimes found in normal tissues. However, their expression differs from that of normal tissues by their higher expression levels in the tumor, by alterations in their protein structure, or by their aberrant subcellular localization within tumor cells. Ideally, antigens included in a cancer vaccine are expressed on the surface of all cancer cells and are immunologically relevant and important in maintaining a malignant phenotype. The first TAA identification was made in the context of melanoma with melanoma antigen family A1 (MAGE-A1) in 1991; its expression is restricted to male germ line cells and trophoblastic cells [21]. MAGE-A1 is a member of a large

gene family, comprising of 25 cancer germ line genes. Cancer-testis antigens (CTA) are one of the most prominent TAAs and include antigens such as the MAGE antigens and NY-ESO-1.

In recent years, with the development of deep sequencing technologies, studies have revealed the presence of antigens resulting from somatic mutations and giving rise to proteins with altered sequence. These mutation-derived antigens, also known as neo-antigens, are tumor-specific and patient-specific. Targeting neo-antigens would overcome self-tolerance, and the induction of low-avidity clones is observed when using non-mutated self-antigens in the vaccine [22, 23]. Since several studies have shown that the mutational load is a predictive biomarker in patients treated with checkpoint inhibitors, adding DC-based vaccines that will directly target neo-antigens during treatment will potentially work synergistically [24–27]. This approach already generated promising results. For instance, a study in stage III melanoma patients, which used DCs pulsed with peptides encoding for neo-antigens after prior treatment with ipilimumab (anti-CTLA-4 antibody), demonstrated that the DC vaccines induced a diverse neo-antigen-specific T cell receptor repertoire (see 2.5. for other examples). The ultimate aim of a DC-based vaccine is not only to induce an immune response against the antigens included in the vaccine but also to result in antigen spreading leading to a broad immune response capable of recognizing the heterologous cancer cell populations present in the tumor environment.

2.2.2. Antigen loading

Several methods have been used to enhance DC loading with TAAs, in order to increase DC vaccine efficacy. DCs can be loaded with peptides, DNA, RNA, or tumor cells to provide them with the TAAs. An overview of the advantages and disadvantages of each method is given in **Table 1**.

2.2.2.1. DC pulsed with peptides

Loading DCs with short peptides (between 8 and 11 amino acids long), predicted to bind with MHC I and resulting in $CD8^+$ T cell responses, is the most common method to load DCs with antigens in the field of DC vaccination. Since DCs loaded with these peptides can only activate $CD8^+$ T cells, an alternative method is to load DCs with long peptides (between 28 and 35 amino acids long). Due to their length, these peptides are presented by DCs through cross-presentation and are thus capable of targeting both $CD4^+$ and $CD8^+$ T cells. A phase II study is currently being conducted assessing the immunogenicity of a vaccine consisting of DCs matured with poly-ICLC and loaded with NY-ESO and MelanA peptides coupled to an adjuvant called Montanide ISA-51 (NCT023334735).

2.2.2.2. DCs pulsed with DNA/RNA

DCs can be genetically modified to produce and express tumor-associated antigens, by introducing DNA or RNA encoding for these TAAs through nonpathogenic viruses, such as adenoviruses, or through electroporation.

mRNA has a short half-life and can be loaded directly on DCs without using vectors or requiring knowledge of the patient's haplotypes. The electroporation method has been shown to be the most efficient way to introduce mRNA into DCs, by temporarily increasing cell permeability

Antigen-loading method	Advantages	Disadvantages
Peptides	• Easy to manufacture • Easy for immune monitoring	• Limited to tumor types with known/identified TAAs • MHC restriction
RNA	• No integration in host genome • Encoding for specific antigen • Different antigens can be encoded by one construct • Possible to encode for maturation agents	• Variable expression • Vulnerable to degradation • Limited to tumor types with known/identified TAAs
DNA	• Antigen presentation in high levels	• Integration in host genome • Limited to tumor types with known/identified TAAs
Tumor lysates	• No need to identify the TAAs in advance • Presentation of multiple epitopes resulting in both CD4+ and CD8+ responses • Prolonged antigen presentation	• The presence of immunologically irrelevant antigens/self-antigens can induce T cell tolerance • Co-delivery of immunosuppressive factors (IL-10, TGF-β) hampering DC maturation and T cell-stimulating capacity • Labor intensive
DC/tumor cell fusion	• No need to identify the TAAs in advance • Presentation of multiple epitopes resulting in both CD4+ and CD8+ responses • Prolonged antigen presentation	• Low fusion efficiency • Limited availability of autologous tumor cells

Table 1. Overview of the advantages and disadvantages of different antigen loading methods used to generate DC-based vaccines.

through application of an electric field, thus facilitating mRNA entry into the cells, without the need for additional agents [28]. A transfection with mRNA enables presentation of multiple antigens, as well as the loading with maturation agents and cytokines simultaneously. Transfection of tumor-derived RNA in DCs was used to treat melanoma patients, and antitumor T cell responses were reported in about 50% of the patients [29]. Besides delivering TAAs to DCs, RNA transfection can also deliver maturation agents to DCs in order to enhance their T cell stimulatory capacity [30], thereby overcoming the need to mature the DCs in vitro through the addition of different maturation stimuli and shortening the time needed to generate the vaccine.

Plasmid DNA transfection provides a more stable gene material, thus offering longer expression time. One of the most efficient ways to transfer DNA into DCs is through nucleofection, a nonviral, electroporation-based method that allows the DNA to directly enter the nucleus [31]. A study showed transfection of tumor DNA into DCs to be feasible and to result in DCs capable of presenting antigens to T cells resulting in the activation of antigen-specific T cells [32]. However, a major drawback to use DNA in the generation of a DC-based vaccine is the risk of genome integration of the DNA. This risk can be circumvented by using mRNA to load the DCs with the desired antigens.

2.2.2.3. DC pulsed with tumor lysates

Generating tumor lysates can be done through multiple freeze–thaw cycles or by irradiating tumor cells with UV rays [33]. These methods have been shown to induce both apoptosis and necroptosis of the tumor cells and result in the release of DAMPs, such as heat-shock proteins (HSP) and high-mobility group box 1 (HMGB1). These factors are known to induce the so-called immunogenic cell death that results in subsequent DC maturation, thereby promoting effective immune responses. DCs, matured with LPS and IFN-γ and subsequently loaded with tumor lysates, were clinically tested in ovarian cancer patients. Vaccination with these DCs resulted in robust IL-12 production and potent $CD4^+$ and $CD8^+$ T cell responses [34]. A phase III study in glioblastoma using DCs loaded with autologous tumor lysates (DC-VAX-L) is currently ongoing (NCT00045968). A pilot clinical trial testing a personalized vaccine was conducted using autologous DCs pulsed with oxidized autologous whole tumor cell lysate. Vaccination induced T cell responses to autologous TAAs and previously unrecognized neo-epitopes, associated with prolonged survival [35].

2.2.2.4. DC/tumor cell fusions

DCs can be fused to tumor cells using polyethylene glycol as a fusogenic agent or by electrofusion. The advantage of this technique is that all the TAAs that are expressed by the tumor can be processed by the DCs and presented in the context of both MHC classes I and II, resulting in the activation of $CD4^+$ and $CD8^+$ T cells [36, 37]. A clinical study in multiple myeloma patients has shown this approach to be safe and feasible and to result in effective T cell responses and disease stabilization in a majority of the patients [38].

The optimal antigen loading method has not yet been identified, and all of the current methods have advantages and disadvantages (see **Table 1**), but it is clear that delivering antigens in the context of both MHC I and MHC II is of critical importance in order to induce a sustained antitumor response.

2.3. DC maturation

In order to induce a potent antigen-specific T cell response, immature ex vivo generated DCs need to be fully matured before their readministration to the patients. The readministration of immature DCs has been shown to induce tolerance instead of immunity against the target antigen(s) [39, 40]. Studies comparing the immunogenicity of immature and mature DCs show that maturation is necessary for the induction of immune responses in patients [41, 42]. Mature DCs are characterized by a high expression of MHC class I and II molecules on their surface, expression of different costimulatory molecules, secretion of pro-inflammatory cytokines and chemokines necessary for T cell activation, and migration toward the draining lymph nodes. The production of inflammatory cytokines by DCs is essential in order to activate $CD8^+$ T cells. The amount of IL-12 produced by DCs was shown to correlate with the induction of strong antitumor responses. Two clinical trials, one in melanoma [33] and one in malignant glioma [42], have shown that high IL-12 concentration derived from DCs was predictive for a favorable clinical outcome. Initially, DC vaccines consisted of DCs generated ex vivo that were loaded with tumor antigens but without the inclusion of a maturation stimulus to activate the DCs. Although safety and feasibility were established, encouraging different research

groups to further explore this strategy, this first generation of DC vaccines actually showed limited success [43]. The lack of maturation was thought to be a major reason for failure. These observations led to great efforts put into developing different types of maturation stimuli that would increase DC-based vaccine efficiency resulting in profound clinical effects. Moreover, the timing of maturation of the DCs seems to be an important factor to consider. Several studies have described "DC exhaustion," characterized by a loss of IL-12 secretion capacity and T cell activation capacity after gaining full maturation [44, 45].

2.3.1. TNF-α, IL-1β, IL-6, and PGE_2 cocktail

In early years the golden standard to maturate DCs ex vivo in the vaccine field was the addition of a cocktail of the pro-inflammatory cytokines TNF-α, IL-1β, and IL-6, with prostaglandin E_2 to cultures of immature DCs [46]. This cocktail has been shown to increase the expression of MHC I and II molecules, CD40, and CCR7 but on the other hand also failed to stimulate IL-12p70 production. Although PGE_2 has been shown to promote migration of DCs toward lymphoid tissues through the upregulation of CCR7 expression on DCs, some studies have suggested that PGE_2 might be responsible for the lack of IL-12 production and for inducing Tregs and MDSCs, leading to suboptimal immune responses [47, 48]. This prompted a search for alternative maturation cocktails.

A combination of TNF-α, IL-1β, INF-α, IFN-γ, and poly-IC creates so-called α-type 1 polarized DCs, characterized by an improved IL-12p70 production. DCs matured with this cocktail and loaded with glioma-associated antigen were used to treat patients with malignant glioma and resulted in an upregulation of type 1 cytokines and chemokines, including interferon-α and CXCL10. Trials assessing α-type 1 polarized DCs are currently ongoing in several cancer types (NCT02151448, NCT00970203, NCT01876212).

2.3.2. Toll-like receptors

Immune cells are capable of sensing pathogens via pattern recognition receptors such as the Toll-like receptors (TLRs). Activation of TLRs, by different pathogens or danger signals, on DCs induces the activation of different signaling pathways responsible for the upregulation of costimulatory molecules and production of pro-inflammatory cytokines. Many vaccine adjuvants, targeting these receptors, have been developed in the last two decades. Poly-IC is a synthetic TLR3 ligand analog, widely used in clinical trials, including trials with DC vaccines. TLR3 ligand used as an adjuvant increased DC maturation, interferon secretion, and tumor suppression [49–51]. TLR4 recognizes lipopolysaccharide (LPS) structures present on the membrane of Gram-negative bacteria. LPS combined with IFN-γ results in the activation of highly immunogenic DCs, eliciting strong antigen-specific CTL responses and a high IL-12/IL-10 ratio in vitro [52, 53]. A nontoxic derivative of LPS called monophosphoryl lipid A (MPL) is a TLR4 agonist used in many vaccines as a cancer vaccine adjuvant [53–56].

Simultaneous activation of different TLRs has also been shown to mediate synergistic effects on DCs resulting in strong upregulation of IL-12 production, thus enhancing and sustaining a Th1-polarizing capacity. It was shown that DCs matured with poly-IC, a synthetic TLR3 ligand analog and/or R848, are able to produce high levels of IL-12p70. The addition of PGE_2 improved

the migratory capacity of the DCs while maintaining their IL-12p70 production capacity upon T cell encounter in vitro [57]. Synergy induced by TLR combinations is further discussed in [58].

The use of TLR agonists to mature DCs is discussed in detail further in this chapter.

2.3.3. CD40L

CD40L, a member of the TNF superfamily, is expressed on activated T cells and interacts with CD40 present on activated DCs and other APCs. The CD40/CD40L pathway plays a role in T cell priming and differentiation. Furthermore, CD40 engagement with CD40L is necessary for DC activation and results in an upregulation of costimulatory molecules and secretion of cytokines such as IL-12. Delivering CD40L to DCs mimics T cell help and allows DC licensing in order to induce $CD8^+$ cytotoxic T cells. A phase II study using DCs loaded with tumor RNA and CD40L RNA combined with sunitinib (a tyrosine kinase inhibitor) in metastatic renal cell cancer patients showed the vaccine to be well tolerated, and clinical benefits were experienced in 62% of the patients [59]. A phase III trial assessing the overall survival benefit of using this approach is currently ongoing (NCT01582672). DCs matured with a combination of CD40L and IFN-γ, resulting in an enhanced IL-12p70 production, and loaded with gp100 were used to vaccinate melanoma patients. The amount of IL-12p70 was positively correlated with the generation of gp100-specific $CD8^+$ T cells [42].

2.3.4. TriMix

TriMix is a maturation cocktail consisting of mRNA encoding for CD40L and CD70 and a constitutive active form of TLR4. The aim of this mix is to mimic the physiological activation of antigen-presenting cells (APCs). It facilitates (i) DC activation, (ii) DC maturation, and (iii) activation of cytotoxic $CD8^+$ T cells by DCs. The introduction of CD70 provides a costimulatory signal to naïve T cells expressing CD27, thus supporting T cell proliferation. This formula has been shown to result in maturation and increased T cell stimulatory capacity of DCs. The additional benefit is the use of RNA technology to deliver these components simultaneously with the antigen-encoding mRNA, through electroporation, thus avoiding incubation of DCs with different cytokines that can result in exhausted DCs. This mix has been shown to enhance secretion of IL-12 and several other pro-inflammatory cytokines by DCs and to stimulate differentiation of naïve $CD4^+$ T cells into effector Th1 cells [30, 60–62]. A phase I trial in advanced pretreated melanoma patients showed these TriMix DCs to be safe and immunogenic, resulting in a 27% objective response rate [63]. The addition of ipilimumab (an anti-CTLA-4 monoclonal antibody) to the TriMix DCs in a phase II trial in advanced pretreated melanoma patients resulted in a 6-month disease control rate of 51% and overall tumor response rate of 38% [64].

2.4. Optimal delivery route

Importance of the delivery route lays in the ability of the injected DCs to travel to lymph nodes. The lymph nodes are the rendezvous point for DCs and T cells to interact. Several delivery routes have been tested including intradermal, subcutaneous, intravenous, intraperitoneal, intranodal, and intratumoral delivery of ex vivo generated DCs. When injected intradermally,

a majority of the DCs seem to remain at the injection site, with only less than a few percentages of injected cells reaching the draining lymph nodes. Nevertheless, this small amount of DCs was sufficient to induce antigen-specific responses [65]. DCs delivered intratumorally show retention at the tumor site with little migration to the lymph nodes [66]. In a phase I trial with metastatic melanoma patients, autologous peptide-pulsed DC vaccines were injected via different routes (intranodal, intravenous, and intradermal). This study concluded that the intranodal route seems to be superior for T cell sensitization [67]. Furthermore, according to a meta-analysis of clinical trials of DC-based vaccines in pancreatic cancer and renal cell carcinoma, the choice of the administration route seems to influence therapeutic efficacy. Indeed, lymph node-targeting routes (subcutaneous, intradermal, and intranodal) were shown to be superior over the intravenous route [68]. Nevertheless, the intravenous route has been shown to be an effective administration method for DC vaccines, in the context of some pancreatic carcinoma and melanoma trials. Combining several administration routes is an alternative strategy. The combination of intradermal with intravenous delivery of TriMix DCs has shown to elicit durable clinical responses in advanced melanoma patients [63].

2.5. Combination therapy

2.5.1. Checkpoint inhibitors

The expression of different inhibitory immune checkpoints can affect the efficacy of a DC vaccine by hampering tumor-specific T lymphocytes to exert their function. In recent years, several monoclonal antibodies against checkpoint inhibitors have been developed, with the aim to recover T cell cytotoxicity. The best characterized checkpoint receptors are CTLA-4 and PD-1/PD-L1. Clinical evidence in patients with melanoma suggests that combining anti-CTLA-4 monoclonal antibodies with DC therapy is more effective than either agent alone [69]. It was shown that treatment of patients with advanced melanoma with DCs electroporated with mRNA encoding for TriMix and loaded with four melanoma-associated antigens combined with ipilimumab resulted in an overall response rate of 38% and a 6-month disease control rate of 51% [64]. Treatment with DCs loaded with neo-antigens of patients who underwent ipilimumab treatment showed to elicit antigen-specific $CD8^+$ T cells [70].

Although this approach showed promising results, anti-CTLA-4 monoclonal antibodies are associated with a high percentage of immune-related toxicity. Alternatively, PD-1 monoclonal antibodies, which inhibit PD-1/PD-L1 interactions on tumor cells, seem to have a more favorable toxicity profile. The combination of PD-1 inhibitors with DC therapy is currently being tested in several clinical trials for several cancer types (NCT01067287, NCT02528682, NCT03152565, NCT03014804, etc.) [8].

2.5.2. Chemotherapy

Antitumor immune responses induced by DC therapy can be further enhanced by endogenous production of immune stimulatory cytokines. Lymphodepleting chemotherapy can create an optimal cytokine environment for expansion of antitumor immune cells (T cells and NK cells), by eliminating negative immune cell populations and lowering tumor burden. The recovery phase following lymphodepletion creates an opportunity to use DC vaccines combined with adoptive T cell therapy. Furthermore, some chemotherapeutic agents have been

shown to induce immunogenic cell death of tumor cells, which can further potentiate anti-tumor immune responses elicited by DC therapy [71]. AML patients treated with autologous AML/DC fusion cells after chemotherapy resulted in a lack of relapse in 72% of the patients, at a median follow-up of 57 months [72]. Other examples of synergy between chemotherapy and DC therapy can be found in [73, 74]. Several clinical trials assessing the combination of DC vaccine with chemotherapy (with or without other interventions) are currently ongoing in several cancer types (NCT00082641, NCT00338377, NCT00617409, NCT02649582, etc.) [8].

2.5.3. Targeted therapy

Increasing the effectiveness of DC vaccines can be achieved by modulating the tumor microenvironment through a decrease in immune suppressive cells at the tumor site (**Figure 1**).

Regulatory T cells (Tregs) play a role in the induction of peripheral tolerance, by downregulating activation of autoreactive T cells. In most tumor types, the presence of Tregs correlates with a poor clinical outcome [75]. Depletion of Tregs can be achieved by using monoclonal antibodies targeting CD25 (IL-2 receptor α chain). The use of daclizumab combined with antigen-pulsed DCs not only in a trial in metastatic cancer patients resulted in the depletion of Tregs but also in a suppression of tumor-specific CTLs (due to the expression of CD25 on both Treg and effector T cells) [76]. Another CD25-targeting strategy is the use of denileukin diftitox (also known as ONTAK), a recombinant IL-2-diphtheria toxin antigen. Although a study showed ONTAK to be able to deplete Tregs, while maintaining antigen-specific CTLs in renal cell carcinoma patients [77], contradictory evidence demonstrates the induction of tolerogenic DCs and depletion of NK cells when using ONTAK [78]. A study assessing the combination of ONTAK with DC therapy has recently been completed in stage III and IV melanoma (NCT00056134), and one trial is currently ongoing in ovarian cancer (NCT00703105). A non-CD25 targeting therapy is the inhibition of indoleamine 2,3-dioxygenase (IDO), an immune regulatory enzyme that supports Treg function. A trial assessing the combination of 1-methyl-D-tryptophan (an IDO inhibitor) with DC therapy in metastatic breast cancer patients has recently been completed (NCT01042535). Other non-CD25 targeting drugs such as sunitinib and dasatinib (tyrosine kinase inhibitors) are known to inhibit Treg activity. The combination of sunitinib with DC therapy is currently being tested in a phase III trial in patients with advanced renal cell carcinoma (NCT01582672).

MDSCs are a heterogeneous population of immature progenitor cells, known to suppress T cell function. MDSCs have been shown to negatively regulate immune responses in cancer [79]. It was shown that MDSCs can impair the activity of DC vaccines, by decreasing the ability of DCs to mature, take up antigens, migrate, and induce IFN-γ production by T cells [80]. The targeting of MDSCs can be done through different ways. Beside the effect of sunitinib on Treg, this tyrosine kinase receptor can also deplete MDSCs. Cyclooxygenase-2 (COX-2) inhibitors were shown to decrease expression of MDSC-attracting chemokine CCL2 and increase expression of CXCL10, thereby promoting attraction of CTLs. A phase II trial is currently assessing the safety and feasibility of α-type 1 polarized DCs with the addition of a COX-2 inhibitor called celecoxib in patients with peritoneal surface malignancies (NCT02151448). Many other therapies can decrease MDSC-mediated immune suppression such as VEGF inhibitors, all-trans retinoic acid, lenalidomide and chemotherapeutic drugs such as gemcitabine. Clinical trials combining these agents with DC based vaccination are ongoing.

2.6. Conclusions

Although it is shown that ex vivo DC vaccination strategies are safe, well tolerated, and capable of inducing tumor antigen-specific immune responses in a substantial number of vaccinated patients, a lot of challenges remain. Generating autologous ex vivo DCs is a time-consuming and labor-intensive process with significant logistic challenges and high production costs [8]. In addition, only specific types of tumor have been studied in the ex vivo setting, mainly due to practical limitations. These include the lack of appropriate tumor antigens or the absence of sufficient tumor material when tumor lysates are used for antigen loading of DCs [81]. Therefore, the in situ modification of DCs represents an attractive alternative strategy.

3. In situ DC vaccination

To overcome the limitations associated with the ex vivo DC manipulation, different strategies to manipulate DC in vivo are currently under investigation. For example, by targeting in vivo

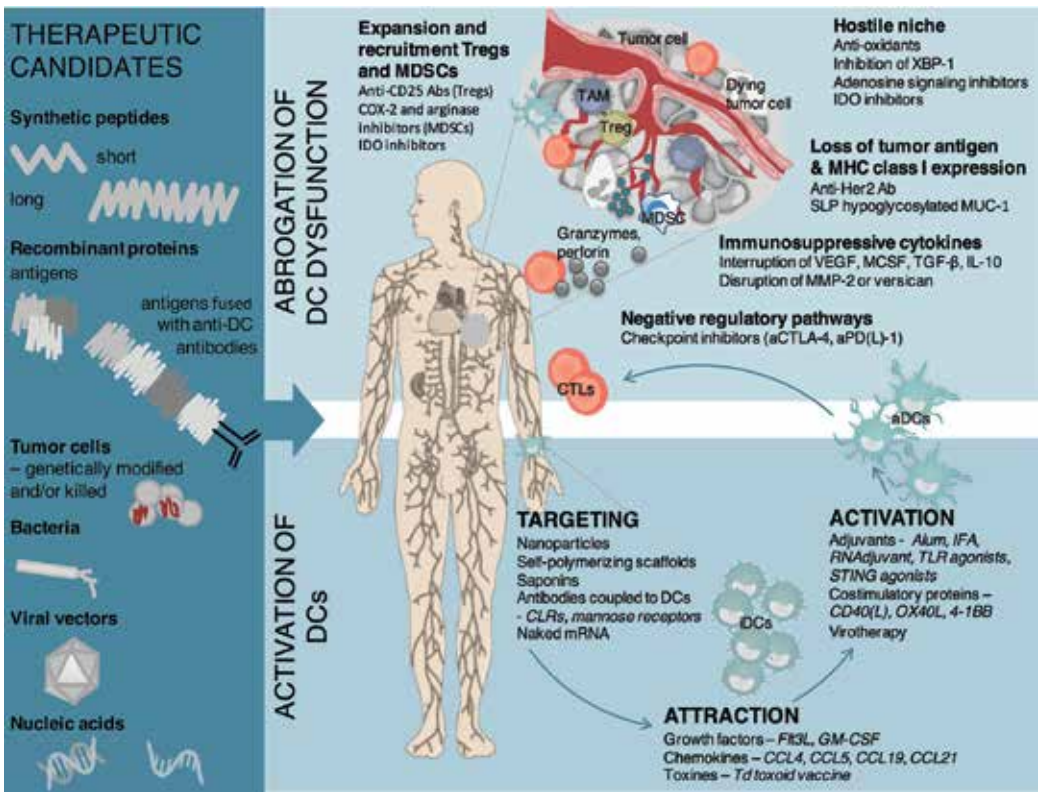

Figure 1. Overview of different therapeutic agents and targets to modulate dendritic cell function in the tumor microenvironment. To abrogate DC dysfunction (upper panel), different therapies can be envisaged to inhibit tumor-promoting characteristics, thereby remodeling the TME, to restore the functionality of the DCs. In the lower panel, it is shown how DCs can be targeted in vivo to deliver DC-activating cargo, to increase the number of DCs, and to promote their capacity to activate effector T cells, leading to the eradication of the tumor nodule (s). Various therapeutic formats can be used to achieve this, and these are listed in the left panel. DC = dendritic cell, aDC = activated DC, iDC = immature DC, Treg = regulatory T cell, MDSC = myeloid-derived suppressor cell, TAM = tumor-associated macrophage.

DCs and exploiting their natural sentinel functions, the need to identify tumor-specific antigens is circumvented. Therefore, this strategy can take advantage of the complete antigenic repertoire of the tumor and is not limited to predefined TAAs. In this way, it is feasible to develop an off-the-shelf product that can elicit strong antitumor immune responses against tumor-specific neo-antigens in a wide variety of cancer types. Different strategies to attract, expand, activate, and target DCs in situ will be discussed here.

3.1. Attraction of dendritic cells

In order to elicit an effector T cell response, appropriate numbers of DCs are required at the tumor site to take up the antigens and present these to the T cells. Indeed, Lavin et al. showed a decrease in $CD141^+$ DCs accompanied by a low number of activated $CD8^+$ T cells in the tumors of patients with early-stage lung adenocarcinoma [8]. Rather than focusing on attempts to deliver high numbers of ex vivo generated DCs to patients, new strategies are being developed to attract DCs to the site of interest in vivo through the administration of different growth factors or chemokines [82]. The most relevant approaches to expand and/or attract DC subsets in vivo are highlighted in this section.

3.1.1. Growth factors

One approach is the use of Fms-related tyrosine kinase 3 ligand (Flt3L), a key growth factor in the generation of DCs from hematopoietic progenitors present in the bone marrow. Systemic administration of recombinant human Flt3L (CDX-301, Celldex Therapeutics) to healthy donors increases the frequency of different types of DC subsets. Treating cancer patients with Flt3L can facilitate on the one hand the isolation of different subsets in sufficient numbers for multiple rounds of ex vivo DC vaccination. On the other hand, the increased frequency of DC subsets may improve uptake of TAAs and increase migration toward lymph nodes to induce successful immune responses [83]. The latter has been tested in a clinical trial (NCT02129075), where stage IIB–IV melanoma patients were vaccinated with a combination of systemic recombinant hFlt3L (CDX-301), to mobilize DCs, and a fusion protein of a human monoclonal antibody with specificity for a DC receptor, DEC-205, linked to the tumor-associated antigen NY-ESO-1 (CDX-1401) together with adjuvant poly(IC:LC). It has been shown that CDX-301 greatly expanded peripheral blood DCs and evidence of priming T cell immunity to the vaccine antigen was demonstrated [8].

Other trials are evaluating the effect of Flt3L administration in combination with stereotactic radiotherapy in a phase II clinical trial in patients with advanced NSCLC (NCT02839265) or in combination with adenoviral TAA expression in a phase I study in glioblastoma patients (NCT01811992) [9].

Multiple vaccine platforms include granulocyte-macrophage colony-stimulating factor (GM-CSF) in their formulations [9]. GM-CSF is known for its function in DC recruitment and maturation and also facilitates the homing of CTLs to the tumor site. Treatment with GM-CSF-secreting genetically modified tumor cells (GVAX-Pancreas NCT00084383 or Melanoma-GVAX-NCT01435499) resulted in promising antigen-specific protective immune responses, particularly when administered together with supporting drugs such as cyclophosphamide or innate immune ligands (STINGVAX) [8]. This is discussed in a later section of this chapter.

3.1.2. Chemokines

Chemokines participate in the antitumor immune response by regulating the trafficking and positioning of lymphocytes as well as by regulating different effector functions. Moreover, chemokines can act as a natural adjuvant in vaccination protocols for the treatment of various malignancies and infectious diseases [12].

CCL4 (macrophage inflammatory protein-1β, MIP-1β) is a potent chemoattractant for T lymphocytes, NK cells, as well as immature DCs. Spranger et al. have demonstrated the migratory capacity of DCs in response to recombinant murine CCL4 [84]. Complementary, Luo X et al. proved the chemotactic activity to $CD4^+$ T cells, $CD8^+$ T cells, NK cells, and immature DCs by transfecting CT26 cells with the MIP-1β gene. When AdhMIP-1β was injected in situ, lymphocytes were recruited to the tumor site. This intratumoral administration of AdhMIP-1β elicited a striking increase in tumor-specific CTL activity [85]. In metastatic melanoma patients, it was shown that an increased $CD8^+$ T cell infiltration is associated with enhanced expression of CCL3, CCL4, CCL5, CXCL9, and CXCL10 and an increased likelihood to respond to ipilimumab [86].

CCL5 (regulated upon activation, normal T cell expressed and presumably secreted, RANTES) has a strong chemotactic activity toward multiple immune cells, including DCs, macrophages, monocytes, NK cells, leukocytes, and T cells by binding on CCL5 receptors (CCR1, CCR3, CCR4, and CCR5).

Several preclinical studies have shown that co-immunization strategies, combining CCL5 as a vaccine adjuvant with DC growth factors or TAA, potently induce antitumor immune responses. Moreover, it was shown that ectopic expression of CCL5 at the tumor site attracts and activates different types of immune cells, such as DCs, $CD4^+$ Th1 cells, $CD8^+$ T cells, and NK cells [87]. However, therapies based on the induction of immune responses by CCL5 should also consider surface expression of CCL5 receptors on the tumor. The level of CCL5 expression by tumor or tumor stromal cells is critical for determination of its beneficial or detrimental activities. The goal of CCL5-based tumor vaccines is to maximally activate immune effector cells and minimally mobilize tumor cells [87].

CCL19 (Epstein–Barr virus-induced molecule 1 ligand chemokine, ELC) is produced by a subset of DCs and possibly by other nonlymphoid cells, in T cell areas of the lymphoid tissue. CCL19 is produced by fibroblastic reticular cells and is essential for the formation and maintenance of the T cell zone in lymphoid organs, where both T cells and DCs are recruited from the periphery and meet each other. The ability of CCL19 to attract T cells, B cells, DC, macrophage progenitor cells, and NK cells is mediated through the CCR7 receptor.

In an advanced lung carcinoma model (CC-10Tag), it was shown that intranodal administration of recombinant CCL19 led to a significant reduction in tumor burden coupled with extensive mononuclear infiltration [88]. Using CAR-T cells engineered to express IL-7 and CCL19, complete regression of preestablished solid tumors and prolonged survival in different mouse tumor models was observed. In addition, an increased infiltration of DCs and T cells into the tumor tissues was established by treating the animals with CAR-T cells expressing IL-7 and CCL19 [89].

CCL21 (secondary lymphoid tissue chemokine, SLC, Exodus-2, thymus-derived chemotactic agent 4, 6CKine) is evaluated intratumorally in preclinical models of lung, melanoma, and prostate cancer, leading to a significant increase in $CD4^+$ and $CD8^+$ T lymphocytes and DCs infiltrating both the tumor and the draining lymph nodes resulting in T cell-dependent anti-tumor responses [82].

3.1.3. Toxins

Alternatively, tetanus/diphtheria (Td) toxoid vaccine can be used to mobilize DCs. Most people have received Td toxoid vaccines in their childhood, so by treating them with Td toxoid vaccine a $CD4^+$ T cell memory response is initiated, promoting the migration of DCs to the lymph nodes and activating an immune response. Indeed, preconditioning of tumors with Td toxoid vaccine in glioblastoma patients, receiving autologous monocyte-derived DC vaccines loaded with GBM antigen pp65, significantly improved the survival and antigen-specific T cell responses [8].

3.2. Activation of dendritic cells

Besides the observation that there is a decrease in the number of cross-presenting DCs accompanied by a low number of activated $CD8^+$ T cells in tumors of patients with early-stage lung adenocarcinoma, DCs isolated from cancer patients often lack the expression of maturation markers, have an immature phenotype, and fail to activate T cells [8]. Therefore, a successful cancer vaccine still requires a powerful adjuvant in order to properly activate the DCs and to meet the minimal criteria for engaging the immune system [9]. Choosing a suitable adjuvant is important, as it can potentially override immunosuppression and allow the vaccine to maximize its therapeutic potential [9]. In the following sections, we will discuss some of the most used and powerful adjuvants able to activate DCs.

3.2.1. Aluminum salts (alum)

Alum, the first adjuvant to be used in human vaccines, is thought to function by adsorbing and then slowly releasing antigens in vivo to enhance the immune response. Alum has been reported to activate the inflammasome pathway particularly in DCs. Supplementing alum vaccines with other adjuvants and cytokines like Montanide (NCT00031733) and IL-12 in patients who have undergone surgery for stage II/III/IV melanoma has been reported to elicit Th1 antitumor responses [9].

3.2.2. Incomplete Freund's adjuvant (IFA)

Montanide adjuvants are an iteration of IFA that function by forming depots to concentrate vaccines at the injection site and facilitate slow release of antigens to enhance uptake by APCs. Different phase I/II studies are ongoing to assess the benefit of using Montanide in combination with different TLR agonists and standard chemotherapy in a variety of cancer types including melanoma, prostate cancer, and glioma (NCT02425306, NCT01079741, NCT02126579, NCT02293707, NCT02193347, NCT02795988) [9].

3.2.3. RNAdjuvant

CureVac AG, a biopharmaceutical company focusing on mRNA-based drugs, developed RNAdjuvant. This is a noncoding synthetic RNA (CV8102) that activates a set of pattern recognition receptors and induces upregulation of IFN-inducible genes at the injection site. Intratumoral therapy with CV8102 led to a dose-dependent tumor growth inhibition resulting in complete tumor eradication in nearly 50% of CT26 tumor-bearing mice. Moreover, combination of intratumoral CV8102 and systemic anti-PD-1 treatment led to significantly enhanced antitumoral responses compared to monotherapy. At the moment, a phase I, dose-escalation study of intratumoral CV8102 is ongoing to evaluate safety and tolerability. A second phase I trial is ongoing in patients with melanoma and squamous cell carcinoma of the head and neck combining intratumoral CV8102 with anti-PD-1 blockade.

3.2.4. TLR agonists

TLR agonists activate and mature DCs and have the potential to reverse T cell anergy, thereby overcoming immune suppression.

Hiltonol (poly-ICLC) is a stabilized dsRNA therapeutic viral mimic or "danger signal" that activates multiple elements of innate and adaptive immunity via signaling through TLR3. It is a stand-alone immunomodulator, but when properly combined with antigen, it generates a comprehensive Th1 immune response. Hiltonol is tested in numerous clinical trials, in patients with nonmelanoma skin cancer, glioma, lymphoma, ovarian cancer, prostate cancer, B and T cell lymphomas, etc. (NCT02423863, NCT01188096, NCT01976585, NCT03162562, NCT03262103, NCT00880867). Another TLR3 agonist is rintatolimod. Besides its use as therapeutic adjuvant in clinical trials for HIV-1 infection and chronic fatigue syndrome, rintatolimod is also tested in phase I/II studies in $Her2^+$ breast cancer patients which are treated with Her2 peptides and a combination of GM-CSF and rintatolimod (NCT01355393). It is also used in combination with IFN-α to treat patients with metastatic colorectal cancer (NCT01545141, NCT03403634), and a phase I/II trial in patients with recurrent ovarian, fallopian tube, or primary peritoneal cancer receiving an autologous vaccine composed of autologous oxidized tumor cell lysate (OC-L) administered in combination with rintatolimod (NCT01312389) is ongoing.

Bacillus Calmette-Guérin (BCG) activates TLR2 and TLR4 in macrophages and DCs. This vaccine was primarily developed for the prevention of tuberculosis and is nowadays the standard treatment for patients with in situ or non-muscle invasive bladder cancer. Currently, successful trials using BCG in combination with topical treatment of 5% imiquimod in melanoma patients are ongoing. Glucopyranosyl lipid A(G100), a synthetic TLR4 ligand, has showed success in early clinical trials in eliciting Th1-polarized antitumor immunity (NCT02501473) [90]. Picibanil (OK-432) is a lyophilized preparation of *Streptococcus pyogenes* and is approved in Japan for the treatment of cervical, gastric, and oral cancer [9, 90]. eTheRNA immunotherapies are evaluating the use of intranodally administered TriMix mRNA in a phase Ib clinical study in melanoma patients.

The only known natural ligand to activate TLR5 is flagellin, a constituent protein of bacterial flagella. Preclinical in vitro and in vivo data indicate that formulations using liposomal engrafted synthetic peptide containing flagellin fragments can induce DC maturation.

One phase I clinical trial has been completed using entolimod (CBLB502, a pharmacologically optimized flagellin derivative) in patients with late-stage solid tumors (CT01527136). The treatment was well tolerated with only common adverse events such as fever, transient hypotension, and hyperglycemia. Another TLR5 agonist, M-VM3 (Mobilan), a recombinant non-replicating adenovirus encoding human TLR5 and its ligand flagellin, is currently in two clinical trials (NCT02654938, NCT0284499) for prostate cancer [90].

Stimulation of TLR7/TLR8, receptors for single-stranded RNA, significantly augments DC maturation, Th1 cellular immunity, cross-presentation of antigens, and humoral immune responses. One of the three FDA-approved commercialized small-molecule TLR7/TLR8 agonists is imiquimod, formulated as a dermal cream, for HPV-mediated external genital warts, superficial basal cell carcinoma, and actinic keratosis. The imiquimod 5% cream was tested in a randomized controlled trial (NCT0066872) in patients with nodular and superficial basal cell carcinoma and demonstrated to be superior to excision surgery. Another promising lipid-modified imidazoquinoline is 3 M-052. It is evaluated as an adjuvant in many vaccine models and showed promising preclinical results in mouse melanoma and prostate tumor models.

Unmethylated CpG oligodinucleotides, a TLR9 agonist, used in peptide vaccines has been shown to boost antitumorigenic T cell responses. MGN1703, a covalently closed natural DNA molecule, is a TLR9 agonist which elicits significant IFN-α induction and broad activation of human immune cells in vitro. This molecule is taken into a phase II study (NCT01208194) to treat patients with metastatic colorectal cancer, in combination with standard chemotherapy and bevacizumab (a monoclonal anti-VEGFA antibody). Patients who received MGN1703 showed a superior progression-free survival compared to placebo. The compound is also tested in a phase I clinical trial to determine the highest tolerable dose in combination with ipilimumab (anti-CTLA-4) in patients with advanced solid tumors (NCT02668770) and in patients with small-cell lung cancer (NCT02200081).

Another pattern recognition receptor is stimulator of interferon genes (STING), and upon activation of this pathway, an interferon response is induced. STING ligands are cytosolic double-stranded DNA molecules, host signaling second messenger cGAMP, and pathogen-derived cyclic dinucleotides (CDNs) [9]. Corrales et al. showed a regression of local as well as distal tumors in different mouse models, and the induction of lasting memory responses against tumor rechallenges could be achieved upon treatment with CDNs [91]. STING agonists are also combined with other therapeutic agents such as the STINGVAX vaccine platform, which uses GM-CSF-secreting cells along with modified STING agonists. Hanson et al. showed that nanoparticulate STING agonists are potent lymph node-targeted vaccine adjuvants. In addition, synthetic STING agonists, such as ADU-S100, are already tested in patients with advanced solid tumors (NCT02675439). Besides the specificity of the STING agonists, DNA vaccines, such as VGX-3100, may also have the inherent potential to activate the STING pathway.

Another ligand of the PRR is *Listeria monocytogenes* (Lm), and attenuated Lm strains promote antigen cross-presentation and antitumoral immune responses. Overall, att-Lm strains have the capacity to deliver TAAs and also act as adjuvants for boosting DC vaccines by neutralizing immunosuppressive factors (NCT02575807), recruiting DCs by expressing GM-CSF (NCT01417000), or inducing DC activation through TLR and STING signaling [9].

3.2.5. Costimulatory proteins

Several vaccine strategies have been designed to leverage CD40-mediated DC activation, including vaccination with tumor cell lines modulated to overexpress CD40L (NCT00458679, NCT02719015, NCT02466568), anti-CD40 antibody (NCT02376699, NCT02482168, NCT01103635), and recombinant CD40L protein (NCT00001145) [9]. Preclinical data showed that the intranodal and intratumoral delivery of TriMix mRNA results in the activation and maturation of DCs, leading to robust antitumor responses and prolonged survival in different murine tumor models. Currently, a phase I clinical trial (NCT03394937) is ongoing in melanoma patients to assess safety and tolerability of the intranodal delivery of TriMix mRNA. Another phase I clinical trial is planned in early-stage breast cancer patients, who will be treated intratumorally with TriMix mRNA prior to surgery. Other costimulatory proteins involved in the expansion, function, and survival of T cells are OX40L and 4-1BB. Preclinical results in a variety of induced and spontaneous tumor models suggest that targeting 4-1BB with agonist antibodies can lead to tumor clearance and durable antitumor immunity. Clinical trials of two agonist antibodies, urelumab and utomilumab, are ongoing. Both antibodies have demonstrated promising results in patients with lymphoma and are being tested in combination therapy trials with other immunomodulatory agents. However, important considerations should be given to 4-1BB-mediated toxicities.

OX40 ligand (classically expressed on activated APCs) is a costimulatory membrane-bound protein, and OX40 signaling can be induced by different technologies such as OX40-specific agonistic antibodies (phase I clinical trial NCT01644968 in metastatic cancer patients), OX40L-Fc fusion proteins (NCT02221960), RNA aptamers and transfected tumor, and DCs. In analogy to the mouse tumor models, anti-OX40 monoclonal antibodies (mAb) may further benefit from combination strategies. There is one combination trial combining anti-OX40 mAbs with radiation and cyclophosphamide (NCT01303705) in cancer patients with metastatic prostate cancer. Other combination trials analyzing the potential of combining anti-OX40 with anti-CTLA4, anti-PDL1, and anti-CD20 mAbs are ongoing (NCT02205333). Currently, a first-in-human, phase I dose-escalation study is ongoing evaluating the safety and tolerability of escalating intratumoral doses of mRNA-2416 (encoding OX40L) in patients with relapsed/refractory solid tumor malignancies or lymphomas.

3.2.6. Virotherapy

Oncolytic viruses (OVs) are attenuated, mutated, or naturally benign viruses that preferentially target and lyse cancer cells while leaving normal, non-transformed cells relatively unharmed. The antiviral immune response that follows OV infection occurs within the vicinity of the tumor; overturns tumor-associated immune evasion mechanisms; enhances DC activation, maturation, and TAA uptake and presentation; and thus has the potential to establish a robust antitumor specific immune response. Currently, there are numerous examples of these OVs including reovirus, vesicular stomatitis virus, vaccinia virus, Newcastle disease virus, measles virus, poliovirus, herpes simplex virus, coxsackievirus, adenovirus, and Maraba virus. Interestingly, the therapeutic administration of OVs drives two contrasting immunities, namely, antiviral and antitumor [92]. It has been demonstrated that a "prime boost" immunization strategy—sequential immunization with different strains of oncoviruses expressing the same TAA—negates the risk of generating "distracting" antiviral immunity in mice and is now being

evaluated in clinical trials (NCT02285816) [9].OVs have the natural capabilities to alter the maturation status of DCs, but other studies on engineered OVs (e.g., adenovirus, HSV, arbovirus, poxvirus) have also focused on enhancing the interaction of OVs with DCs by encoding growth factors (GM-CSF and Flt3L), chemokines (CCL2), cytokines (IL-12, RANTES, and IFN-β), and defensins (β-defensin-2) within the viral genome. For example, an oncolytic adenovirus encoding MIP-1α and Flt3L has been constructed to promote DC recruitment and expansion in vivo, which ultimately had a strong synergistic effect on the infiltration of tumors by DCs and T cells. The administration of IL-12 and GM-CSF-expressing adenovirus (Ad-ΔB7/IL12/GMCSF) in combination with DCs in B16-F10 melanoma tumor-bearing mice also showed increased DC migration to draining lymph nodes due to the upregulation of $CCL21^+$ lymphatic vessels around tumor tissues. In addition, GM-CSF-expressing adenovirus ONCOS-102 is used in a phase I clinical trial (NCT01598129) in patients with advanced solid tumors. Another example is JX-594, also known as Pexa-Vec. Intravenous delivery of this GM-CSF-expressing vaccinia poxvirus with a deletion of the thymidine kinase gene resulted in increased tumor-infiltrating $CD8^+$ T cells and reduced metastasis in hepatocellular carcinoma (NCT00554372). Intratumoral injections of HSV-1 expressing GM-CSF, also known as talimogene laherparepvec (T-VEC), have been shown to trigger the development of antitumor immunity in metastatic melanoma patients (NCT00769704). This is achieved through DC stimulation (attraction and maturation) via GM-CSF, resulting in enhanced priming of antigen-specific T cells [92]. Currently, T-VEC is being evaluated in combination with checkpoint blockade drugs (NCT01740297) [9].

3.3. Targeting DCs in vivo

In order to directly target DCs in vivo, different strategies can be followed to deliver maturation agents or antigens to the DCs. Nanoparticles can be loaded with adjuvants and antigens, enabling them to activate lymph node-resident DCs and induce potent immune responses. Examples of biologics that can be incorporated in nanoparticles are immune-activating cytokines and growth factors (IL-12, IL-2, GM-CSF), neutralizing antibodies against immunosuppressive cytokines (TGFβ), stimulatory ligands (TLR agonists), and factors selectively targeting specific DC (subsets) along with antigens [93–95].

Another approach to deliver your DC modulatory agent is to use self-polymerizing scaffolds, which drain to the lymph node and become immunogenic particles in vivo. Saponins, plant-derived glycosides that form stable immune-stimulating complexes along with cholesterol and phospholipids, are also a safe candidate for delivering cargo to the DCs in vivo. They are being explored for their antitumorigenic potency in combination with novel platforms such as nanoparticles, oncolytic viruses, and chemotherapy agents [9]. Another option to target the DCs and activate them is the use of antibodies specifically binding to DCs. The antigen is bound to antibodies directed against surface receptors of DCs leading to uptake of the antigen. Endocytic antigen uptake through C-type lectin receptors like DEC-205 has been shown to induce cross-presentation. CDX-1401, a vaccine comprising of DEC-205 fused with tumor antigen NY-ESO-1, has been proven therapeutic and safe against advanced malignancies and is being evaluated in patients with ovarian carcinoma, leukemias, and melanoma (NCT02166905, NCT01834248, and NCT02129075). An example of another DC targeting antibody is a vaccine developed by Celldex targeting mannose receptors expressed on APCs in combination with TLR agonists [9]. However, if these antibody–antigen conjugates are not

accompanied by adjuvant to stimulate the immune system, tolerance rather than immunity might occur [81]. The challenge of this approach will be to match the DC surface target and the selected adjuvant with the desired immune outcome, all in the context of an altered immune system [96]. Besides the abovementioned approaches, Van Lint et al. already demonstrated the feasibility of injecting "naked" (non-packaged) mRNA in vivo (either intranodally or intratumorally). It has been shown that naked mRNA is preferentially taken up by cross-presenting CD8α$^+$ DCs [97, 98]. This approach circumvents the necessity of using different kinds of delivery vectors or targeting molecules.

4. Conclusion

In conclusion, it is of utmost importance that DCs used for vaccination are equipped to stimulate effector T cells, and this can only be achieved when these DCs are fully matured and activated. Many challenges remain that must be considered to improve the efficiency and clinical outcome of DC-based immunotherapy in cancer. These include the choice of the different synergistic immunotherapies, selection of ideal adjuvants, administration route, and timing of the different treatments. It is likely that the future of cancer immunotherapy will be a combination of different immunotherapy platforms. One arm will focus on inhibiting tumor-induced immunosuppression, while the second arm will aim to activate antitumor immunity.

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Since their discovery in 1973 by Zanvil Cohn and Ralph Steinman (Nobel Prize in Physiology and Medicine, 2011), dendritic cells (DCs) continue to intrigue research scientists as their new markers, subpopulations and properties are being detected and the relevant literature is being doubled each year. This book combines the most comprehensive reviews of several critical aspects of DC biology and function written by a group of international experts in the field. The first section briefly discusses recent advances in DC subtypes, phenotypes, and functions in different diseases. The following sections look closely at DC phenotyping, DC–NK cell interplay, and roles of DC in bone loss and repair and parasitic infections. The final section on DC and cancer includes perspectives on DC vaccination based on modifications and therapeutic applications.

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