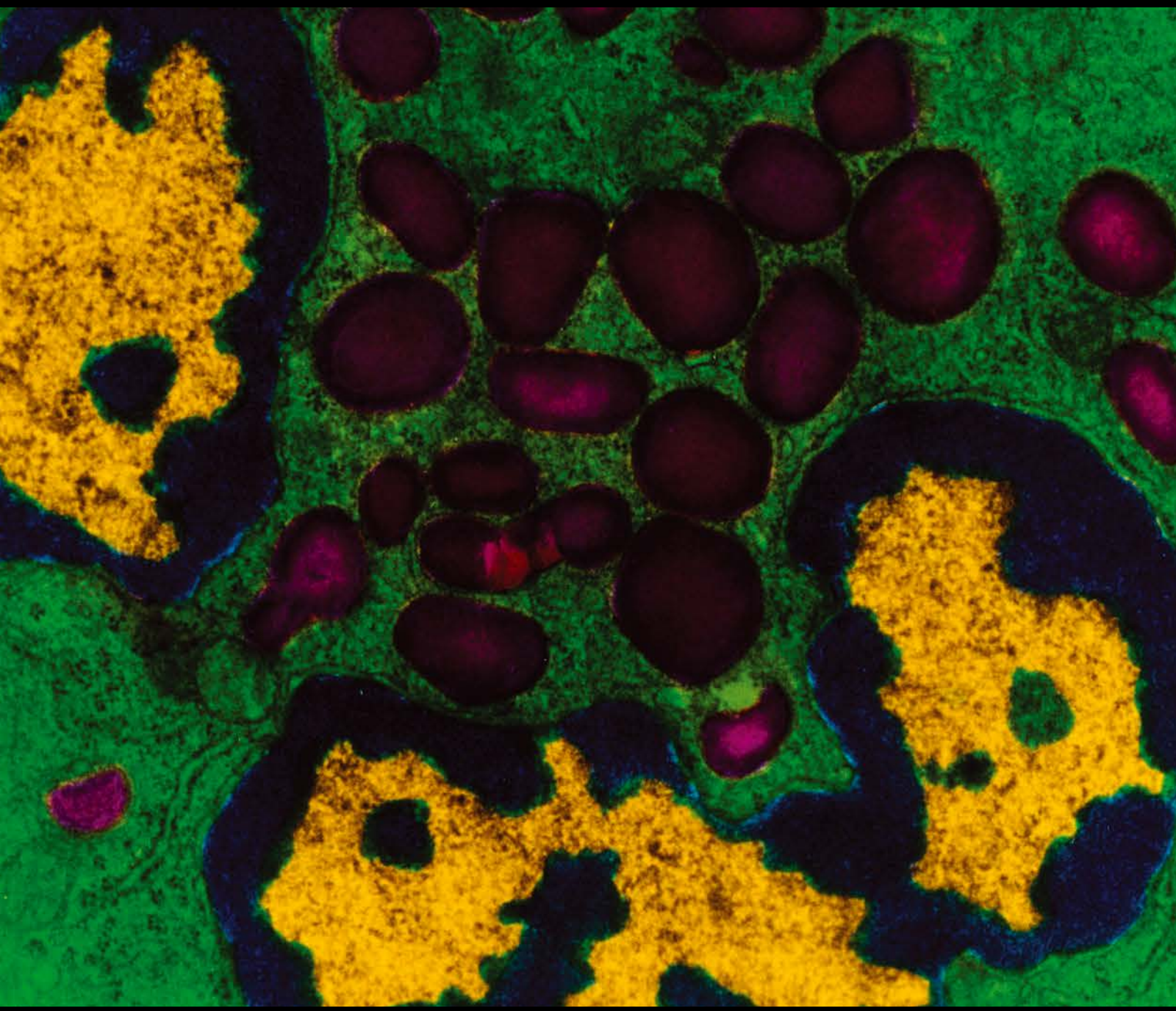
Mediators of Inflammation

Dendritic Cells as Vaccines: Key Regulators of Tolerance and Immunity

Guest Editors: Jurjen Tel, Daniel Benitez-Ribas, Edith M. Janssen, Evelien L. J. Smits, and Joannes F. M. Jacobs



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Editorial Board

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Editorial

Dendritic Cells as Vaccines: Key Regulators of Tolerance and Immunity

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Dendritic cells (DCs) are a specialized family of professional antigen presenting cells that serve as a bridge linking the innate and adaptive arms of our immune system. DCs sense pathogens or interact with harmless antigens or non-pathogenic bacteria thereby tightly regulating the balance between tolerance and immunity. Despite their indispensable role in eliciting immune responses, DCs are a rather rare and heterogeneous type of immune cell, which differ in phenotype and function depending on maturation status, subsets, and age as well as their localization and microenvironment. Although scarce in numbers, cultured or naturally occurring DCs have been extensively investigated in clinical trials for both their capacity of priming antigen specific cytotoxic and helper T cells and humoral responses and their potential to induce immunological memory, which are capacities that distinct them from other, nowadays, exploited forms of immunotherapy.

Cancer immunotherapy has been designated the scientific breakthrough of the year in 2013. This has a broader implication for DC research in general, as DC based therapy can also be used to induce tolerance in autoimmune or immune-based diseases or to induce or improve immunity in, for instance, virally infected individuals. In this special issue we present two original research articles as well as five review papers on the therapeutic potential of the use of DC subsets for DC based immunotherapy in cancer, autoimmune disorders, and infectious diseases.

In their paper "Linking CD11b$^+$ Dendritic Cells and Natural Killer T Cells to Plaque Inflammation in Atherosclerosis" M. Rombouts et al. performed extensive immune profiling in mice to investigate risk factors for plaque inflammation during atherosclerosis. They demonstrate that circulating CD11b$^+$ cDC and NKT cells show great potential to reflect the inflammatory status in the atherosclerotic plaque. This may provide biomarkers with which atherosclerotic lesion progression can be monitored and may provide leads for immune cell based interventions.

J. Klarquist et al. provide an oversight of the changes in DC composition, maturation, and functionality in patients with systemic lupus erythematosus (SLE) and mouse models of spontaneous SLE. Based on the similarities between human and murine DC subsets as well as their reported relevance to disease, they suggest that mouse models provide a useful platform for the identification, dissection, and targeting of the DC intrinsic and extrinsic processes that facilitate the development, progression, and possibly a cure for SLE.

In the paper entitled "Immunity and Tolerance Induced by Intestinal Mucosal Dendritic Cells", J. Aliberti describes the tolerogenic potential of DC in the digestive tract under steady-state conditions. The various DC subsets orchestrate

tolerogenic responses towards commensal gut flora and they orchestrate powerful immune responses directed against invading pathogens. Failure to successfully complete this task may result in inflammatory bowel disease, food allergy, or celiac disease. Insight into the various DC subsets in the gut and the factors that influence their function may provide novel druggable targets as a basis for novel therapies.

Immunological tolerance remains a challenge in clinical organ transplantation and in management of autoimmune diseases. Tol-DCs are being regarded as a powerful tool to induce immune homeostasis in autoimmune diseases and as such are currently explored in clinical trials. In the review entitled "Metabolism Is Central to Tolerogenic Dendritic Cell Function" W. J. Sim et al. provide a thorough overview of how metabolic reprogramming of DCs drives differential cellular function and how this specifically contributes to pathologies. Furthermore, they describe and link tolerogenic DCs with immunosuppressive cytokines, for example, IL-10, and how these drive the shift in metabolism during TLR stimulation. Finally, they provide an overview on how pharmacological manipulation of the DC metabolism can be exploited for the generation of DC vaccines.

As the field of tolerogenic DC treatments moves forward, the need has arisen for the development of standardized protocols for the generation and application of DCs to allow comparison between different treatments and streamline the time from bench to bedside. A. T. Brinke et al. outline the efforts of the European A FACTT (Action to Focus and Accelerate Cell Based Tolerance Inducing Therapies) network that aims to harmonize DC production protocols, functional quality control parameters, immune monitoring parameters, and therapeutic regulations in order to accelerate the implementation of cell based tolerance inducing therapies in the clinic.

Currently, blood DC subsets are explored for the first time in clinical trials for treating metastatic cancer patients. S. P. Sittig et al. probed the potential of blood DC subsets to polarize and stimulate T cells. They specifically compared human plasmacytoid DCs (pDCs), BDCA1$^+$ myeloid DCs (mDCs), and BDCA3$^+$ mDCs and their ability to respond to TLR ligation and prime naive CD4$^+$ T helper cells in an allogenic antigen unspecific and autologous antigen specific fashion. Although they clearly observed differences in the activation profile of the distinct DC subsets, all activated DC subsets were efficient in eliciting the production of IFN-γ by naive CD4$^+$ T helper cells. Their findings further establish all three human blood DCs, despite their differences, as promising candidates for immunostimulatory effectors in cancer immunotherapy.

In the review "Pathogen-Associated Molecular Patterns Induced Crosstalk between Dendritic Cells, T Helper Cells, and Natural Killer Helper Cells Can Improve Dendritic Cell Vaccination," T. Oth et al. describe the optimization of ex vivo generated DC vaccines by using rationally designed combinations of interferon gamma and different pathogen-associated molecular patterns for maturation. In this way, a cellular interplay is stimulated between key players of the antitumor response, DC, T helper 1 cells, natural killer cells, and cytotoxic T cells. Activation of multiple effector cell types might be the key to curative cancer vaccination. In this regard, interleukin 12-p70 is an important factor that stimulates efficient immunity. Attention should be paid to the generation procedure of the cellular vaccine so that the DC will still be able to produce interleukin 12 following injection. The outcome of DC vaccination might probably be further enhanced by making it part of a combination therapy that combines immune activation with attacking the immunosuppressive tumor microenvironment.

In summary, this special issue illustrates the function of various DC subsets and their contribution to tissue homeostasis. A better comprehension of the DC subsets and the networks they operate on may provide novel biomarkers to diagnose, prognosticate, and monitor disease. In addition, it may provide insights into improving the effectiveness of DC based immunotherapy.

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Review Article

Dendritic Cells in Systemic Lupus Erythematosus: From Pathogenic Players to Therapeutic Tools

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System lupus erythematosus (SLE) is a multifactorial systemic autoimmune disease with a wide variety of presenting features. SLE is believed to result from dysregulated immune responses, loss of tolerance of CD4 T cells and B cells to ubiquitous self-antigens, and the subsequent production of anti-nuclear and other autoreactive antibodies. Recent research has associated lupus development with changes in the dendritic cell (DC) compartment, including altered DC subset frequency and localization, overactivation of mDCs and pDCs, and functional defects in DCs. Here we discuss the current knowledge on the role of DC dysfunction in SLE pathogenesis, with the focus on DCs as targets for interventional therapies.

1. Introduction

Systemic lupus erythematosus is a chronic autoimmune inflammatory disease that affects multiple organ systems, prototypically characterized by high levels of circulating autoantibodies and glomerulonephritis. Clinical symptoms also encompass musculoskeletal, dermatological, neuropsychiatric, pulmonary, gastrointestinal, cardiac, vascular, endocrine, and hematologic manifestations. The reported incidence of SLE nearly tripled over the last 40 years due to improved detection of mild disease [1], but SLE prevalence estimates still vary considerably, ranging from 10 to 150 cases per 100,000, depending on geography, race, and gender [2–5]. In the United States, the prevalence of SLE is higher among Asians, African Americans, African Caribbeans, and Hispanic Americans compared with Caucasians [6–9]. Similarly, in European countries SLE prevalence is higher among people of Asian and African descent [5–9]. Interestingly, SLE is reported infrequently in Africa [10]. Mortality rates are relatively low, at 10–50 per 10,000,000 of the general population and show correlation with renal and cardiovascular manifestations as well as infection [11]. Importantly, patients commonly experience profound fatigue and joint pain and a decreased quality of life [12–15].

The precise etiology of SLE remains unclear and likely varies, considering its diverse clinical manifestations. Nevertheless, SLE is believed to result from dysregulated immune responses, loss of tolerance of CD4 T cells and B cells to ubiquitous self-antigens, and the subsequent production of anti-nuclear and other autoreactive antibodies. This dysregulation is associated with high serum levels of type I IFN, observed in greater than 70% of patients [16, 17]. Current "standard of care" treatments encompass high-dose corticosteroids, antimalarials, and immunosuppressive drugs that are associated with significant adverse side effects. As these treatments suppress symptoms and do not cure the disease, new therapies are needed.

Contemporary treatment strategies have been shifting emphasis toward the identification of immunological processes, both soluble and cellular, in order to redirect aberrant immune responses. Dendritic cells have recently been recognized as important players in the induction and progression of autoimmune diseases, including SLE [18]. Human and mouse studies have associated lupus development with

altered DC subset frequency and localization, overactivation of mDCs or pDCs, and functional defects in DCs [19, 20].

However, full dissection of the relative contribution of the causes and the consequences of the dysfunctionality in the different DC subpopulations is needed to understand the processes that govern SLE development, progression, remission, and relapses, in order to design interventional treatments that have the potential to redirect the immune system and eventually lead to a cure for this disease.

2. DC Populations in Humans

DCs are a heterogenous population of professional antigen presenting cells, which bridge innate and adaptive immunity. In the absence of exogenous triggers, DCs contribute to the clearance of dying cells and the maintenance of tolerance. During infection, or in the context of autoimmunity, however, DCs play a pivotal role in the activation of CD4 and CD8 T cells. DCs were initially identified by Ralph Steinman and lack typical lineage markers for T cells (CD3), B cells (CD20), and NK cells (CD56) while expressing high levels of MHC class II [35, 36]. Within this population comparative studies have identified a small number of subsets that have homologues in several mammalian species [37, 38].

2.1. Myeloid DCs: BDCA1$^+$ DCs and BDCA3$^+$ DCs. Myeloid DCs are considered "conventional" or "classical" DCs and are characterized by expression of CD11c and CD11b and lack of CD14 and CD16. Within this population we currently distinguish two populations based on the expression of the markers CD1c/BDCA1 and BDCA3/CD141 [39].

The BDCA1$^+$ DCs are the major myeloid DC population and are found in blood, lymphoid organs, and most tissues. BDCA1$^+$ DCs express a wide variety of pattern recognition receptors including TRL1–8, lectins, and cytokines, allowing them responsiveness to a diverse array of environmental cues. BDCA1$^+$ DCs are strong stimulators of naïve CD4 T cell responses, which can be shaped differently depending on which innate stimuli are present [37].

The BDCA3$^+$ DCs make up >10% of the mDCs and have been found in lymphoid and nonlymphoid tissues as well as blood and bone marrow. BDCA3$^+$ DCs express high levels of TLR3, XCR1, and CLEC9 and have been shown to display an increased capacity to phagocytose dying cells and cross-present cell-associated antigens to CD8 T cells compared to other DCs subsets [34, 40, 41].

2.2. Plasmacytoid DCs. pDCs lack the classic mDC markers CD11b and CD11c and express high levels of CD123, CD303 (BDCA2), and CD304 (BDCA4). pDCs are known for their capacity to produce vast amounts of type I IFNs in response to viruses and/or virus-derived nucleic acids predominantly via engagement of TLR7 and TLR9. pDCs have been shown to prime CD4 T cells and cross-prime CD8 T cells, especially in the context of infection [42]. Several studies implicate pDCs in the induction and maintenance of tolerance through the induction of regulatory T cells (Tregs) [43–45].

2.3. Monocyte-Associated DCs. There are currently several populations of DCs that are thought to develop from monocytes rather than common DC precursors. These cells display a variety of phenotypes and functions, but there is no consensus on their exact classification or their role *in vivo*.

CD14$^+$ DCs are observed in several nonlymphoid tissues, including the skin. These cells express CD11c but lack BDCA1 or BDCA3. The CD14$^+$ DCs express low levels of costimulatory molecules or chemokine receptors that promote migration. While these cells have been suggested to be poor at stimulating naïve T cells, they have been found to support the formation of T follicular helper cells and to provide direct help to B cells [46–49].

Inflammatory DCs (iDCs) have been suggested to originate from classic CD14$^+$ blood monocytes under inflammatory conditions. These cells may express some of the myeloid DC markers and seem prone to produce proinflammatory cytokines. *In vitro* studies suggest that different types of inflammatory stimuli give rise to populations with distinct proinflammatory phenotypes. TNFα/iNOS expressing inflammatory DCs have been found in skin lesions of patients with psoriasis and atopic dermatitis [50, 51].

SlanDCs encompass a subset of monocytes with high expression of MHC class II, CD16, and 6-sulpho LacNAc (slan). SlanDCs were shown to express TRL7 and TLR8 and to produce IL-12, IL-23, and TNF, preferentially promoting Th1 and Th17 cell differentiation. This population has been isolated from the inflamed skin of psoriatic patients and SLE patients with cutaneous lupus, the colon, and draining lymph nodes of patients with inflammatory bowel diseases, as well as CSF samples and inflammatory brain lesions of patients with MS [52–55]. Interestingly, SlanDC infiltration in tumors is associated with tolerance and poor prognosis, indicating either diversity within the slanDC population or heterogeneity in its function.

2.4. Tissue DCs. Nonlymphoid tissue resident DCs are present in most tissues in steady state and have been associated initially with induction of tolerance to self-antigens [36–38, 56–58]. These cells migrate at a very low rate to the draining LN under steady state conditions but show significant increased migration under inflammatory conditions. Several studies have identified networks of tissue resident DCs in the skin, lung, gut, and liver [59, 60]. Each of these networks consists of several subpopulations with different capacities for phagocytosis, antigen processing and presentation, migration, and the type of immune response they promote. Due to accessibility, skin DCs, especially Langerhans cells (LC), have been the most studied tissue-DC in the context of SLE.

2.5. DC Activation of T Cells. One of the defining features of DCs is the expression of class I and class II major histocompatibility proteins and the processing and presentation of peptide antigens to T cells. DCs predominantly present self-antigens in low quantities resulting in immunologic tolerance. Once activated, however, DCs mature in a process that usually involves migration to a draining lymph node and the priming of T cells [61–63]. The factors governing the functional result of T cell priming are multifactorial,

Table 1: pDCs in SLE.

Markers used to identify subset	Reference	Frequency	Phenotype	Function
BDCA2$^+$ CD123$^+$	Tucci et al. [82]	↓ in blood, correlated with LN and ↑ in kidney (more than other DC subsets)		
BDCA2$^+$ (blood) and BDCA4$^+$ (kidney)	Fiore et al. [78]	↓ in blood in active disease and ↑ in kidney (more than other DC subsets)	DCs in kidney were immature (DC-LAMP$^-$), localized to tubulointerstitium, in clusters, and lacked dendrites	
BDCA2$^+$ Lin.$^-$ HLA-DR$^+$	Migita et al. [77]	↓ in blood		
CD123^{high} CD11c$^-$ CD16$^-$ HLA-DR$^+$	Henriques et al. [80]	↓ in blood in active disease		
BDCA2$^+$CD123^{high}	Kwok et al. [90]	*Normal* in blood	↓ IFNα production by PBMC per pDC upon CpG stimulation	
BDCA2$^+$ BDCA4$^+$ CD123$^+$	Jin et al. [79]	↑ in blood per total PBMC	*Normal* HLA-DR, CD86, CD83, CCR7	↑ T cell proliferation in MLR
BDCA2$^+$ CD11c$^-$	Gerl et al. [81]	na	*Normal* HLA-DR, CD86, CD83, CCR7, CD40, BAFF, CCR1, and CCR5 and ↓ CMKLR1	↑ basal and CCL19-specific migration
BDCA-2$^+$ CD4$^+$ CD11c$^-$ Lin$^-$	Hagberg and Rönnblom [86]		↓ SLAMF5/CD84, SLAMF7/CRACC/CD319, *normal* SLAMF1, SLAMF2/CD48, SLAMF3/CD229, SLAMF4/CD244, and SLAMF6/CD352	

including the relative concentration of surface peptide/MHC, costimulatory molecule expression, and cytokine release. Ultimately, the combination of these signals will result in either T cell anergy, deletion, or activation, proliferation, and differentiation [64–66].

A wide variety of cell surface costimulatory proteins expressed by DCs can signal both activation (41-BB, CD40, CD70, CD80, CD83, CD86, GITRL, ICOSL, LTBR, and OX40L) and inhibition (PDL1, PDL2) of an engaged T cell (reviewed in [67, 68]). In addition, secretion of pro- and anti-inflammatory cytokines by DCs contributes to the outcome of T cell priming. DCs can produce a wide variety of cytokines; which cytokines are produced depends upon environmental signals as well as upon the DC subtype. Cytokine production is driven by input from paracrine and autocrine cytokine signaling, as well as input from innate pattern recognition receptors (PRRs) including toll-like receptors (TLRs). The combination of these signals not only influences whether a T cell becomes activated, but also plays a key role in directing T cell differentiation toward various effector fates.

3. Role of DCs in SLE Development and Progression

Although it is not certain how immunological tolerance is broken in SLE, DCs are thought to play key roles [30]. Perhaps the most prominent model proposes that the initial injury is due to a build-up of dying cells, a result of either dysregulated apoptosis or insufficient clearance of dying cells by DCs and other phagocytes [22, 23, 69]. Indeed, high levels of apoptotic cells are found in SLE patient serum, germinal centers, and inflamed tissues, such as the skin and kidney [24, 27]. Mounting evidence indicates that self-RNA and self-DNA from these dying cells induce the unremitting output of type I IFN by pDCs [21] via engagement of TLR9 or TLR7 [31, 70] and potentially via other cytosolic nucleotide sensing pathways such as *RIG-I/IPS1* and STING (*TMEM173*) [28, 71, 72]. Type I IFNs produced by DCs promote their own activation and maturation in an autocrine manner, including increased IFN output and increased surface expression of CD80, CD86, and MHC class II, making them better at activating T cells [21, 25, 26, 73]. Furthermore, type I IFNs directly promote B cell activation, antibody production, and T cell survival and expansion [29, 32, 33]. Altogether, these data suggest that DCs are key players in SLE pathogenesis and point to DCs as promising therapeutic targets.

4. DC Abnormalities in SLE Patients

Several reports indicate that the frequency, composition, and phenotype of DCs in SLE patients differ from those of healthy individuals (see Tables 1 and 2). However, it is difficult to compare results between laboratories, given differences in disease activity and manifestations, the effect of various drug treatments on DC development and phenotype, and the variations in analytical parameters.

Table 2: DCs in SLE.

Markers used to identify subset	Reference	Frequency	Phenotype	Function
BDCA1$^+$	Fiore et al. [78]	↓ blood in active disease and ↑ kidney in active disease	DCs in kidney were immature (DC-LAMP$^-$), localized to tubulointerstitium	
BDCA3$^+$	Fiore et al. [78]	↓↓ blood and ↑ kidney in active disease	DCs in kidney were immature (DC-LAMP$^-$), localized to tubulointerstitium, with elongated processes	
BDCA1$^+$ CD11c$^+$ BDCA4$^-$ CD19$^-$	Jin et al. [91]	↓ in blood per total PBMC	↓ CD83, especially in active disease, *normal* HLA-DR, CD86, and CCR7	
HLA-DR$^+$ Lin$^-$ CD4$^+$	Scheinecker et al. [76]	↓ in blood	↓ CD40$^+$, B7$^+$, and CD11c$^+$	↓ T cell proliferation in MLR
BDCA1$^+$ CD11c$^+$	Tucci et al. [82]	*Normal* in blood, relatively few in kidney		
CD11c$^+$ Lin$^-$	Crispín et al. [83]	↑ in blood (though not significant)	↑ CD86$^+$, CD80$^+$, *normal* HLA-DR$^+$, and CD40$^+$	*Normal* T cell proliferation in MLR, moDCs fail to increase costimulatory molecule expression upon activation
CD11c^{high} CD14$^-$	Gerl et al. [81]	na	↑ CD86, BAFF, *normal* HLA-DR, CD83, CD40, CCR7, CCR1, and CCR5 and ↓ CMKLR1	
Adherent, monocyte-derived DCs (MDDCs)	Ding et al. [93]	na	↑ CD86, CD80, HLA-DR, and CD1a and ↓ CD83 after 5–7 d culture	↑ T cell proliferation in MLR
CD14$^+$ sorted, monocyte-derived DCs (MDDCs)	Köller et al. [92]	na	↓ HLA-DR after 8–10 d culture, *normal* CD86, CD83, CD80, CD40, CD54, and CD33	↑ antigen-specific T cell proliferation and normal MLR
M-DC8 (slanDCs)	Hänsel et al. [53]	↑ in skin of patients with cutaneous LE and "strong inflammation" SLE	*In situ* TNF production in cutaneous LE	↑ TNFα production by healthy donor slanDCs in response to SLE serum compared with control serum

Studies have shown reduced [74–81], normal [80, 82], and increased [83] levels of CD11c$^+$ mDC frequencies in PBMC from lupus patients compared to healthy controls. Similarly, pDC levels were found to be unaffected, reduced [74–78, 84, 85], or increased [79, 86]. Decreased frequencies of pDCs or mDCs were most often associated with active disease and to a lesser degree with nonactive disease [75]. Interestingly, studies showing peripheral pDCs decreases observed a concomitant infiltration of pDCs in nephritic kidneys, suggesting that active pDCs may have migrated to the sites of inflammation [78, 82]. Similarly, Fiore et al. showed that besides pDCs, BDCA1$^+$ DCs and BDCA3$^+$ DCs were increased in the renal tubulointerstitium of patients with lupus nephritis [78]. Increased numbers of pDCs and inflammatory/slanDCs are also found in cutaneous lesions of lupus patients, further suggesting migration of DCs to target organs [87, 88]. It is likely that DCs that reside in or have been recruited into the affected tissues will display different characteristics than those circulating in the periphery. Consequently, these populations should be included in further assessments in order to understand their contribution to disease pathogenesis and allow for a rational design of DC-targeting therapeutics.

5. SLE-Associated Dysfunction in Primary DCs

The few published maturation and functionality studies with primary human DCs have given conflicting results. Earlier reports indicated that DCs from SLE patients have normal or even reduced levels of costimulatory molecules and are poor stimulators of allogeneic T cells in mixed lymphocyte reactions. Scheinecker et al. reported that in SLE patients B7$^+$ and CD40$^+$ DCs were reduced and that DC-enriched APC from SLE patients displayed a diminished T cell-stimulatory capacity in both the allogeneic and the antigen-specific MLR, as compared with healthy individuals [76]. On the other hand, Mozaffarian et al. showed increased CD80/CD86 and

reduced PDL-1 expression on mDC during disease flares and an upregulation of PDL-1 during remission [89]. Similarly, Gerl et al. [81] published that monocytes and mDCs from SLE patients expressed higher levels of CD86 and BAFF, but not CD83 and CD40. Upon further assessment of their migratory capacity, they found that pDCs and mDCs from SLE patients had normal expression of CCR1, CCR5, and CCR7 but reduced expression of the chemokine receptor ChemR23 (CMKLR1). However, pDCs from the SLE patients showed an increased basal and CCL19-specific migration *in vitro*.

Assessment of peripheral monocytes, total DCs, $BDCA1^+$ DCs, and $CD14^{-/low}CD16^+$ DCs by Henriques et al. showed that a higher percentage of SLE monocytes and $CD14^{-/low}CD16^+$ DCs produced proinflammatory cytokines as well as a higher amount of cytokines produced per cell, particularly in active disease. Data from Kwok et al. [90] seemed to indicate that type I IFN production by pDC upon TLR9 engagement was diminished in SLE patients, leading them to hypothesize that the persistent presence of endogenous IFNα-inducing factors induces TLR tolerance in pDCs of SLE patients, resulting in impaired production of IFNα. Studies by Jin et al. [79, 91] also suggested deficiencies in TLR9 recruitment/signaling and production of proinflammatory cytokines in pDCs from SLE patients; however, they also showed that SLE pDC had an increased ability to stimulate T cells. Importantly, while pDCs from healthy donors induced suppressive T regulatory cell features (Foxp3 expression) in T cell cultures upon addition of apoptotic PMNs, SLE pDCs failed to do so.

These studies indicate that SLE is associated with phenotypic and functional changes in DCs and that these changes can affect different aspects of the DCs' functional program in distinct and divergent ways.

6. SLE-Associated Dysfunction in *In Vitro* Generated DCs

Due to the paucity of DCs in leukopenic SLE patients, many studies have used *in vitro* generated monocyte-derived DCs (moDCs) to gain insight in DC generation, phenotype, and function in the context of SLE.

Initial studies suggest that monocyte-derived DCs had a reduced proinflammatory and T cell stimulatory activity [92] while later studies suggested accelerated differentiation and maturation concomitant with increased activity to maturation stimuli [93]. MoDCs from SLE patients expressed higher levels of HLA-DR and activating FcγRs, but decreased expression of inhibitory FcγR and expression levels correlated with disease severity [92, 94]. In addition, moDCs spontaneously overexpressed activating costimulatory molecules including CD40, CD80, and CD86 and showed increased production of stimulatory cytokines (IL-6, IL-8, and BAFF/BlyS), eventually resulting in an increased capacity to activate T cells in an MLR [93, 95]. Similarly, Nie et al. [96] demonstrated substantial phenotypic and functional aberrations in DCs generated from Flt3-ligand and GM-CSF/IL-4 stimulated bone marrow aspirates. Both immature and mature DCs from SLE donors expressed higher levels of CCR7, CD40, and CD86 and induced stronger T cell proliferation.

7. Nature versus Nurture

Drawing causative relationships between DCs frequencies, maturation status, functionality, and disease is complex as it is not clear whether aberrations in DC frequency and functionality are the driver or a result of the disease. It is likely that genetic alterations in DCs predispose to the development of accelerated maturation and abnormal behavior. Evidence for this intrinsic defect is supported by the observations that moDCs from SLE patients, generated from either PBMC or bone marrow, display accelerated maturation and increased proinflammatory status compared to moDC from healthy donors. On the other hand, serum of SLE patients has been shown to contain pro- and anti-inflammatory stimuli like type I IFN, type I IFN-inducing factors, and IL-10 that alter DC differentiation, maturation, and functionality, even in DCs from healthy donors [97–99]. This raises the question whether the aberrant behavior of DCs in SLE patients is a result from an intrinsic defect, a result of their development in an inflammatory environment, or a combination of these two [97]. To further confound the interpretation of human clinical data, various classic SLE treatments, including antimalarials, corticosteroids, and immunosuppressive drugs significantly affect DC number, maturity, and functionality [100].

8. Mouse Models to Dissect Role of DCs in SLE Pathogenesis

The availability of mouse models provides an exciting opportunity to gain cellular and molecular insight in the role of different DC populations in the development and progression of SLE. There are a variety of spontaneous models, including the F1 hybrid between the New Zealand Black (NZB) and New Zealand White (NZW) strains (NZB/W F1) and its derivatives, the MRL/lpr and BXSB/Yaa strains, as well as inducible models such as the pristane-induced model and chronic graft-versus-host-disease models (cGVHD) [101–104]. In recent years the number of models has been expanded with genetically modified mice, targeted in genes that can promote, resist, and modify lupus susceptibility [105, 106]. All of these models display their own variation of lupus-like disease reminiscent of symptoms observed in patients, including autoantibody production, lymphoid activation and hyperplasia, lupus nephritis, and skin manifestations. Although all of these models have been instrumental in the identification of several main concepts in this diseases, none of the models can completely recapitulate the complexity and variety of human disease. However, careful pairing of models with patient groups with the similar clinical manifestations can ensure the translational relevance of these preclinical models.

Mouse models have several advantages: (i) the relative homology between human and mouse DCs, (ii) the opportunity to genetically or pharmacologically eliminate specific DC populations during specific stages of disease, (iii) access to all target tissues for the assessment of tissue associated or infiltrating DCs, (iv) the opportunity to assess the effects of common treatments on the parameters, and (v) a plethora of biological and pharmacological tools to dissect the relative

contribution of specific molecules and mediators to the development and progression of disease.

9. Similarities between Mouse and Human DCs

Recent genomic, proteomic, and functional analyses of mouse and human DCs have identified high homology between the most abundant DC populations [107]. Like in human DCs, mouse DCs lineages encompass conventional DCs, pDCs, $CD14^+$ DCs, tissue DCs, and monocyte-derived/inflammatory DCs [38, 108].

Conventional mouse DCs encompass three main subpopulations which are found in circulation as well as in secondary lymphoid organs [109]: (1) $CD11c^{high}MHCII^+CD8\alpha^-33D1^+Sirp\alpha^+CD11b^+$ (CD11b DCs), which express most TLRs except Tlr3, display a preference for activation of CD4 T cells, and have high homology with the human $BDCA1^+$ DCs; (2) $CD11c^{high}MHCII^+CD8\alpha^+CD205^+Sirp\alpha^-CD11b^-$ (CD8α DCs), which express Xcl1, CD141, and Clec9A and express mRNAs coding for most TLRs except Tlr5 and Tlr7, and are characterized by high Tlr3 expression; and (3) $CD11c^{high}MHCII^+$ cells that lack CD8α, CD4, and CD11b (generally termed "double" or "triple" negative) DCs that, like CD8a DC, express Xcl1, CD141, Clec9A, and Tlr3 [110–113]. These latter two populations have a high capacity to phagocytose dying cells and cross-present cell-associated or particulate antigens to CD8 T cells. Based on their genomic and functional analysis these two populations are considered to be homologues to the human $BDCA3^+$ DCs.

Like human pDCs, mouse pDCs produce vast amounts of type I IFN in response to viruses via TRL7/9 mediated pathways. Compared to their human counterparts, mouse pDCs show relatively poor capacity for phagocytosis and antigen presentation. However, both populations have been implied in the maintenance of peripheral tolerance [45, 114–116].

Various types of inflammatory and monocyte-derived DCs have been identified in mice as well. Tissue infiltrating $CD14^+$ DC-like cells have been found under inflammatory conditions [117, 118]. Inflammatory DCs have been shown to arise after a wide variety of immunological insults, including pathogenic infection, experimental sterile inflammation, and models of inflammatory diseases such as RA, colitis experimental autoimmune encephalomyelitis, and allergic asthma (reviewed in [119]).

10. The Role of DCs in Mouse SLE Models

Recent studies indicate an important role for DCs in the development and progression of SLE-like disease in mouse models. Similar to human disease, DCs from lupus-prone mice display a range of alterations in their numbers and their functionality [120–123]. Splenic DCs from NZB/W F1 showed enhanced maturation and a stronger ability to attract B cells and present antigens to T cells than DCs from control mice. pDCs from SLE-prone mice showed increased type I IFN producing capacity upon TLR9 stimulation and increased cell survival compared to pDCs from C57BL/6 mice. Enhanced mDC and pDC activity has also been reported in male BXSB/Mp mice that express an extra copy of Tlr7 on the Y chromosome.

Importantly, depletion studies have now shown causal relationships between DC subsets and disease manifestations. Constitutive depletion of pDCs in lupus-prone mice either through genetic ablation of IRF8, a transcription factor required for pDC and CD8αDC development, or by diphtheria toxin treatment of mice expressing the diphtheria toxin receptor on pDCs resulted in markedly reduced type I IFN production, a reduced IFN signature, reduced autoantibody production, and reduction in the severity of kidney pathology glomerulonephritis [124–126]. Importantly, transient pDC depletion during the early stages of disease was sufficient to significantly alter the course of the disease, suggesting a more prominent role for pDCs in the induction of the disease than in disease pathogenesis at later stages of disease [125]. Diphtheria toxin treatment of CD11c-DTA MLR.Fas^{lpr} mice resulted in reduced T cell differentiation, plasmablast numbers, and autoantibody levels. Interestingly, these mice developed interstitial kidney infiltrates but failed to progress to glomerular or interstitial nephritis, suggesting that DCs play a role in the development of tissue damage [127]. In line with this observation, this group also showed that CD11c depletion, but not LC depletion, resulted in significantly reduced dermatitis, demonstrating that DCs other than LCs control dermatitis in this model [127].

Besides the opportunity to assess the relative and temporal contribution of different DC populations to the development of specific disease manifestations, mouse models also allow for the identification of specific processes in DCs which affect disease development. Targeted deletion of regulatory molecules associated with SLE susceptibility in humans, including Shp1, A20, Blimp-1, Lyn, or Eat-2, specifically in $CD11c^+$ cells resulted in increased DC activity and development of inflammatory and autoimmune phenotypes characterized by the production of autoreactive antibodies and several manifestations of SLE, including severe glomerulonephritis [128–132].

Together these observations indicate that mouse models provide a useful platform for the identification, dissection, and targeting of DC intrinsic and extrinsic processes that facilitate the development, progression, and possibly a cure for SLE.

11. DC Targeted Therapies for SLE

Based on the general role of DC in the regulation of peripheral tolerance to self-antigens, the dysregulation of DCs observed in SLE, and the emerging evidence of the contribution of DCs in the initiation and perpetuation of SLE pathogenesis, it is not surprising that DC-targeting therapeutic strategies have become a topic of interest. Particularly, strategies that would promote self-antigen presentation in a tolerogenic context could be promising for the generation of an abortive or suppressive environment for the autoreactive T and B cells and restoration of peripheral tolerance [133, 134].

In recent years several *ex vivo* models have been established for the generation of human DCs with stable tolerogenic functions (reviewed in [135]). Generally, these resulting tolerogenic monocyte-derived DCs express low levels of positive costimulatory molecules and high levels of immune suppressive mediators (PDL-1, IL-10, etc.). Upon pulsing with specific antigens these DCs are anticipated to promote antigen-specific tolerance via the induction of T cell anergy, T cell apoptosis, skewing of T cell phenotypes to more Th2 or regulatory phenotypes, and the expansion of regulatory T cells.

Tolerogenic DC therapy is still in its infancy and little data is available on its *in vivo* potential. The first studies showed that transfer of antigen-loaded tolerogenic DCs could induce antigen-specific regulatory CD8 T cells and inhibit effector functions in antigen-specific CD8 T cells [136, 137]. A clinical trial in patients with type I diabetes using DCs treated with antisense oligonucleotides to silence costimulatory molecules was less successful, and although the treatment was well tolerated, only very limited tolerance outcomes were reported [138]. A subsequent trial in T1D patients indicated that transfer of IL-10 and TFGβ1 generated tolerogenic DCs pulsed with pancreatic islet cells induced antigen-specific T cell hyporesponsiveness and was associated with better glycemic control [139]. Similarly, transfer of a single dose of tolerogenic DCs, derived by *ex vivo* treatment with NF-κB inhibitors, into patients with active RA resulted in a modest improvement in disease activity 3 and 6 months after injection [140]. Currently there are several trials addressing the therapeutic potential of tolerogenic DCs in multiple sclerosis, rheumatoid arthritis, type I diabetes, and allergic asthma [141].

To date no tolerogenic DC transfer studies have been published in preclinical models or SLE patients. However, *in vitro* data indicate that tolerogenic DCs can be generated from SLE patients [83, 142, 143] and that apoptotic cells can be used as source to load the DCs with autoantigens [143]. The insight obtained from currently ongoing tolerogenic DC treatment strategies in other chronic inflammatory diseases will help to identify critical parameters such as dose, route, and duration of treatment leading to the most efficacious outcome [144, 145]. However, a better understanding of the role of DCs in disease pathogenesis is critically needed in order to select the type of tolerogenic DC that can successfully counteract the dysfunctional adaptive immune responses that maintain the disease.

Competing Interests

The authors declare that they have no competing interests.

Acknowledgments

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Research Article

Linking CD11b$^+$ Dendritic Cells and Natural Killer T Cells to Plaque Inflammation in Atherosclerosis

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Atherosclerosis remains the leading cause of death and disability in our Western society. To investigate whether the dynamics of leukocyte (sub)populations could be predictive for plaque inflammation during atherosclerosis, we analyzed innate and adaptive immune cell distributions in blood, plaques, and lymphoid tissue reservoirs in apolipoprotein E-deficient (ApoE$^{-/-}$) mice and in blood and plaques from patients undergoing endarterectomy. Firstly, there was predominance of the CD11b$^+$ conventional dendritic cell (cDC) subset in the plaque. Secondly, a strong inverse correlation was observed between CD11b$^+$ cDC or natural killer T (NKT) cells in blood and markers of inflammation in the plaque (including CD3, T-bet, CCR5, and CCR7). This indicates that circulating CD11b$^+$ cDC and NKT cells show great potential to reflect the inflammatory status in the atherosclerotic plaque. Our results suggest that distinct changes in inflammatory cell dynamics may carry biomarker potential reflecting atherosclerotic lesion progression. This not only is crucial for a better understanding of the immunopathogenesis but also bares therapeutic potential, since immune cell-based therapies are emerging as a promising novel strategy in the battle against atherosclerosis and its associated comorbidities. The cDC-NKT cell interaction in atherosclerosis serves as a good candidate for future investigations.

1. Introduction

Increasing evidence from animal and human studies points to a nonnegligible role for the innate and adaptive immune system in the development of atherosclerosis, still the leading cause of cardiovascular morbidity and mortality in our Western society [1–4]. In fact, it is estimated that approximately 30% of all deaths worldwide can be attributed to cardiovascular disease [5]. Therefore, there is a great need for the discovery of new biomarkers that may help in the early detection of patients at risk as well as the development of new therapies leading to the stabilization or regression of atherosclerotic plaques.

Recent findings suggest that a mismatch in the distribution, phenotype, and/or function of dendritic cells (DC), the main orchestrators of the immune response, contributes

to the susceptibility to and the course of atherosclerosis [6–10]. There are two major subpopulations of DC, namely, conventional DC (cDC) and plasmacytoid DC (pDC). In mice, cDC subtypes comprise cDC type 1 (cDC1), encompassing lymphoid-tissue-resident CD8α^+ cDC and their migratory counterparts CD103$^+$ cDC, and CD11b$^+$ cDC2 [8, 10, 11]. The specific and highly conserved transcription factor *Zbtb46* can be used to distinguish cDC and their progenitors from other immune cells [12]. Mouse atherosclerotic plaques contain both subtypes of cDC, but CD11b$^+$ cDC are most abundant and have been shown to rapidly increase during atherogenesis. Moreover, they are described to promote atherosclerosis [13, 14]. In contrast, CD103$^+$ cDC protect against atherosclerosis as they have been shown to support the homeostasis of regulatory T cells (Tregs) in a mouse model of atherosclerosis [15]. In humans, cDC are also segregated into two subtypes, namely, BDCA-3$^+$ cDC (cDC1) and BDCA-1$^+$ cDC (cDC2) [8, 10]. In addition to cDC, few pDC can be detected in murine and human atherosclerotic lesions, though their exact role in the disease process is still a subject of discussion [16–19].

Murine and human unstable plaques are associated with increased levels of T cells. Activated CD4$^+$ effector and memory T cells with a T helper (Th) 1 profile are among the earliest cells to accumulate in atherosclerotic lesions [20]. Natural killer T (NKT) cells represent another subset of T cells that recognize glycolipid antigens presented on CD1d molecules (on antigen-presenting cells) and share surface receptors in common with NK cells. In the past few years NKT cells have become of great interest given the fact that lipid accumulation is a prominent aspect of atherogenesis. Moreover, bidirectional interactions between NKT cells and DC ensure amplification and control of the subsequent innate and adaptive immune responses. Most of the experimental data from animal models attribute a proatherogenic role to NKT cells [21–23]. In humans, however, the pathophysiological role of these cells is less clear.

Although multiple immune cells are involved in atherosclerosis, most studies focus on a single cell type due to technical limitations. Detailed immune cell phenotyping requires the use of multilaser flow cytometers [24]. We previously described a protocol and a gating technique to identify and isolate immune cells from human atherosclerotic plaques using multiparametric flow cytometry [14]. In this study, local and systemic immune cell distributions in murine and human atherosclerosis were characterized simultaneously using flow cytometry and real-time qPCR. The distribution of DC (subsets), NK(T) cells, T cells, and monocytes/macrophages was analyzed both in blood and plaques. Previous research has shown that disturbed flow, caused by carotid ligation, induces rapid and dynamic leukocyte accumulation in the arterial wall [25]. However, adaptive immunity may not be solely driven from within the plaque but may also be driven from plaque-draining lymph nodes or even the periphery (e.g., the spleen). Therefore, possible associations between all the compartments were investigated. Additionally, we assessed the expression of different chemokine receptors during disease development to determine whether the homing functionality of immune cells correlates with changes in immune cell dynamics or plaque development.

2. Materials and Methods

2.1. Mice. Male and female ApoE$^{-/-}$ mice were fed a Western-type diet (WD, 4021.90, AB Diets) starting at an age of 6 weeks (wk). Mice were sacrificed with sodium pentobarbital (250 mg/kg, i.p.) before onset of atherosclerosis (0 wk of WD) or after 6, 12, and 24 wk of WD. These time points represent healthy artery, fatty streak, fibroatheroma, and advanced atherosclerotic plaques in mice. Analysis of total plasma cholesterol was performed by using a commercially available kit (Randox) following the manufacturer's instructions. Age-matched nonatherosclerotic C57BL/6J control mice on chow feeding were used to adjust for changes related to ageing rather than atherosclerosis. The animals were housed in a temperature-controlled room with a 12-hour light/dark cycle and had free access to water and food.

2.2. Patients. To characterize the immune cells in human atherosclerosis, 72 patients that were eligible for endarterectomy at the carotid (n = 35; 49%) and femoropopliteal level (n = 37; 51%) were recruited from the clinical departments of Thoracic and Vascular Surgery of the Antwerp University Hospital and ZNA Middelheim. From 57 (79%) of the included patients, peripheral blood samples were collected as well to study the systemic immune cell distribution. High sensitivity C-reactive protein (hs-CRP) levels were measured in serum from 35 patients by the clinical lab of the Antwerp University Hospital. Patient characteristics are shown in Table 1.

2.3. Ethics Statement. The mouse protocols were approved by the Antwerp University Ethics Committee on Animal Experiments (permit number: 2013-68). The animals received human care and were treated according to the national guidelines for animal protection, and the "Guide for the Care and Use of Laboratory Animals" (National Institutes of Health, 1985). Protocols involving patients were approved by the local Ethics Committee (number 12/25/212), and all research was based on written informed consent with proper arrangements for the protection of the confidentiality of personal data of the individuals concerned.

2.4. Cell Isolation and Flow Cytometry from Murine Blood and Tissues. After sacrificing the mice, blood was obtained by cardiac puncture. Single cell suspensions of the aorta draining mediastinal lymph nodes (LN) [26] and the spleen were prepared by passage through a 40 μm cell strainer. Erythrocytes were lysed using a red blood cell lysing buffer (Hybri-Max, Sigma-Aldrich). Remaining leukocytes were counted using a hemocytometer and labelled with anti-mouse monoclonal antibodies (Supplemental Table S1 in Supplementary Material available online at http://dx.doi.org/10.1155/2016/6467375) at 4°C in FACS buffer (PBS + 0.1% BSA (Sigma-Aldrich) + 0.05% NaN_3 (Merck)) in the presence of CD16/32 Fc-receptor blocker (BioLegend). Cells were analyzed on a BD Accuri C6 cytometer (BD Biosciences). Debris and dead cells were excluded based on forward scatter, side scatter, and positive staining for propidium iodide (Life Technologies). The gating

Table 1: Patient characteristics.

Variable	Carotid artery plaque ($n = 35$)	Femoral artery plaque ($n = 37$)	Significance[†]
Age (years)	72 ± 2	71 ± 2	NS
Male gender (%)	60	68	NS
Degree of artery stenosis (%)	82 ± 2	87 ± 1	NS
Risk factors (%)			
Family history	20	46	$P < 0.05^{*}$
Hypertension	71	78	NS
Hypercholesterolemia	80	75	NS
Diabetes mellitus	42	27	NS
Smoking	46	70	$P < 0.01^{**}$
Obesity	29	22	NS
Prior vascular intervention	46	68	NS
Medication (%)			
Acetylsalicylic acid	98	92	NS
NSAIDs	3	3	NS
Beta-blockers	46	65	NS
Calcium channel blockers	34	22	NS
ACE inhibitors	43	35	NS

[†]Significant differences between plaque locations; NS: no significance.

strategy is depicted in Figure 1. Data analysis was performed with FCS Express 4 (De Novo Software).

2.5. Cell Isolation and Flow Cytometry from Human Atherosclerotic Plaques and Peripheral Blood. Atherosclerotic plaques were collected in RPMI 1640 medium (Life Technologies) and kept at room temperature until processing. Cell isolation was performed as described [14]. Briefly, within 2 h after surgery the plaque specimens were dissected into small pieces, followed by an enzymatic digestion with 2.5 mg/mL collagenase IV (Life Technologies) and 0.2 mg/mL DNase I (Roche) for 2 h at 37°C. After digestion, the residue was filtered over a 40 μm cell strainer. PBMC were isolated from blood samples by Ficoll (GE Healthcare) density gradient centrifugation. After isolation, cells from plaque and blood were blocked with mouse gamma globulins and stained with an optimized 9-color panel of mouse anti-human monoclonal antibodies (Table S1). To eliminate the abundance of cell debris and extracellular lipids in the digested plaque suspensions, we used a gating strategy as described previously [14]. All measurements were performed on the FACSAria II (BD Biosciences). Data acquisition and analysis were done using FACSDiva 6.1.2 (BD Biosciences).

2.6. Gene Expression Analysis. The aortic adventitia of mice was partially digested and removed from the rest of the vessel following incubation in an enzyme digestion solution composed of 781.25 U collagenase II and 14.0625 U elastase (Worthington) in 2.5 mL PBS for 10 minutes at 37°C [27]. Total RNA was extracted from the aorta, stripped from the adventitial layer, using a TRIzol-based RNA isolation protocol (Ambion). RNA concentrations were measured with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies). RNA was reverse-transcribed with the SuperScript II Reverse Transcriptase kit (Life Technologies). Quantitative gene expression analysis was performed on a 7300 Real-Time PCR System (Applied Biosystems) using SYBR green technology (SensiMix, GC Biotech). The parameters for PCR amplification were 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 60°C for 1 min, and 72°C for 30 s. Melting curves were checked for amplification of a single, specific product. Used primer pairs are summarized in Table S2. All data were analyzed using qBase+ 3.0 (Biogazelle).

2.7. Histological Analysis. After sacrificing ApoE$^{-/-}$ mice, the proximal ascending aorta and brachiocephalic artery were collected, embedded in Neg-50 (Thermo Scientific), and snap-frozen in liquid nitrogen. Atherosclerotic plaque size, stenosis, and necrotic core (acellular area with a threshold of 3000 μm^2) were analyzed on haematoxylin-eosin (H-E) stained 5 μm cryosections (Table 2). All images were acquired with Universal Grab 6.1 software using an Olympus BX40 microscope and were quantified with ImageJ software (National Institutes of Health).

2.8. Statistical Analysis. All data are presented as mean ± SEM. Multiple comparisons of means were performed for the analysis of all mouse data using one-way ANOVA followed by Dunnett's Multiple Comparison test or two-way ANOVA followed by Bonferroni's Multiple Comparison test where appropriate. Differences between human plaques derived from the carotid and femoral artery were tested with the independent Student's t-test. Variables that failed normality were logarithmically transformed or analyzed with the nonparametric Mann-Whitney U test. Correlations between local and circulating cells in atherosclerosis were described using Spearman's rank-order correlation coefficient. Statistical analysis was performed using Prism 5.0 (GraphPad) or

Table 2: Cholesterol and plaque parameters of ApoE$^{-/-}$ mice during atherogenesis.

	0 wk	6 wk	12 wk	24 wk
Cholesterol (mg/dL)	232 ± 11	625 ± 97***	658 ± 40***	698 ± 45***
Stenosis A_{prox} (%)	0 ± 0	1.2 ± 0.4	13.8 ± 2.5***	23.2 ± 2.3***
Stenosis A_{br} (%)	0.8 ± 0.8	1.8 ± 1.1	51.3 ± 7.7***	61.5 ± 2.8***
Necrotic core A_{prox} (%)	0 ± 0	0 ± 0	0 ± 0	3.5 ± 1.1**
Necrotic core A_{br} (%)	0 ± 0	0 ± 0	1.4 ± 0.7	5.5 ± 1.3***

Data from proximal ascending aorta (A_{prox}) and brachiocephalic artery (A_{br}), mean ± SEM, 0 wk, n = 9–11, 6 wk, n = 11-12, 12 wk, n = 10-11, and 24 wk, n = 11-12; $^{**}P < 0.01$ and $^{***}P < 0.001$.

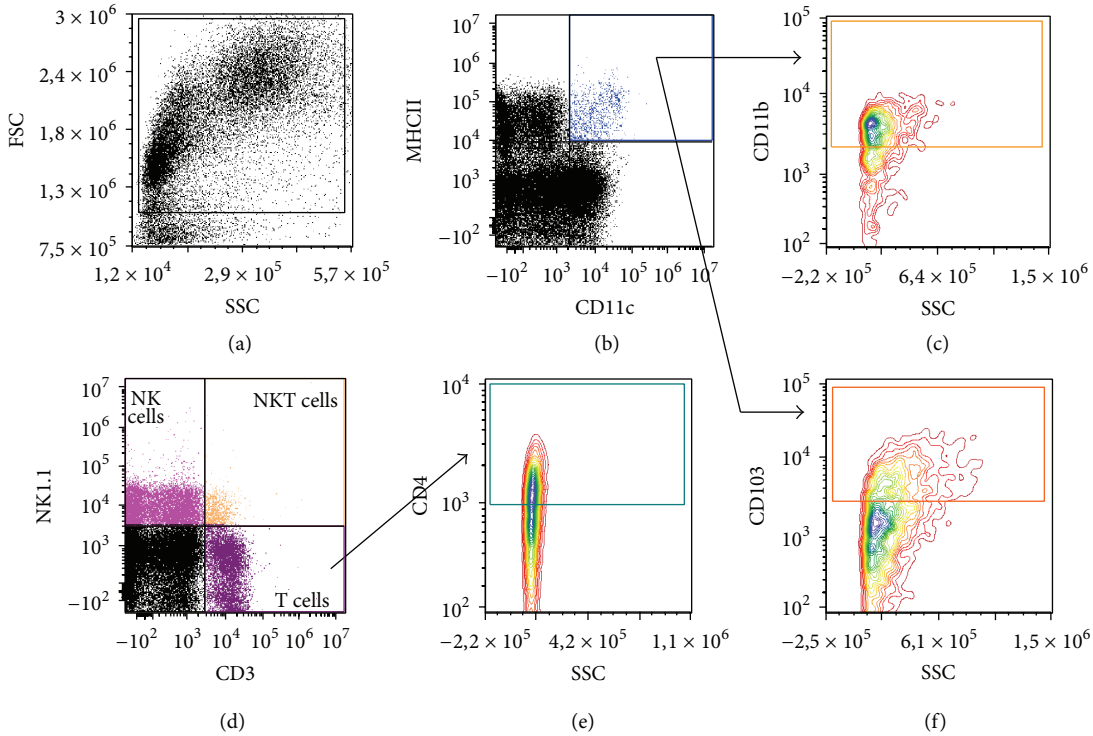

Figure 1: Gating strategy for the analysis of immune cells in murine atherosclerosis. Gates are set on isotypes to correct for nonspecific binding. (a) Plots are pregated on FSC and SSC to define the total percentage of leukocytes from cell debris. (b) The total cDC population was identified based on the expression of CD11c and MHCII. (c, f) Based on their expression of CD11b (c) and CD103 (f) two cDC subsets were identified. A distinction was made between circulating Ly-6C^{high} and Ly-6C^{low} monocytes and tissue resident macrophages (plots not shown). (d) Lymphocyte subsets were identified as T cells (CD3$^+$NK1.1$^-$), NK cells (CD3$^-$NK1.1$^+$), and NKT cells (CD3$^+$ NK1.1$^+$). (e) Th cells were defined as CD4$^+$ cells within the total T cell population.

R version 3.1.2 [28]. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Analysis of Immune Cells in Blood, Plaque, and Lymphoid Tissue of ApoE$^{-/-}$ Mice. During atherosclerotic plaque development in ApoE$^{-/-}$ mice the percentage of total DC increased in the spleen but no distinct changes were observed in blood or LN (Figure 2(a)). Interestingly, at all locations and time points, CD11b$^+$ cDC (or cDC2) represented the most predominant subset. Furthermore, a significant drop of this cDC subset was seen at all locations after 12 wk of WD (Figure 2(b), red bars). Regarding the other cDC subset, CD103$^+$ cDC or cDC1, we found no significant difference over time (Figure 2(c)). No significant changes were seen in the frequency of NK cells (data not shown). Additionally, the percentage of Ly-6C^{low} resident monocytes and their inflammatory counterparts, Ly-6C^{high} monocytes, in blood is decreased after 12 wk of WD. The percentage of both subtypes increased between 12 and 24 wk of WD, which was most pronounced in the Ly-6C^{high} subset (Figure 2(e)).

Regarding cells of the adaptive immunity, the percentage of T cells gradually declined in all studied compartments. The reduction in T cells was most pronounced after 24 wk of WD in blood, spleen, and mediastinal LN compared with mice sacrificed before the onset of atherosclerosis (Figures 2(e)–2(g)). With regard to T cell subsets, the percentage of

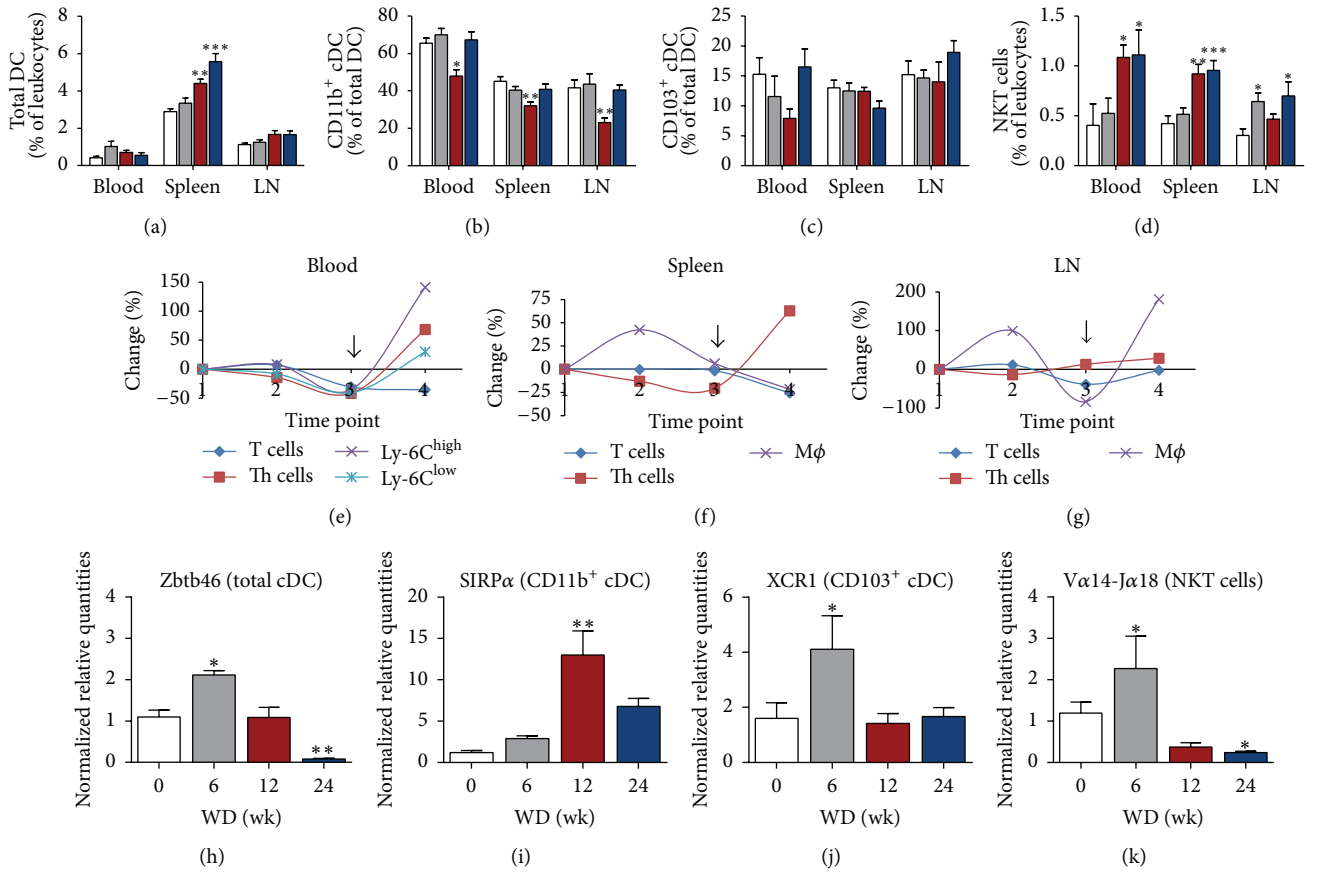

FIGURE 2: Flow cytometry and gene expression results of leukocyte(s) (subsets) in ApoE$^{-/-}$ mice during atherogenesis. Bar graphs representing mice sacrificed after 0 wk (white bars, n = 7–12), 6 wk (grey bars, n = 9–12), 12 wk (red bars, n = 9-10), and 24 wk (blue bars, n = 10–12) of WD. (a) Percentages of the total DC population. (b) Percentages of the CD11b$^+$ cDC subset within the total DC population. (c) Percentages of CD103$^+$ cDC within the total DC population. (d) Percentages of NKT cells within the total leukocyte population. (e–g) Graphs showing fluctuations (as percentage change over time) in T cells, Th cells, and monocytes/macrophages in blood (e), spleen (f), and mediastinal LN (g) at different time points (1 = 0 wk, 2 = 6 wk, 3 = 12 wk, and 4 = 24 wk of WD). The arrow at time point 3 (= 12 wk of WD) indicates dramatic leukocyte changes in all compartments. (h–k) Normalized expression levels of Zbtb46 (h), SIRPα (i), XCR1 (j), and Vα14-Jα18 (k) mRNA in aortic tissue samples; $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

total CD4$^+$ Th cells significantly decreased after 12 wk of WD in blood and spleen as compared to mice at 0 wk of WD (Figures 2(e) and 2(f)). Interestingly, percentages of NKT cells, known as T lymphocytes with innate effector functions, are increased after 12 wk (in blood and spleen) and 24 wk of WD (in blood, spleen, and LN) (Figure 2(d)). All graphs indicate a clear turning point in immune cell dynamics at 12 wk of WD (Figures 2(e)–2(g), arrows). At this time point, a substantial increase was observed in the size and areas of the atherosclerotic plaques in the brachiocephalic and proximal aorta (Table 2). Between 12 and 24 wk of WD systemic immune activation is induced as evidenced by an increase in the majority of immune cells above baseline in all locations investigated.

Expression levels of DC (subset) genes within plaque-containing aortic tissues were measured with qPCR. The expression of Zbtb46, a transcription factor used to distinguish the total cDC population from other immune cells, was increased after 6 wk of WD, returned to baseline after 12 wk of WD, and was reduced below baseline levels after 24 wk of WD (Figure 2(h)). SIRPα and XCR1 gene expression was used to discriminate between CD11b$^+$ cDC and CD103$^+$ cDC, respectively [29]. The expression level of SIRPα increased 11-fold in mice after 12 wk of WD compared to mice sacrificed before the start of the WD (Figure 2(i)). A significant increase (2.6-fold) was also observed in the expression of XCR1 after 6 wk of WD (Figure 2(j)). The same is true for the amount of NKT cells, as detected by Vα14-Jα18 mRNA (2-fold increase, Figure 2(k)).

3.2. The Number of Circulating CD11b$^+$ cDC and NKT Cells Is Highly Indicative for Plaque Inflammation in Mice during Atherosclerosis. In mouse plaques, strong features of inflammation could be detected, including the expression of T-bet, the main director of Th1 lineage commitment [30]. The relative mRNA expression of T-bet was significantly increased (3-fold) after 6 wk of WD feeding compared to mice that had not yet received a WD (Figure 3(a)). Furthermore, the expression of different chemokine receptors, involved in homing of leukocytes to inflammatory sites or lymph

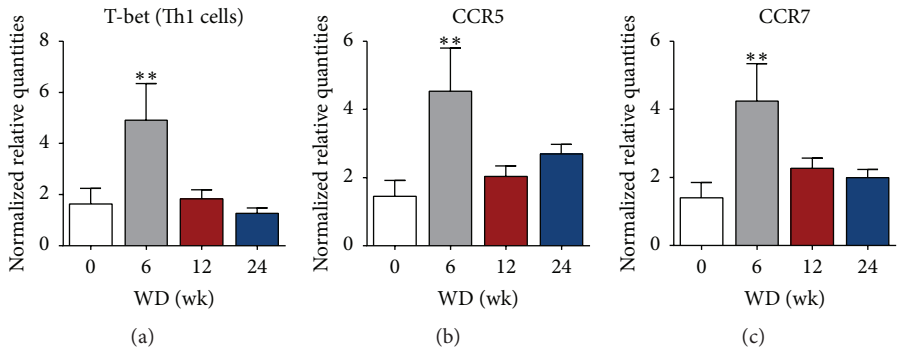

Figure 3: Features of inflammation in mouse aortic plaques. ((a), (b), (c)) Relative mRNA levels of T-bet (a), CCR5 (b), and CCR7 (c) in plaque-containing aortic tissue measured by real-time qPCR. 0 wk, $n = 6$, 6 wk, $n = 5$, 12 wk, $n = 11$, and 24 wk, $n = 14$-15; $^{*}P < 0.05$ and $^{**}P < 0.01$.

nodes, was investigated. Plaque mRNA expression for C-C chemokine receptor type 5 (CCR5) and CCR7 was significantly higher (3-fold) after 6 wk of feeding on the WD and returned to baseline when lesions were progressing (Figures 3(b) and 3(c)). In aortic plaques, the expression of T-bet is positively correlated with CCR5 and CCR7 expression at 6 wk and 12 wk of WD (Table 3). Furthermore, there is a strong correlation between the degree of stenosis in the proximal aorta and the mRNA level of CCR7, Vα14-Jα18, Zbtb46, and SIRPα in mice sacrificed at 6 wk of WD. No correlations were found between the expression levels of CCR5/7 and the numbers of circulating T cells, $CD4^+$ Th cells, B cells, or NK cells after 6 wk on the WD. In mice from the 12 wk of WD group, Vα14-Jα18 expression is correlated to T-bet, CCR5, CCR7, Zbtb46, SIRPα, and XCR1 (Table 3).

Because blood provides a conduit between all organs and tissues, correlations between local and circulating cells in atherosclerosis were also described using Spearman's rank-order correlation coefficient (Table 3). Plaque development and inflammation were most pronounced in mice that had a low number of circulating NKT cells at 6 wk of WD. At this time point, a strong inverse correlation was determined between NKT cell numbers in blood and the expression of T-bet, CCR5, CCR7, Zbtb46, and XCR1 in plaque-containing aortic tissue. In line with these findings, the degree of stenosis in the proximal aorta was inversely correlated to circulating NKT cell numbers. Similar observations were seen for the number of circulating $CD11b^+$ cDC and the expression of the same genes in the aorta at 12 wk of WD.

3.3. $CD11b^+$ cDC and NKT Cell Dynamics in $ApoE^{-/-}$ Mice Are Distinct from Healthy Controls. To correct for changes related to age rather than atherosclerosis, we compared the number of $CD11b^+$ cDC and NKT cells in $ApoE^{-/-}$ mice fed an atherosclerotic diet and age-matched healthy wild-type mice fed a chow diet at two time points which represent early and advanced atherosclerotic lesions. Percentages of circulating $CD11b^+$ cDC are higher in $ApoE^{-/-}$ mice as compared to healthy wild-type controls in blood, spleen, and mediastinal LN during early lesion (6 wk of diet) formation (Figures 4(a)–4(c)). In the case of advanced plaques (24 wk of diet), the $CD11b^+$ cDC percentage is also significantly higher in the blood of $ApoE^{-/-}$ mice (Figure 4(a)). The opposite is seen for the NKT cells: at the initiation of the disease (6 wk of diet) NKT cell numbers in blood (Figure 4(d)) and mediastinal LN (Figure 4(f)) of $ApoE^{-/-}$ mice are low as compared to healthy controls. There is no difference in the NKT cells percentage in the spleen between $ApoE^{-/-}$ and control mice at the onset of atherosclerosis but they increase with enhanced atherosclerosis (Figure 4(e)). Hence, high numbers of $CD11b^+$ cDC and low numbers of NKT cells at 6 wk of diet are attributable to the induction of atherogenesis in $ApoE^{-/-}$ mice.

3.4. Analysis of Immune Cells in Human Atherosclerotic Plaques and Blood. To analyze different leukocyte subsets in plaque and blood samples from advanced atherosclerosis patients, we used a gating strategy as depicted in Figure 5(a). As observed in mice and similar to our previous data [14] we observed predominance of $CD11b^+$ cDC within the $CD45^+$ population in the plaques, compared to the $CD16^+$ monocyte-derived (mo)DC subset. In contrast to the plaque, the $CD16^+$ moDC was the predominant subset in the blood compared to the $CD11b^+$ cDC (Table 4). Clec9A was used as a marker for the human equivalent of $CD103^+$ cDC in mice. However, due to their low numbers [14], we refrained from studying this cDC subset in subsequent analyses in this study. Within the $CD45^+$ population, atherosclerotic plaques predominantly contained NK cells. Furthermore, relatively high mean percentages of NKT cells and T cells were also observed, both in blood and plaque, as compared to the DC (subsets) and monocytes/macrophages (Table 4).

To extend the evaluation of atherosclerotic plaque composition we compared the immune cell distribution in plaques from distinct anatomical locations (carotid versus femoral artery). To correct for size differences between plaques from femoral and carotid artery, the number of cells per gram tissue was calculated. Comparing between plaque locations, the number of cells per gram was significantly higher in carotid plaques for the total DC population (311±70 versus 121 ± 24; $P = 0.014$), $CD16^+$ moDC subset (20 ± 6 versus 5 ± 1; $P = 0.029$), $CD11b^+$ cDC subset (224 ± 51

Table 3: Spearman's rank correlation coefficients (ρ) of associations between gene expression results within plaques or between plaques and circulating NKT cells or CD11b$^+$ cDC.

				6 wk of WD				
	T-bet	CCR5	CCR7	Vα14-Jα18	Zbtb46	SIRPα	XCR1	Stenosis
T-bet$^{(p)}$	—	0.900	0.700	0.200	−0.200	−0.400	0.900	0.354
Stenosis$^{(p)}$	0.354	0.354	0.707	0.707	0.755	0.755	0.354	—
Vα14-Jα18$^{(p)}$	0.200	0.500	0.300	—	0.400	0.200	0.500	0.707
NKT cells$^{(b)}$	−1.000	−0.800	−1.000	−0.400	−1.000	−0.500	−0.800	−0.657
CD11b$^+$ DC$^{(b)}$	−0.400	0.000	−0.100	0.500	0.000	0.000	0.000	−0.251
				12 wk of WD				
	T-bet	CCR5	CCR7	Vα14-Jα18	Zbtb46	SIRPα	XCR1	Stenosis
T-bet$^{(p)}$	—	0.855	0.855	0.891	0.758	−0.818	0.952	−0.261
Stenosis$^{(p)}$	−0.261	−0.515	−0.393	−0.370	−0.381	0.345	−0.200	—
Vα14-Jα18$^{(p)}$	0.891	0.721	0.879	—	0.636	−0.830	0.939	−0.370
NKT cells$^{(b)}$	−0.456	−0.535	−0.426	−0.322	−0.116	0.274	−0.377	−0.189
CD11b$^+$ DC$^{(b)}$	−0.717	−0.733	−0.717	−0.583	−0.650	0.633	−0.733	0.400

p, plaque; b, blood.
The magnitude of the correlation coefficient determines the strength of the correlation: $|\rho| > 0.7$ strong correlation; $0.5 < |\rho| < 0.7$ moderate correlation; $|\rho| < 0.5$ weak correlation.

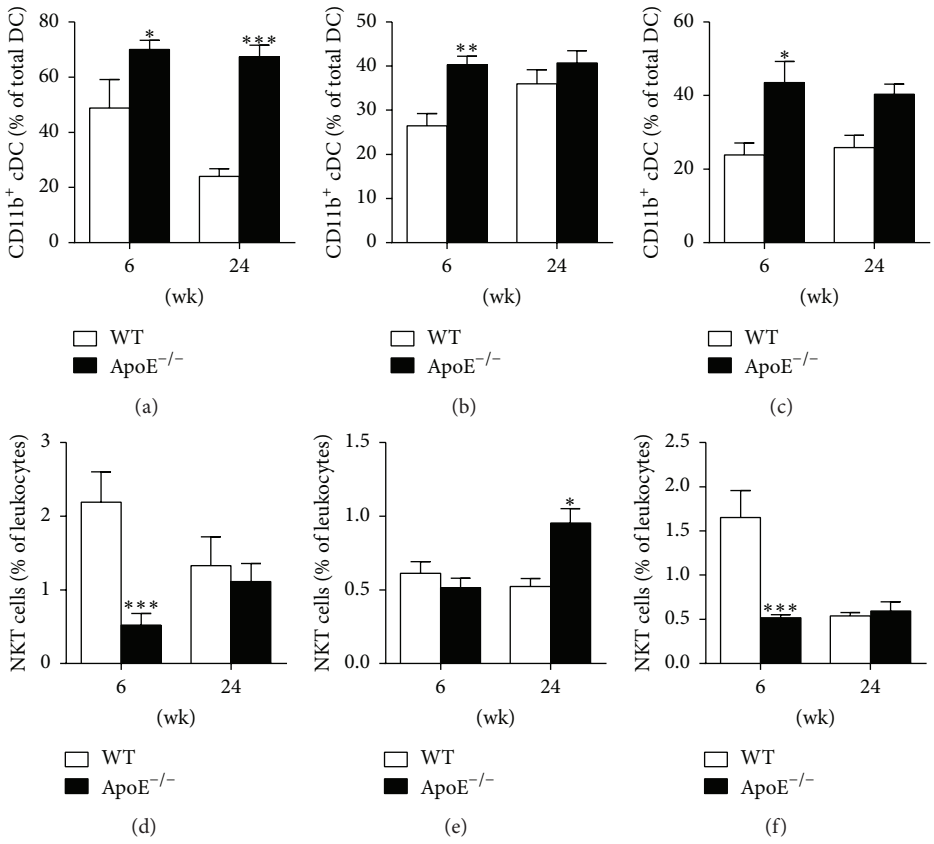

Figure 4: Comparison of CD11b$^+$ cDC and NKT cell fluctuations between ApoE$^{-/-}$ mice and age-matched healthy controls. (a–c) Percentages of circulating CD11b$^+$ cDC in blood (a), spleen (b), and mediastinal LN (c) of ApoE$^{-/-}$ mice fed a WD and healthy wild-type controls fed a chow diet sacrificed at 6 and 24 wk of diet. (d–f) Circulating NKT cell numbers in blood (d), spleen (e), and mediastinal LN (f) of ApoE$^{-/-}$ mice fed a WD and healthy wild-type controls (on chow diet) sacrificed at 6 and 24 wk of diet. WT (white bars), $n = 4$–6; ApoE$^{-/-}$ (black bars), $n = 9$–12; $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

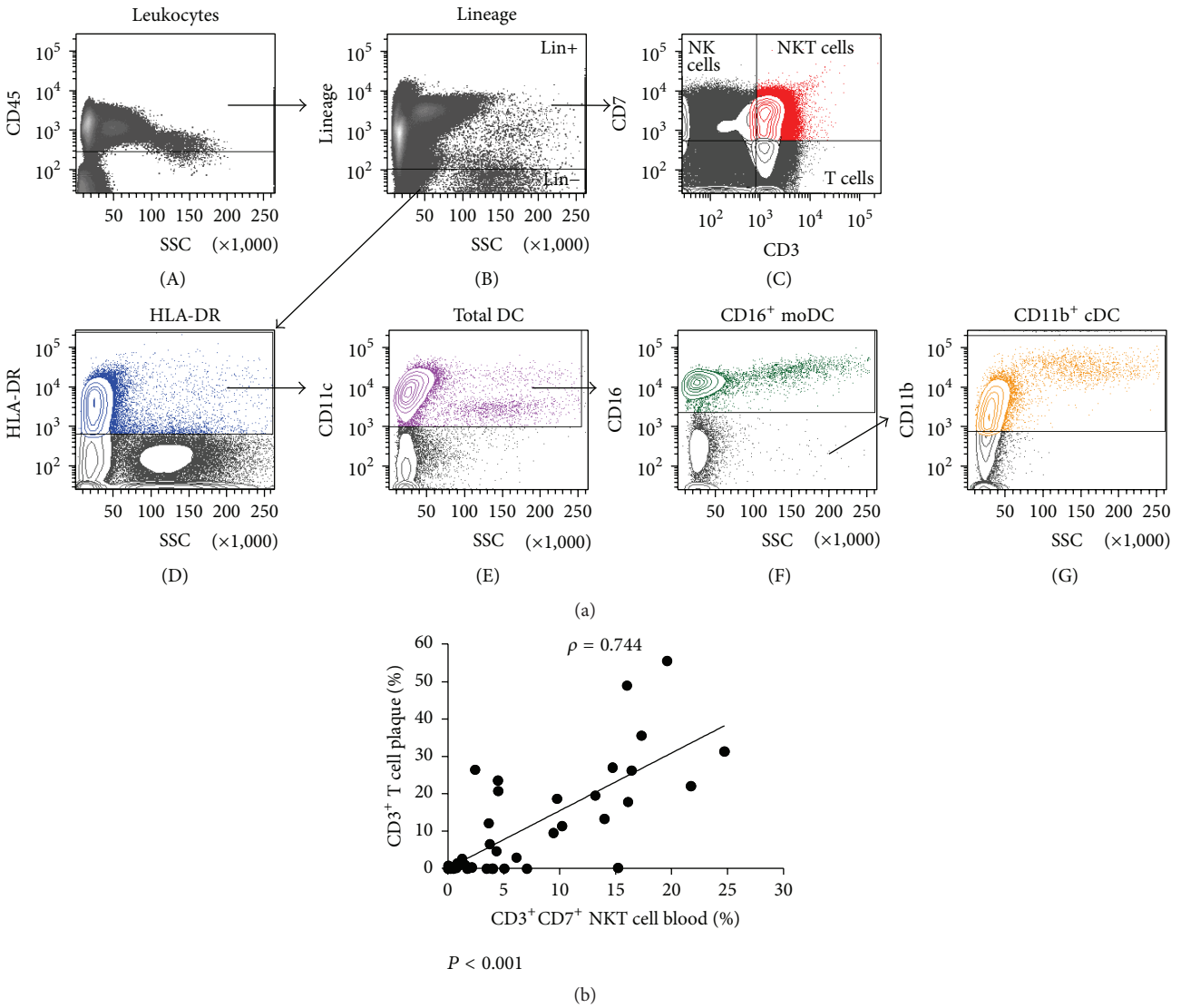

Figure 5: Leukocyte subsets and correlation analysis in human plaques and blood. (a) Gating strategy for the analysis of leukocyte(s) (subsets) in human plaque and blood samples. After staining the leukocyte population using a CD45 pan leukocyte antibody (A), lineage markers (= a CD3, CD14, CD19, CD20, and CD56 cocktail) were used to separate the DC (lineage−) from the other immune cells (lineage+) (B). Within the lineage+ cells we identified T cells, NK cells, and NKT cells, which were defined as $CD3^+$, $CD3^-$ $CD7^+$, and $CD3^+$ $CD7^+$, respectively (C). cDC were then identified as positive for HLA-DR (D) and CD11c (E). CD16 was used for the staining of monocyte-derived DC (F). Subsequently, $CD11b^+$ cDC (G) were gated from the CD16-negative population. Within the lineage+ population we identified monocytes in blood as $HLA\text{-}DR^+$ $CD11c^+$ $CD11b^+$ $CD14^+$ and macrophages in plaques as $HLA\text{-}DR^+$ $CD11c^+$ $CD11b^+$ $CD68^+$ (plots not shown). (b) Spearman's rank correlation plot showing the relationship between $CD3^+CD7^+$ NKT cell numbers in blood and the percentage of total $CD3^+$ T cells in plaques obtained from the same endarterectomy patient ($n = 57$).

versus 85 ± 19; $P = 0.014$), NKT cells (1652 ± 623 versus 127 ± 38; $P = 0.01$), and T cells (1551 ± 556 versus 208 ± 65; $P = 0.029$). In contrast, the macrophage (403 ± 212 versus 10 ± 3; $P = 0.260$) and NK cell numbers per gram (1344 ± 221 versus 1092 ± 271; $P = 0.477$) did not significantly differ between the two locations.

3.5. In Advanced Human Atherosclerosis the Number of Circulating NKT Cells Is Predictive for Plaque Inflammation. We investigated whether correlations could be found between the immunological parameters in blood and advanced plaques collected from the same endarterectomy patient. We could find a strong predictive role for NKT cells which strengthens our observations in mice. Remarkably, the percentage of NKT cells in blood correlates strongly with the percentage of total T cells in the plaque ($\rho = 0.744$; $P < 0.001$) (Figure 5(b)). A linear regression analysis was performed to explore which of the human plaque variables can be predicted by blood variables. In all models, the logarithm of the plaque variables was entered as outcome variable. Here, we could see that the predictive value of the percentage of NKT cells in blood to predict the T cell (and NKT cell) load in the plaque is very

Table 4: Flow cytometric analysis of immune cells in human atherosclerotic plaques and blood.

	Blood (% within $CD45^+$ population)	Plaque (% within $CD45^+$ population)
Total DC		
$CD11c^+$ DC	6.5 ± 0.9	2.0 ± 0.2
DC subsets		
$CD11b^+$ cDC	2.7 ± 0.4	1.4 ± 0.2
$CD16^+$ moDC	3.7 ± 0.5	0.3 ± 0.1
Other leukocytes		
$CD14^+$ Mo	0.4 ± 0.1	NA
$CD68^+$ Mφ	NA	1.1 ± 0.8
$CD3^+$ T cell	10.3 ± 1.8	5.9 ± 1.0
$CD7^+$ NK cell	9.5 ± 1.7	14.1 ± 1.3
$CD3^+CD7^+$ NKT cell	8.9 ± 1.8	6.7 ± 1.5

Mo, monocyte; Mφ, macrophage; NA, not applicable.

Table 5: Coefficient of determination (R^2) values for each of the plaque variables calculated from a multiple linear regression model with all the risk factors and blood parameters as independent variables ($n = 57$ plaque and blood samples). This is the amount of variance in the outcome (plaque variable) that can be explained by all the risk factors and blood variables together.

Plaque variable (%)	R^2
Total DC	0,484670
$CD11b^+$ cDC	0,434602
$CD16^+$ moDC	0,534353
Macrophages	0,500945
T cell	0,802104
NK cells	0,744789
NKT cells	0,727062

strong ($P < 0.001$). This was also the case for the prediction of the NK cell and macrophage load ($P < 0.05$). Subsequently, for each of the plaque variables, a multiple linear regression model was fitted with all the risk and blood parameters as independent variables. For these models, the coefficient of determination (R^2) was calculated (Table 5). This shows that T cells ($R^2 = 0.802104$), NK cells ($R^2 = 0.744789$), and NKT cells ($R^2 = 0.727062$) in plaques are predictable by the combination of risk factors and blood variables. Next, stepwise backward model building was performed, starting with a model including all the plaque variables with $R^2 > 0.6$, to obtain multiple regression models with only the most significant predictors for each plaque variable. Strikingly, for both the percentages of T cells and NKT cells in plaques, the NKT cell numbers in blood are the most significant predictors. In addition, partial R^2 values were calculated to describe how strongly the cells in blood contribute to the prediction of cells in the plaque, based on all blood parameters and risk factors from a patient. The NKT cell numbers in blood strongly improve the prediction of the amount of both NKT cells (partial $R^2 = 0.36$; $P = 8.9 \times 10^{-9}$) and T cells (partial $R^2 = 0.20$; $P = 1.1 \times 10^{-7}$) in plaques, even if all other risk factors are accounted for. For the prediction of NK cells in plaques, the contribution of NKT cells in blood was not significant (partial $R^2 = 0.006$; $P = 0.34$).

C-reactive protein (hs-CRP) has been endorsed by multiple guidelines as a biomarker of atherosclerotic cardiovascular disease risk [31, 32]. However, in this study, hs-CRP levels in blood do not correlate with the percentages of NKT cells in blood or plaques from the same patient (data not shown), although this may be due to the fact that there were only few data points available for hs-CRP ($n = 35$).

4. Discussion

To date, only a few studies reported the analysis and association of circulating inflammatory cells and advanced atherosclerosis. Most of the existing data come from subclinical atherosclerosis and asymptomatic patients [33]. The aim of the present study was to analyze the frequency of immune cells in blood, plaque, and associated lymphoid tissues (i.e., mouse spleen and aorta-draining LN) and to investigate whether fluctuations in leukocytes are associated with or can be predictive for plaque growth and inflammation.

The most pronounced changes during atherosclerosis in mice occur early in plaque development in cells of the innate immune system. Early atherogenesis is marked by an elevation in plasma cholesterol levels followed by (oxidative) modification of low density lipoproteins, a well-known trigger of inflammation. Antigen-presenting cells are needed at this time to encounter these "foreign" antigens; hence more DC are present in the circulation and draining lymph nodes. As atherosclerosis progresses the number of $CD11b^+$ cDC declines significantly at 12 wk of WD in all locations investigated, suggestive of massive recruitment to the growing lesions in the aortic wall. Indeed, we observed an increase in the relative expression level of SIRPα in the aorta at the same time, together with a substantial increase in plaque size in the brachiocephalic artery and proximal ascending aorta. Recruitment of immune cells to sites of inflammation, infection, or injury is stimulated by chemokines and their receptors [34–37]. CCR5 directs recruitment of immune cells to inflammatory sites like atherosclerotic lesions, while CCR7 can mediate monocyte/macrophage egress from lesions and controls the subsequent migration of immune cells from the plaque to secondary lymphoid organs [37]. We observed a strong inverse correlation between circulating $CD11b^+$ cDC numbers and CCR5/7 expression in mouse aortic plaques at 12 wk of WD. Accordingly, mice that have a low number of $CD11b^+$ cDC in their circulation, as is the case at 12 wk of WD, have high expression levels of CCR5/7 in their plaques. This indicates a high degree of leukocyte trafficking to and from the plaque. In addition, we have also seen an inverse correlation between circulating $CD11b^+$ cDC and the expression levels of T-bet, Vα14-Jα18, and Zbtb46, which points to an increased inflammatory status in the plaque.

Additionally, this study revealed that plaque development and inflammation were most pronounced in mice that have a low number of circulating NKT cells at 6 wk of WD. At this time point, expression of inflammation markers, including T-bet, chemokines (CCR5/7), and cDC (Zbtb46), as well as the

degree of stenosis in the proximal ascending aorta, correlated with NKT cell numbers in blood, pointing to a very significant role of NKT cells in the initiation of atherosclerosis. In line with these findings, previous research demonstrated that the contribution of NKT cells on atherosclerosis is transient and limited to early fatty streak lesions [38, 39]. Similar to the observations made by Aslanian et al., we detected Vα14-Jα18 mRNA in early lesions (6 wk of WD) but found no accumulation of Vα14-Jα18 after the 6-week time point (12 and 24 wk of WD) [38]. Consistent with results from a study by Major et al., we found that NKT cell numbers are low in blood and LN of ApoE$^{-/-}$ mice compared with age-matched wild-type mice at an early stage of atherosclerosis development (6 wk of diet) [40]. As the lesion progresses to advanced atherosclerosis, the total DC number increased in the spleen after 12 wk and even more after 24 wk on the WD, due to systemic immune activation [41]. Additionally, this could also be the result of extramedullary hematopoiesis. When the bone marrow can no longer handle the production and differentiation of hematopoietic cells, it will outsource the production of circulating leukocytes, including DC. Of all organs, the spleen is an ideal outsource destination in ApoE$^{-/-}$ mice [42, 43].

Taken together, these data suggest that inflammatory processes, with an emphasis on CD11b^{+} cDC and NKT cells, are crucial in the early development of atherosclerosis before any morphological changes (plaque development) are visible. Based on these data, we propose 12 wk as a preferred time point for intervention, especially when assessing the effects of immunomodulatory therapies for preventing the development and progression of atherosclerosis. This is in agreement with Jeon et al., who reported a peak at 12 wk of diet in inflammatory mediators ICAM-1, CCR2, IL-6, IL-12p40, and IL-17 [41].

Parallel to mice, we also observed predominance of the CD11b^{+} cDC subset in human plaques when compared with CD16^{+} moDC, while the latter is the main subset in blood. In a recent study, CD11b^{+} cDC were described to promote atherosclerosis development by limiting the expansion of Tregs [44]. In consonance with the drop in circulating CD11b^{+} cDC in mice, we and others have shown previously that circulating CD11b^{+}/BDCA-1^{+} cDC numbers are reduced in patients with coronary artery disease [45, 46]. In this study, we only enrolled patients with symptomatic advanced atherosclerosis and were therefore not able to draw a comparison with asymptomatic patients. However, to our knowledge, we are the first to report a direct correlation between NKT cell numbers in blood and the load of T cells (and NKT cells) in the atherosclerotic plaques. Both CD1d expressing cells and NKT cells were previously shown to be present in advanced human atherosclerotic plaques [47]. Here, we demonstrated that the percentage of NKT cells in blood strongly improves the prediction of both T cells and NKT cells in the plaque, independent of all the other risk factors. In line with these findings, Levula et al. applied gene set enrichment analysis and real-time qPCR to human advanced atherosclerotic plaques from carotid and femoral arteries as well as aortas. 26 genes, out of a total of 29 genes, of the NKT pathway were significantly upregulated in atherosclerotic plaques versus nonatherosclerotic controls [48]. Furthermore, in humans, it was reported that circulating NKT cell numbers are reduced in patients who experienced previous cardiovascular events compared with either asymptomatic atherosclerosis patients or young healthy individuals [47]. Unfortunately, data on leukocyte cell numbers in the arterial wall during early atherogenesis are virtually nonexistent as patients mostly present themselves in the clinic when serious blockages are already present. Nevertheless, in the search for better or additional biomarkers that can alert physicians for the presence of inflammatory plaques, circulating NKT cells should be further explored, as also proposed for type 2 diabetes and cancer [49, 50]. We need to, however, remain cautious. Even in healthy individuals NKT cell numbers can fluctuate substantially and NKT cell subsets may play different functional roles in atherosclerosis [51]. Future studies, including a higher number of patients and different stages of atherosclerosis, will need to clarify the true potential of NKT cells as biomarkers (or even cellular therapy) for inflammatory, and thus unstable, atherosclerotic plaques.

Finally, we could not find a correlation between circulating hs-CRP levels and the percentages of NKT cells in blood or plaques, although this is most likely due to the small sample size and the associated large variation in hs-CRP levels. CRP is increased in individuals with an overlap to other risk factor pathways such as obesity, low social class, and smoking. Studies on the added value of CRP in risk prediction of cardiovascular disease show that hs-CRP levels can confirm the presence of plaques but do not provide insight on the degree of stenosis or the inflammation in the plaque [33].

To date, the value of circulating leukocyte profiles as biomarker of atherosclerosis is underappreciated [33]. Despite the fact that cDC and NKT cells are quantitatively minor components of the immune system, they do appear to play a major role in modulating the course of the disease. We believe that a profound analysis of circulating leukocytes, in particular CD11b^{+} cDC and NKT cells, may thus provide a helpful tool to assess the inflammatory and immune status of an atherosclerosis patient.

5. Conclusion

We provide an extensive quantitative description of systemic and peripheral immune cell dynamics over the entire life span of atherosclerotic lesion development in ApoE$^{-/-}$ mice. Based on the crucial shift in leukocyte trafficking at 12 wk of WD, we propose this to be a preferred time point for therapeutic intervention, aimed at targeting the dysregulated immune response in atherosclerosis. Furthermore, our results show that circulating NKT cells may carry biomarker potential reflecting atherosclerotic lesion progression and/or inflammation, both in mice and humans. Because of its predictive value, the DC-NKT cell axis in atherosclerosis could provide potential as a tool for better patient risk stratification and/or a target for plaque stabilization, especially when determining the optimal timing for therapy.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Immunity and Tolerance Induced by Intestinal Mucosal Dendritic Cells

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Dendritic cells present in the digestive tract are constantly exposed to environmental antigens, commensal flora, and invading pathogens. Under steady-state conditions, these cells have high tolerogenic potential, triggering differentiation of regulatory T cells to protect the host from unwanted proinflammatory immune responses to innocuous antigens or commensals. On the other hand, these cells must discriminate between commensal flora and invading pathogens and mount powerful immune response against pathogens. A potential result of unbalanced tolerogenic versus proinflammatory responses mediated by dendritic cells is associated with chronic inflammatory conditions, such as Crohn's disease, ulcerative colitis, food allergies, and celiac disease. Herein, we review the dendritic cell population involved in mediating tolerance and immunity in mucosal surfaces, the progress in unveiling their development in vivo, and factors that can influence their functions.

1. Introduction

The digestive tract is in direct contact with foreign antigens and microorganisms. The ability of the immune system to keep tolerance to commensals while remaining capable of responding to injury or infection with pathogenic microorganisms is essential for tissue homeostasis. Any disturbances in this balance either by genetic, environmental, or infectious causes can lead to chronic inflammatory and/or autoimmune diseases. The mucosal immune system should sense pathogens versus innocuous dietary antigens or commensal microorganisms. While a strong and protective response is required to eliminate pathogens, tolerance is essential for harmless antigens or nutrients, thus avoiding inflammatory responses.

During oral tolerance systemic immune effector function including delayed type hypersensitivity response and IgE antibody production are affected [1, 2]. Furthermore, intestine-resident effector cells also undergo tolerance. Impairment of oral tolerance seems to be associated with coeliac disease, characterized by an aberrant Th1-mediated DTH triggered by dietary gluten [1, 3]. Similarly, IgE-mediated food allergies can be derived from the break of tolerance to food antigens [1, 4].

Along the same lines, break of tolerance at the large intestine is thought to trigger hyperreactivity to commensal bacteria resulting in inflammatory bowel diseases, including Crohn's disease [5]. Interestingly, tolerance to commensal flora does not exert a systemic effect [6, 7]. Moreover, IgA production is maintained, thus supporting commensalism, because of the noninflammatory properties of IgA [8, 9].

The induction of oral tolerance has been the object of several studies. It is well accepted that clonal deletion and/or T cell anergy are components of the mechanism of action of oral tolerance, however induction of regulatory T cells (Treg's) has become widely known as its central component [10]. The induction of FoxP3+ Treg cells requires CD103+ dendritic cells (DCs). Herein, we will review the development/differentiation of mucosal resident DC subsets and their relative contribution to tolerance and immunity.

2. Subsets and Function

Intestinal DCs are located throughout the villus lamina propria and in intestinal lymphoid tissue (Peyer's Patches, solitary isolated lymphoid tissue, and mesenteric LN), where they play a central role in sampling and processing luminal as well as peripheral self-antigen for presentation to

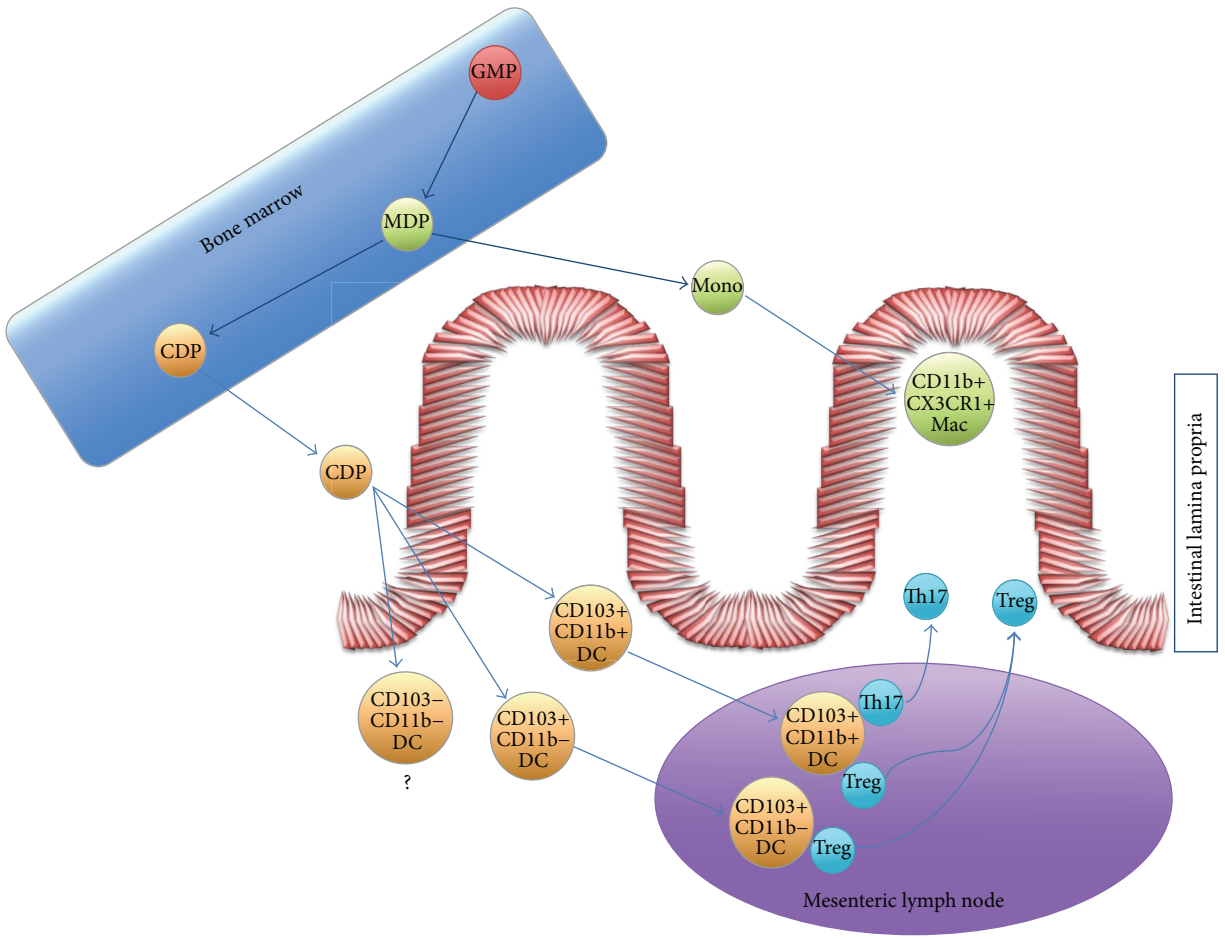

Figure 1: Intestinal mucosal dendritic cell and macrophage development and function. Bone marrow resident Granulocyte Macrophage Progenitors (GMP) give rise to Macrophage DC Precursors (MDP). In turn, CDP give rise to peripheral blood monocytes (Mono) and Common DC Progenitors (CDP). Monocytes will migrate to the lamina propria differentiating into CD11b+CX3CR1+ macrophages that directly sample antigens from the intestinal lumen. On the other hand, CDP will give rise to three subpopulations of intestinal lamina propria DCs: CD11b+CD103+, CD11b−CD103+, and CD11b−CD103−. The former two subsets are responsible for sampling antigen and priming naïve T cells into regulatory T cells (Treg) or IL17-producing T cells (Th17).

T cells [10]. A seminal study by Rescigno et al. [11] showed that CD11c+ cells send transepithelial dendrites from the lamina propria that penetrate through tight junctions and capture *Salmonella* from the lumen. Lamina propria contains two major populations of CD11c+ mononuclear phagocytes: $CD11c^{hi}$CD103+CD11b+CX3CR1− cells (DCs) and $CD11c^{int}$CD103−CD11b+CX3CR1+ cells (macrophages) [6, 9, 12–15]. CX3CR1+ macrophages, rather than the CD103+ DCs, are sampling the intestinal luminal content by extending transepithelial dendrites [11, 13, 16–18]. Exposure to TLR-ligands [13] and microbes [18] induces transepithelial dendrites formation [17]. CD103+ DCs have not been observed extending transepithelial dendrites [17].

DCs (CD11c+CX3CR1− cells) can be further subdivided into three major subsets based on the expression of CD11b and CD103, with CD11b+CD103+, CD11b−CD103+, and CD11b−CD103− [19, 20] (Figure 1). Lymphoid tissue resident DCs include plasmacytoid DCs (pDCs) and CD8α+ and CD11b+ conventional DCs (cDCs). They can be found along the lymphoid organs associated with the intestine, including PPs, isolated lymphoid follicles, and MLNs. Nonlymphoid tissue DCs, found in the parenchyma of tissues, are also known as migratory DCs. Under steady-state conditions, migratory DCs promote the expansion of regulatory T cells, required for tolerance to self-antigens [21, 22] (Figure 1). On the other hand, during inflammatory response to infection, these cells promote protective T cell responses [23, 24]. The expression of the chemokine receptor CCR7 and its ligands CCL21 and CCL19 control whether migratory DCs move into draining LNs [25].

Classic process of DC maturation occurs upon exposure to microbial stimuli or proinflammatory cytokines. Typically, morphological, phenotypic, and functional changes are observed. Such modifications are essential for effective naïve T cells priming and activation. On the other hand, migratory DC maturation is associated with tolerance induction rather than activation and proliferation, despite upregulation in MHC II and CD40 [20]. Importantly, the signals that

trigger and modulate such maturation processes are poorly understood.

Induction of tolerance versus immunity by intestinal DC is, at least in part, mediated by retinoic acid receptors (RAR) signaling [26–29]. Thus, exposure to RA triggers expression of gut-homing receptors along with enhancing expansion of FoxP3+ T cell and IgA B cell differentiation. On the other hand, antagonists of RAR inhibit expansion of such cells [30–32]. Induction of gut-homing receptors on primed T cells as well as FoxP3+ T cell differentiation in vitro is best achieved in the presence of migratory (CD103+CD11b− or CD11b+) DCs among other DC subsets [4, 5, 7]. RALDH2 is one of the enzymes that metabolize retinal to RA, CD103+ DCs express high levels of the gene encoding it – *aldh1a2*. Consistently, CD103+ DCs triggered RAR-dependent signaling in responding T cells [33]. Small intestine-lamina propria and MLN resident CD103+ DCs trigger RAR signals and induce expression of CCR9 in responding T cells [34]. All DCs trigger limited RAR signaling in T cells; however high levels of CCR9 induction are a key function associated with small intestine-lamina propria and MLN CD103+ DCs. On the other hand, the CD103+CD11b+ subset seems critical for the induction of proinflammatory Th17 cells [19, 20] given its high induction of IL6 in response to microbial stimulation [35].

3. Mucosal Dendritic Cell Precursors and Homing Markers

The interaction of FMS-like tyrosine kinase 3 (Flt3) with its ligand (Flt3-L) is critical for the generation of CD103+ DCs [36], both in mice and humans [37, 38]. Pre-B cells as well as myeloid and monocytic lineages show upregulated Flt3 mRNA, while Flt3-L mRNA expression is ubiquitous [39]. Both Flt3 and Flt3-L show high conservation in mice and humans. Treatment of mice with human Flt3-L leads to activation of mouse Flt3 [40] triggering bone marrow hyperplasia along with hematopoietic stem and progenitor cell proliferation. Interestingly, FLT3-L showed a positive bias in the expansion of CD103+ DCs [13].

Macrophage and DC bone marrow precursors give rise to monocytes and common DC progenitor [41]. Common DC progenitors are comprised within lineage (lin)- negative, Flt3-L+ cell subset [42, 43] (Figure 1). PDCs and cDCs are both derived from the common DC precursor within this lin- Flt3+ compartment [44, 45]. The common DC precursor is GM-CSF receptor α+ [45]. The transcription factor IRF8 is required for development and activation of pDCs and CD8α+ DCs [46–48] and PU.1 is important for all conventional (nonplasmacytoid) DCs [49, 50]. The expression of PU.1 is induced by Flt3 signaling [51]. Intestinal CD103+CD11b− DCs are developmentally related to the CD8α+ lymphoid DC subset, since both subsets are dependent on the presence of the transcription factors IRF8, Id2, and BATF3 [52].

Most CD103+ small intestine-lamina propria DCs have been shown to develop directly from a circulating FLT3+ common DC precursor and not from CD103− small intestine-lamina propria DCs [53] (Figure 1). Interestingly, a great proportion of MLN resident CD103+ DCs are thought to be derived from a migratory population arriving from small intestine-lamina propria that plays a critical role in presenting orally derived soluble antigen to T cells (Figure 1). Presumably, these cells seize antigens locally in the small intestine and subsequently migrate into the MLN. On the other hand, CD103− MLN DCs appear to be derived from a blood population that populate and expand the MLN and is involved in the T cell priming to systemic antigens [53]. Importantly, CD103+ DCs are present in normal and inflamed human MLN and display similar phenotypic and functional properties to their murine counterparts [6].

CCR7, a chemokine receptor which is required for DC migration from peripheral tissues into the draining LN, is required for accumulation of CD103+ DC in the MLN, as CCR7-deficient hosts have reduced numbers of MLN CD103+ DCs [54–56].

4. Intestinal Mucosal Dendritic Cell Responses to Infection

Intestinal flora is composed of trillions of resident bacteria that can provide beneficial effects to the host [57]. For example, bacterial metabolites including vitamins and short chain fatty acids are relevant for the host development, including lymphoid populations in the intestine. Moreover, resident bacteria mediate resistance against pathogen infection [58]. Several host immune-regulatory mechanisms have evolved to prevent inappropriate activation of inflammatory responses in response to the commensal flora, including the hyporesponsiveness of intestinal epithelium and resident macrophages to bacterial Toll-like receptor ligands [59, 60]. However, intestinal microbiota can potentially trigger (or enhance) an inflammatory response. Chemically induced and spontaneous colitis are reduced or abolished in antibiotic-treated mice and germ-free mice [61–65] and *Bacteroides* species and members of the Enterobacteriaceae family including *Klebsiella pneumoniae* and *Proteus mirabilis* can promote colitis [66, 67].

Activation of inflammatory responses by flora is mediated by host pattern-recognition receptors [68]. Inflammasome, a multiprotein complex that leads to caspase-1 initiated proteolytic processing of pro-interleukin-1β and pro-IL18 into their active forms [69]. In the intestine, *Salmonella* triggers resident phagocytes to produce IL-1β in an NLRC4-dependent manner leading to neutrophil recruitment [70].

The role of the NLRP3 inflammasome in intestinal inflammation is controversial. On one hand, mice lacking NLRP3 or caspase-1 were shown to be less susceptible to chemically induced colitis [71, 72]. On the other hand, it was shown that these same animals had increased susceptibility and worsened pathology [73, 74]. Along the same lines, the role of IL-1β in colitis is also controversial. While IL-1β blockage improves intestinal inflammation in different animal colitis models [75, 76], another study showed that genetic deficiency of IL-1β leads to increased susceptibility to experimental colitis [8]. Although it is not clear what the reasons for such differences in results are, one potential explanation is the composition of gut flora [71]. For instance,

Escherichia coli trigger NLRP3 inflammasome in bone marrow derived macrophages to produce IL-1β [77, 78].

5. Mucosal Tolerance and Dendritic Cells

Several commensal *Bacteroides* and Bifidobacteria strains can directly induce monocyte-derived DCs to acquire a tolerogenic phenotype [79]. Polysaccharide A from *Bacteroides fragilis*, a Gram-negative anaerobic commensal bacterium, can also associate with CD11c+ cells in MLNs and drive a mixture of Th1 systemic responses and IL10-producing Treg cells in the colonic LP [80]. Segmented filamentous bacteria induce differentiation of both mucosal Th17 and FoxP3+ Treg cell. These effects are associated with the modulation of APC function in the lamina propria [19, 81, 82]. Antigen presentation by CD103+ DCs can be tolerogenic [5, 7] or immunogenic [83], dictated by the microenvironment [83–85]. Those conditions should be crucial for the development of novel therapeutic approaches using CD103+ DCs in triggering mucosal immunity or tolerance.

Under steady-state conditions, lamina propria-resident CD103+ DCs are tolerogenic. However, inflammation induces MLN CD103+ DCs into a proinflammatory phenotype. For instance, MLN CD103+ DCs purified from colitic mice triggered Th1 responses along with high levels of IL6 production [83, 86]. During intestinal inflammation, MLN CD103+ DC acquires these proinflammatory properties with no phenotypical and ontogenetic changes.

Naturally occurring CD4+CD25+Foxp3+ Treg cells are thymus-derived and are important to modulate a wide range of immune-mediated pathologies, including autoimmunity, colitis, and chronic infection. However, inducible Treg cells arising from the naïve pool are particularly beneficial in the intestine. The balance of triggering protective immunity to invading pathogens while retaining tolerance to dietary antigen and the commensal flora is critical. These cells can be generated in the periphery from the naive T cell pool after, for example, the oral administration of antigen or the targeting of peptide ligands to DCs in vivo [87].

Some specific nutrients are known to have notable effects on the modulation of mucosal immunity. Moreover, mucosal DCs are constantly exposed to dietary antigens. Vitamin A, whose only source in mammals is through the diet, mediates several functions of CD103+ DCs. Its depletion from the diet inhibits Treg differentiation induced by MLN CD103+ DCs as well as inducing gut-homing receptors on T cells [88, 89].

Tryptophan is another example of dietary element that is required for the IDO-dependent tolerogenic effects of mucosal DCs [90] and for generation of ligands of the aryl hydrocarbon receptor (AhR), such as L-kynurenine that regulates the balance between Th17 and Treg cell differentiation [91–93] and has powerful direct anti-inflammatory activity on DCs [94].

Diet-derived lipid mediators can activate anti-inflammatory peroxisome proliferator-activated receptor (PPAR)γ [95]. Short chain fatty acids (including acetate, butyrate, and propionate) are among the most abundant metabolites derived from microbiota-mediated digestion of dietary fiber [96]. Exposure of monocyte-derived DCs to butyrate and propionate prevented proinflammatory cytokine release induced after LPS incubation [97]. In fact, animals deficient for butyrate receptor, GPR109a, are susceptible to the development of colitis and colon cancer [98].

Curcumin is a spice historically used as a medicine in India and Southeast Asia. Exposure of curcumin triggers a tolerogenic activity in DCs, including upregulation of *aldh1a2* and IL10 while promoting FoxP3+ Treg cells [99].

The mucosal neural anatomy is disrupted in inflammatory bowel diseases [100]; intestine is permeated by a complex nervous system. On the other hand, hematopoietic cells are responsive to neurotransmitters and mediators from the enteric nervous system exert immune-regulatory effects [100]. Vasoactive intestinal peptide (VIP) is produced by intestinal enteroendocrine and immune cells and has vasodilator and regulator of epithelial permeability activities [101]. VIP suppresses lipopolysaccharide-induced DC maturation [102] while promoting differentiation of IL10- and TGF-β-secreting Treg cells [103–105]. In agreement with these observations, DCs exposed to VIP to prevent chemically induced colitis [105]. Taken together, these studies revised here point to the complexity of interactions between mucosal DCs, nonimmune cells, the microbiota, and ingested nutrients. All these factors contribute to promoting and maintaining tolerance mediated by intestinal DCs under steady-state conditions. While no single mediator plays a dominant role, redundancy among several pathways and components is evolutionary advantageous to ensure that homeostasis is maintained.

Conflict of Interests

The author declares that there is no conflict of interests regarding the publication of this paper.

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Review Article

Metabolism Is Central to Tolerogenic Dendritic Cell Function

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Immunological tolerance is a fundamental tenant of immune homeostasis and overall health. Self-tolerance is a critical component of the immune system that allows for the recognition of self, resulting in hyporeactivity instead of immunogenicity. Dendritic cells are central to the establishment of dominant immune tolerance through the secretion of immunosuppressive cytokines and regulatory polarization of T cells. Cellular metabolism holds the key to determining DC immunogenic or tolerogenic cell fate. Recent studies have demonstrated that dendritic cell maturation leads to a shift toward a glycolytic metabolic state and preferred use of glucose as a carbon source. In contrast, tolerogenic dendritic cells favor oxidative phosphorylation and fatty acid oxidation. This dichotomous metabolic reprogramming of dendritic cells drives differential cellular function and plays a role in pathologies, such as autoimmune disease. Pharmacological alterations in metabolism have promising therapeutic potential.

1. Introduction

Immune homeostasis is achieved when there is a balance between immunogenicity to nonself or pathogens and tolerance to self. Amongst many lymphocytes involved, dendritic cells (DCs) play an important role in both the innate and adaptive immune response. DCs originate from hematopoietic progenitor cells (HPCs) and contribute to immunity by recognition of pathogenic signals. Upon activation by Toll-like receptor (TLR) binding, DCs migrate from the periphery into lymph nodes during a maturation process. DCs can act as antigen-presenting cells (APCs) by efficiently presenting peptide-major histocompatibility complex (MHC), molecules to antigen-specific T cells which then eliminate pathogens [1]. Protection against pathogenic invasion is important, but it is also critical for immune system to be at the very least nonresponsive to self, a concept known as tolerance. Central tolerance is a deletional process where high affinity reactive T cells are eliminated [2]. Peripheral tolerance is the combination of inducing anergy in self-reactive T cells that escape the thymus and the suppressive action of regulatory T cells [3]. Specific types of DCs, the tolerogenic dendritic cells (tol-DCs), are critical in maintaining tolerance. Defects in self-tolerance play a role in autoimmune diseases and autoinflammatory diseases.

In recent years, cellular metabolism has been identified as a key component in immune cell function. Decades of research have led to the characterization of cellular metabolism as a vast network of biochemical processes important for energy production and cell fate determination [4]. Revolutionary advances in mass spectrometry, high performance liquid chromatography (HPLC), and extracellular flux analysis have opened up the field of immune bioenergetic analysis [5]. Studies have revealed fundamental metabolic differences within human peripheral blood leukocytes and their component subsets [6]. Furthermore, functional activity of these immune cells can be altered with changes in metabolic reprogramming. This review will focus on tol-DCs, metabolic reprogramming by pharmacological agents, and their potential use in the clinic.

2. Immunologic Tolerance

The function of immune system is to defend an organism from pathogenic invasion. Immunologic tolerance refers to an ability to suppress self-reactivity and control the response to prolonged and persistent infection. Tolerance is an active process involving multiple cellular subsets to constantly control self-reactivity. During an ongoing immune response, mechanisms are required to tightly regulate self-reactivity in

a spatial and time dependent manner to reduce collateral tissue damage. Breakdown in tolerance results in serious pathology like autoimmune diseases, allergies, and graft rejections. In mammals, tolerance checkpoints occur mechanistically at two levels: centrally and peripherally.

Central tolerance acts as a first line of defence against autoimmunity. The chief mechanism of central tolerance is the deletion of autoreactive T cells in the thymus. This process is aided by thymic DCs and thymic medullary epithelial cells which present self-peptide-MHC complexes to T cells. T cells first undergo positive selection followed by negative selection during T cell development. Under positive selection, T cells with low T cell receptor (TCR) expression or an inability to react with MHC molecules are removed. Any self-reactive T cells are deleted from the T cell repertoire under negative selection when they react strongly with self-peptide-MHC complexes presented on thymic DCs [7, 8]. Despite an effective mechanism of limiting self-reactivity, T cells with moderate or low affinity may survive central tolerance scrutiny and enter the periphery.

Secondary peripheral mechanisms are required to suppress the activation of any remaining autoreactive cells. DCs are crucial in maintaining tolerance in the periphery. Constitutive ablation of all DCs in mice resulted in the development of spontaneous fatal autoimmunity under steady state conditions [9]. DCs are vital to the induction of T cell anergy in which T cells become functionally inactivated following an antigen encounter. In 2002, Bonifaz et al. showed that antigen delivery by anti-DEC-205 antibodies to DC induced $CD8^+$ T cell tolerance [10]. T cell activation requires T cells to first recognize the appropriate MHC-peptide complex followed by costimulatory signals from DCs to proliferate and differentiate. In the absence of costimulatory signals, self-reactive T cells do not proliferate when they encounter self-peptide-MHC complexes and remain unresponsive towards primary and secondary stimulation, inducing T cell anergy. Self-reactive T cell activation may also be suppressed by regulatory T cells (Tregs). Natural Tregs are thymically derived and self-antigen-specific. Upon recognition of their specific antigen, they can broadly suppress nearby effector T cells in a nonspecific manner. Researchers also demonstrated that anti-DEC-205 antibodies targeted to immature DC or peptide-loaded mature DCs are potent inducers of Tregs yielding T cell tolerance, indicating that these cells may be the actual in vivo effectors [3, 11]. Peripheral tolerance is a dynamic multicellular process and plays a key role in keeping autoreactivity under surveillance.

3. Tolerogenic DCs

DCs form a critical link between the innate and adaptive immune systems and their state of maturation determines an immunological or tolerogenic outcome. In the presence of specific signals, immature DCs (iDCs) are differentiated to Th1/2/17/9 or TfH inducing state, while, in response to tolerogenic signals, iDCs are matured toward an alternate, Treg-inducing state [12]. Once generated, these cells integrate peripheral tolerogenic signals and inhibit T cell autoreactivity, thereby promoting peripheral tolerance and maintenance of immune homeostasis.

Tol-DCs can be characterized by their surface markers and cytokine profile. Generally, tol-DCs express lower levels of surface MHC class II and costimulatory molecules relative to immunogenic DC, a state often referred to as "semimature" [21–24]. These cells can be further characterized by the specific expression of surface immunoreceptor tyrosine-based inhibitory motifs (ITIMs) containing receptors such as Fc gamma receptor IIb (CD32b), Ig-like inhibitory receptors (ILT3/ILT4), and paired immunoglobulin-like receptor (PILR) [25]. They also express immunomodulatory molecules like PD-L1 (ligand of programmed death 1) [26], heme oxygenase 1, human leukocyte antigen G (HLA-G) [27, 28], CD95L, galectin-1 [29], and DC-SIGN (Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Nonintegrin) [12, 30, 31]. Interestingly, a number of genes involved in metabolism seem to play a critical role in tol-DC biology such as indoleamine 2,3-dioxygenase (IDO), IL-27 [32], and nitric oxide (NO) [12, 31]. The production of immunosuppressive factors including IL-10 and transforming growth factor-beta (TGF-b) can contribute to their function by promoting the expansion of Tregs and directly inhibiting T cell response. It is important to note that this phenotype is largely resistant to subsequent activation signals indicating it is not a transient differentiation state.

IL-10 is believed to play a pivotal role in regulating the expression of both immune-inhibitory receptors and cytokines during tolerogenic DC maturation process. A number of studies indicate that IL-10 regulates the expression of ILTs in DCs and monocytes [33, 34]. Treatment of myeloid DCs with exogenous IL-10 displays high levels of ILT3 and ILT4 surface expression [25]. Transduction of ILT3 and ILT4 in DCs results in generation of $Foxp3^+$ Tregs and inhibition of alloproliferation [35]. IL-10 signaling also induces the expression of ILTs orthologue paired PILR-alpha [33, 36]. PILRs block the access of CD8 molecules to MHC-I and it has been demonstrated that PIR-B-deficient DCs result in Cytotoxic T Lymphocytes (CTL) activation and accelerated graft and tumor rejection [37]. IL-10 is crucial in regulating PD-L1 expression on tol-DCs. Ligation of PD-1/PD-L1 leads to the recruitment of SHP phosphatase which plays an important role in inhibiting T cell response [38, 39]. Exogenous addition of IL-10 in DC culture from normal donor upregulated PD-L1 surface expression, while IL-10 blockade with neutralizing antibody reversed upregulated PD-L1 expression in DCs from tumor patients [40]. Blocking PD-L1/PD-1 signaling pathway augmented HIV-specific CD4 and CD8 T cell function in chronic HIV infection [41]. IL-10 has also been shown to upregulate the expression of CD95L [42]. CD95/CD95L mediated apoptosis in T cells is important for the maintenance of peripheral tolerance and termination of an ongoing immune response [43]. IL-10 has also been shown to regulate the expression of IDO, an enzyme of tryptophan catabolism, in tol-DCs. The presence of IL-10 during DC maturation prevented IFN-γ-induced downregulation of IDO, resulting in sustained expression of functional IDO even in mature, IFN-γ-activated DCs [44]. Transduction of IDO gene into DCs suppressed allogeneic

T cell proliferation in vitro [45]. IL-10 also regulates the expression of other tolerogenic DC markers, such as HLA-G, Inhibin Beta A (INHBA), Aquaporin 9 (AQP9), and Signaling Lymphocytic Activation Molecule (SLAM) [33], which are believed to be expressed on many tolerogenic DCs. Their functions as related to tolerance induction are unclear at this point.

There are differing opinions on the role of inhibitory cytokine TGF-b in the induction of DC tolerogenic state. An elegant study by Flavell Laboratory using a dominant negative form of TGF-beta receptor II under the control of CD11c promoter ($CD11c^{-}$ dnTGFbetaRII) showed that DC homeostasis is independent of TGF-beta signaling [46]. However, subsequent studies show that TGF-b signaling does play a role in DC tolerogenesis. TGF-b1 gene modified bone marrow-derived immature DCs display decreased IL-12 secretion and alloantigen-specific T cell unresponsiveness in vitro and in vivo [47]. Pallotta et al. demonstrated that exogenous addition of TGF-b induces the expression of IDO in DCs, supporting spontaneous tolerogenesis [48, 49]. It has also been shown that TGF-b treatment increased the expression of PD-L1 in DCs, resulting in T cell apoptosis and Treg expansion [50]. Signal transducers mediated the increment in PD-L1 and activators of transcription 3 (STAT3) and blockade of STAT3 significantly decrease PD-L1 expression. TGF-b pulsed bone marrow-derived DCs result in a decrease in IL-12p70 production and inhibited allogenic T cells, leading to long-term survival of the graft [51].

Tol-DCs can also be generated ex vivo using several pharmacological agents such as dexamethasone (DEX), rapamycin, vitamin D_3, or Vit D_3/Dex combination [52–54]. These tolerogenic DCs are semimature in phenotype and possess the ability to suppress alloreactive response. DEX is an immunosuppressive glucocorticoid. DEX-polarized tolerogenic DCs are able to inhibit T cell proliferation and cytokine production as well as promote functional Treg expansion [55]. DEX treated DCs decrease expression of costimulatory molecules and increase secretion of IL-10 while decreasing IL-12 [56, 57]. Rapamycin is an inhibitor of the Ser/Thr protein kinase, mammalian target of rapamycin (mTOR), and widely used as an anticancer drug and an immunosuppressant. Rapamycin-treated DCs display a reduction in CD40, CD80, and CD86 expression and T cell hyporesponsiveness [58]. Rapamycin pulsed DCs promote the expansion of functional Treg [59]. Vitamin D_3 is a pleiotropic hormone, which regulates calcium homeostasis, promoting innate immunity while inhibiting adaptive immunity. Exogenous treatment with Vit D_3 results in high expression levels of PD-L1 in DCs, suppressing T cell proliferation [60]. Vit D_3 treated DCs induce ILT3 expression and result in ILT3 dependent $CD4^{+}Foxp3^{+}$ regulatory T cells expansion [61] and alloreactive T cell inhibition [62]. Ligation of Vit D_3 to vitamin D receptor (VDR) significantly increases NF-κB promoter binding affinity and inhibits NF-κB expression and activation, promoting tolerogenic DCs [63]. NF-κB inhibitor, BAY 11-7082, and treated DCs display low expression of MHC class II and CD40 molecules and in vivo injection of BAY 11-7082-treated DC induces IL-10 producing $CD4^{+}$ regulatory T cells [64]. Protein kinase C inhibitors such as bisindolylmaleimide I, GO 6983, and RO 32-0432 inhibit NF-κB activation in DCs, giving rise to tolerogenic DCs [65]. β-catenin activation drives tolerogenic DCs cell fate and it has been shown that disruption of E-cadherin signaling leads to the activation of β-catenin, giving rise to tolerogenic DCs [66]. DC-specific deletion of β-catenin increased proinflammatory cytokine production [67]. Interestingly, all these pathways target different steps in DC differentiation and activation and yet they converge to generate cells with functionally similar characteristics. It is important to determine the underlying biological process driving this common differentiation.

3.1. Metabolism. The role of metabolism in underpinning immune cell function has become an area of active research over recent years. In living organisms, cellular metabolism is critical for the production of energy in the form of ATP, as well as cellular maintenance and proliferation [4]. Glycolysis is a metabolic process that breaks down glucose to rapidly produce ATP. Intermediates of glycolysis can enter the Pentose Phosphate Pathway (PPP), which generates reductive capability and anabolic building blocks. Oxidative products of this pathway can feed back into glycolysis and affect cellular function [68, 69]. In most cells, oxidative phosphorylation (OXPHOS) produces the bulk amount of ATP molecules [70]. Otto Warburg has described the shift away from OXPHOS and towards aerobic glycolysis despite the presence of oxygen as the Warburg effect [71]. The shift in cell metabolism can be explained by the requirement for quick biosynthesis in contrast to efficient energy production. A variety of immune cells have been shown to display different metabolic priorities. With recent advances in metabolic analysis including the extracellular flux analyzers, cellular oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) can be determined by generating bioenergetic profiles of mitochondrial respiration and glycolysis, respectively [6]. The analysis of peripheral blood from healthy donors shows distinct metabolic profiles in monocytes, lymphocytes, and platelets determined by parameters including basal respiration, ATP-linked respiration, proton leak, reserve capacity, and non-mitochondrial respiration. In these studies, differences in the metabolic priorities of immune cells become apparent. Neutrophils seemingly dedicate most energy production to glycolytic metabolism, lymphocytes mainly use oxidative phosphorylation, and monocytes show a degree of both glycolytic and oxidative pathways [72]. Studies looking at differential metabolic programs in lymphocytes demonstrate the importance of metabolism for immune function. In T cells, metabolism defines the immune response, where memory T cells have been revealed to rely on increased FAO and glucose [73]. The involvement of mitochondria during T helper cell (T_H) activation has also become evident. During Ca^{2+}-dependent activation of T_H, an immunological synapse is formed between T_H and APCs. This is associated with a relocalization within the T_H cell structure, bringing mitochondria closer and increasing the influx of Ca^{2+}, indicating mitochondrial involvement in T_H activation [74]. Additionally, Chang et al. have shown the role of T cell metabolism in tumor growth, where a decrease in glycolysis and nutrient

availability for T cells fuels tumor progression [75]. The cellular metabolic state dictates downstream function for many immune cells.

3.2. Metabolism in DC. Changes in cellular metabolism are important in many aspects of DC development and function. During DC maturation, the metabolic profile of precursors and differentiating DCs is different, shifting from glycolysis to OXPHOS [76]. Studies show that this involves reactive oxygen species, as well as an increase in expression of mitochondrial respiratory enzymes, ATP content, and antioxidant capacity [77]. Similarly, resting and activated DCs show differences in metabolic priorities. Maturation of DCs by a variety of TLR stimuli initiates specific signaling cascades, which feed into metabolic regulation. The AKT pathway activates Hexokinase 2 to boost glycolysis and the TCA cycle, resulting in increased PPP activity and the formation of citrate for fatty acid synthesis. The NF-κB signaling pathway is also triggered and can cause stress in the endoplasmic reticulum, which stimulates the constitutively active unfolded protein response (UPR) and promotes fatty acid synthesis [76]. Recently, Cubillos-Ruiz et al. revealed that, in tumor-associated DCs, downstream activation of X-box-binding protein 1 (XBP1) by UPR and the related production of lipid bodies impede DC antigen-presenting function and effectiveness in initiating T cell responses [78]. With this shift towards glycolysis in activated DCs, glycolytic intermediates can promote PPP which support biosynthesis of nucleotides for increased protein output and the generation of NADPH. Glycolytic intermediates can also enter the TCA cycle and support lipid membrane production and macromolecule biosynthesis [79]. Furthermore, during LPS activation, OXPHOS and FAO are decreased due to the upregulation of inducible nitric oxide synthase (iNOS) and inhibition of 5′ adenosine monophosphate-activated protein kinase (AMPK), respectively [71]. Everts et al. show that iNOS can block OXPHOS and NO can shut down mitochondrial function [80]. Therefore, the different metabolic profiles in DCs are a key factor in differential DC functions. This raises the possibility of altering the immune response by influencing DC metabolism for therapeutic purposes in diseases, such as autoimmunity and cancer.

Tol-DCs also show a distinct metabolic profile (Figure 1). In recent studies by Malinarich et al., the expression of metabolic pathway related genes shows fundamental differences within DCs, with a focus on tol-DCs. The studies demonstrate an increased expression of genes related to OXPHOS, especially of electron transport chain complexes II and IV. Additionally, IL-10, a characteristic cytokine of tol-DCs, blocks the shift in metabolism at TLR stimulation to glycolysis and favors OXPHOS [81]. Moreover, high mitochondrial activity and increased ROS production are seen in tol-DCs. This mitochondrial activity is associated with an increase in FAO and the utilization of triglycerides as a carbon source. Inhibition of the pathway leads to a loss of tolerogenic capacity. Inhibition with etomoxir increased responder T cell activation and caused a dramatic decrease in OCR [82]. Similar to resting T cells, the catabolic profile and high-energy demand in tol-DCs may be related to their active

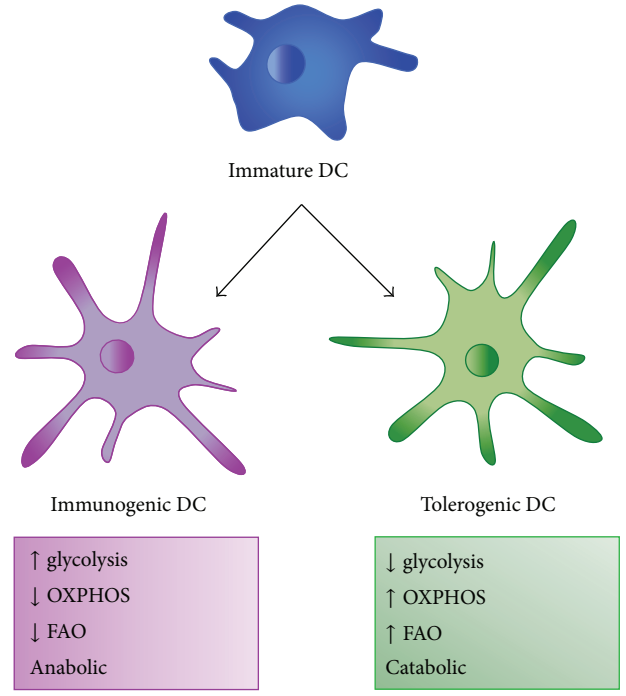

Figure 1: Differentiation of dendritic cells. Immature dendritic cells (DCs) can mature into either activated, immunogenic DCs that induce $T_H1/T_H2/T_H17$ for T cell function and elimination of pathogens or tolerogenic DCs that induce the expansion of T regulatory cells and T cell unresponsiveness for immune tolerance. Immature DCs mature into immunogenic DC with a shift in metabolism towards glycolysis, which is associated with increasing biomass for effector function. Tolerogenic DC, on the other hand, shifts cellular metabolism towards OXPHOS (oxidative phosphorylation) and favors FAO (fatty acid oxidation). This catabolic and highly energetic profile may be related to energy required for active suppression and protein degradation.

suppressive function. Tryptophan breakdown plays a role in peripheral tolerance [83] and the metabolic enzyme arginase-1 has been shown to control DC differentiation in mice [84]. Lying upstream of immune function, the manipulation of DC metabolism may be used for therapeutic purposes.

3.3. Pharmacological Manipulation of DC Metabolism for DC Vaccines. Extensive research on long-term graft acceptance led to the identification of tolerogenic myeloid populations including DCs [85]. Many pharmacological agents such as Vit D_3, DEX or Vit D_3/DEX combination, or rapamycin can be used to generate tol-DCs (Figure 2). Vit D_3 induces changes in proteins involved in iron metabolism, TCA cycle, OXPHOS, and the PPP. A recent paper demonstrated that Vit D_3 acts synergistically with metformin to increase the activation of AMPK pathway [86]. Being a metabolic sensor, AMPK is able to detect fluctuations in the AMP : ATP ratio. Activation of AMPK inactivates acetyl-CoA carboxylase, releasing the inhibition on carnitine palmitoyltransferase-1 (CPT-1). This promotes catabolic metabolism, increasing FAO and OXPHOS [70, 87–89]. Pharmacological activation

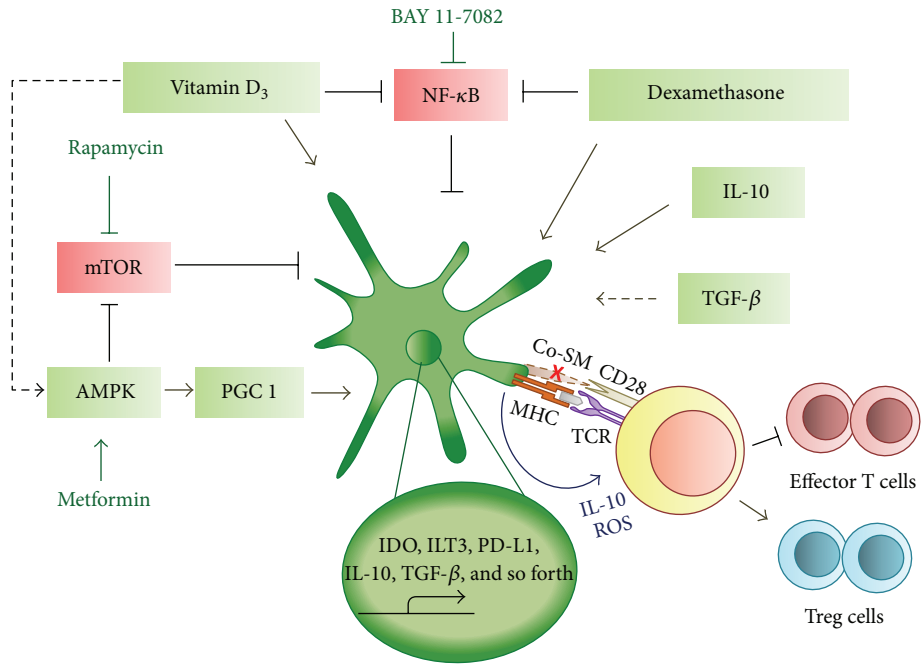

Figure 2: Induction of tolerogenic dendritic cells by cytokines and pharmacological agents. Tolerogenic dendritic cells can be induced by TGF-β (transforming growth factor-beta), IL-10 (interleukin-10), and pharmacological agents dexamethasone, BAY 11-7082, vitamin D_3, rapamycin, and metformin which regulates dendritic cell metabolism. The induction of tolerogenic dendritic cells leads to an increase in IDO (indoleamine 2,3-dioxygenase), ILT3 (Ig-like inhibitory receptor 3), PD-L1 (ligand of programmed death 1), IL-10, and TGF-β transcription and expression. The downregulation of Co-SM (costimulatory molecules) on tolerogenic dendritic cells and the subsequent release of IL-10 and ROS (reactive oxygen species) result in the inhibition of T cells alloproliferation and the expansion of Treg (T regulatory cells), inducing tolerance.

of AMPK signaling also promotes mitochondrial biogenesis, increasing mitochondrial OXPHOS by peroxisome proliferator-activated receptor gamma coactivator (PGC) 1-α [70, 87]. Resveratrol has been shown to enhance PGC-1α activity and is being used for the induction of tolerogenic DCs [70, 90]. Vit D_3 generated tol-DCs are able to suppress TLR-driven activation and glucose consumption [91]. Tolerogenic DCs generated with VDR agonists have been used as DC vaccines in clinical treatment of psoriasis, a Th1 and Th17 cell-mediated autoimmune disease of the skin [13]. Yates et al. demonstrated that infusion of Vit D_3 metabolically modified DCs into TCR-Tg $RAG^{-/-}$ mice that accepted a skin transplant resulted in de novo generation of $CD25^+Foxp3^+$ Tregs and dominant transplant tolerance. Mice that were previously rendered tolerant not only retained their existing grafts but also accepted fresh grafts indefinitely [14].

Glucocorticoid receptor activation leads to a cascade of anti-inflammatory responses in a variety of immune cells. In DCs, DEX administration regulates the MHC-II antigen presentation pathway and the proteins involved in the response to stress [92, 93]. DEX is able to inhibit the expression of iNOS and NO production, often increased in inflammatory diseases [94]. DCs generated with DEX have been used to minimize allograft rejection. Emmer et al. demonstrated that preinjection of LPS-matured DEX-DCs resulted in prolonged survival of a completely MHC-mismatched heart allograft in the transplant recipient [15]. A combination of Vit D_3 and DEX has also been used to generate tol-DCs. These Vit D_3/DEX-DCs display a Vit D_3 induced metabolic profile and stress response similar to DEX-DCs [52]. These DCs are less sensitive to death by nutrient starvation and have robust antioxidative machinery. It has been shown that Vit D_3/DEX generated DCs are more potent in inhibiting allogenic T cell proliferation than Vit D_3 or DEX-DCs [95]. Pedersen et al. demonstrated that infusion of Vit D_3/DEX treated DCs into $CD4^+CD25^-$ T cell transfer SCID mice resulted in suppression of colitis [16].

mTOR is a key regulator of glycolysis and anabolic metabolism [96] and its inhibition with rapamycin has been shown to induce catabolic metabolism and the generation of tolerogenic DCs. A number of studies have highlighted the potential of metabolically modified rapamycin-treated DCs in the induction of transplant tolerance. Injection of alloantigen-pulsed RAPA-DC vaccine into mice before transplantation significantly prolonged a full MHC-mismatched organ allograft [17, 97]. More strikingly, when $CD4^+$ T cells from RAPA-DC vaccinated heart-graft recipient mice were adoptively transferred to naïve mice, resistance to transplant rejection could also be transferred [18].

4. Conclusion and Future Perspectives

Induction of full tolerance remains an elusive goal in clinical organ transplantation and in the management of autoimmune diseases. Based on the promising efficacy in animal models, several groups have begun initiating clinical trials of

Table 1: Dendritic cell therapeutics.

Condition	Organism	Adjuvant	Metabolic modulator	Immune response	References
Psoriasis	Human	$1,25(OH)_2D_3$	Yes	Increased $CD4^+CD25^+$ suppressor T cells	[13]
Skin transplant	Mouse	$1,25(OH)_2D_3$	Yes	Increased $CD25^+Foxp3^+$ Tregs	[14]
Allograft rejection	Mouse	Dexamethasone	Yes	Increased ratio IL-10/IL-12	[15]
Colitis	Mouse	$1,25(OH)_2D_3$ & dexamethasone	Yes	Lower $CD4^+$ $CD25^-$ T cell response	[16]
Heart graft	Mouse	Rapamycin	Yes	Inhibiting T cell IL-2 & IFN-γ production	[17]
Heart graft	Mouse	Rapamycin	Yes	Stimulating $CD4^+CD25^+Foxp3^+$ Treg	[18]
Type-1 diabetes	Human	Antisense oligonucleotides targeting CD40, CD80, and CD86	No	Stimulating $B220^+$ $CD11c^-$ B-cells	[19]
Rheumatoid Arthritis	Human	BAY 11-7082	No	Higher ratio Treg/effector T cells	[20]

tol-DC vaccination to treat autoimmune diseases. A phase I clinical trial has started using tol-DC vaccines to reduce islet-specific inflammation in type-1 diabetes [19, 98]. Several groups are starting DC vaccination clinical trials for treating Rheumatoid Arthritis (RA) using BAY 11-7082-treated DC loaded with citrullinated peptides derived from RA patients [20]. Another DC vaccine trial is underway using autologous DC metabolically modified with dexamethasone and vitamin D_3 and loaded with synovial fluid [99]. Cell based tolerogenic DC vaccinations, including those mentioned above, may prove uniquely effective in restoring immune homeostasis in patients with autoimmune disease. To date, there is no published report on clinical trials using tolerogenic DC vaccines in transplantation, but given the great need for novel tolerogenic strategies in this field, studies may be forthcoming.

Most trials to date have used ex vivo modulated DCs for vaccination. Antigen-pulsed DCs treated with pharmacological agents might not reflect the complexity of natural tol-DCs in vivo. It should be noted that, in the clinic, this approach has shown efficacy and has the advantage that these cells can be generated in sufficient amounts for multiple injections. Loading with specific antigens may improve clinical outcome; however, this poses a challenge in diseases with many autoantigens, such as systemic lupus erythematosus. There has been a growing body of work focused on using antibodies to deliver tolerogenic signals directly to DCs in vivo, thereby bypassing the need for ex vivo manipulation and the potential side effects of immunosuppressive drugs [11, 100, 101]. It remains to be seen if the future of tolerogenic DC vaccination lies in this generation of complex biologic targeting molecules.

There is growing evidence that distinct metabolic reprogramming lies at the heart of DC differentiation, acting as a master regulatory switch in determining immunogenic or tolerogenic DC cell fate. Given the broad availability of pharmacologic compounds, which target this axis, metabolically modulated tol-DCs are promising tools for tolerogenic vaccination in the clinic. DC vaccination to prevent or to inhibit immune activation is highly sought after in allergy, autoimmunity, and transplantation medicine. Our understanding of the mechanism to which metabolism regulates cellular processes in DCs is increasing. Evidence from a multitude of animal models strongly demonstrated the efficiency of metabolically modulated tol-DC vaccines in allergic and autoimmune diseases as well as in transplantation (Table 1). However, for DC-based tolerogenic vaccination in man, only a few clinical trials have been performed for inflammatory and autoimmune disorders (Table 1). Future studies are required to identify the important metabolic targets which can be manipulated to induce tol-DCs for vaccination and facilitate their rapid translation to the clinic. Metabolic modification of DCs yielding phenotypically stable tol-DCs with migratory capacity, combining DC vaccines with autoantigens, and timely and accurate delivery of DC vaccines to target sites are all important approaches with potential to further enhance vaccine efficacy.

Abbreviations

AMPK: 5′ adenosine monophosphate-activated protein kinase
APC: Antigen-presenting cell
DCs: Dendritic cells
DC-SIGN: Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Nonintegrin
DEX: Dexamethasone
ECAR: Extracellular acidification rate
FAO: Fatty acid oxidation
HPCs: Hematopoietic progenitor cells
HLA-G: Histocompatibility antigen, class I, G
HPLC: High performance liquid chromatography
IL-10: Interleukin-10
IL-27: Interleukin-27
ILT3: Immunoglobulin-like transcript 3
ILT4: Immunoglobulin-like transcript 4
iDCs: Immature DCs

IDO: Indoleamine 2,3-dioxygenase
iNOS: Inducible nitric oxide synthase
MHC: Major histocompatibility complex
mTOR: Mammalian target of rapamycin
NO: Nitric oxide
OXPHOS: Oxidative phosphorylation
OCR: Oxygen consumption rate
PBMCs: Peripheral Blood Mononuclear Cells
PD-L1: Ligand of programmed death 1
PPP: Pentose Phosphate Pathway
Tregs: Regulatory T cells
TCR: T cell receptor
T_H: T helper cell
TGF-b: Transforming growth factor-beta
TLR: Toll-like receptors
TNF: Tumor Necrosis Factor
Tol-DC: Tolerogenic DC
TCA: Tricarboxylic Acid
UPR: Unfolded protein response
VDR: Vitamin D (1,25-dihydroxyvitamin D_3) receptor
Vit D_3: Vitamin D (1,25-dihydroxyvitamin D_3).

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Pathogen-Associated Molecular Patterns Induced Crosstalk between Dendritic Cells, T Helper Cells, and Natural Killer Helper Cells Can Improve Dendritic Cell Vaccination

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A coordinated cellular interplay is of crucial importance in both host defense against pathogens and malignantly transformed cells. The various interactions of Dendritic Cells (DC), Natural Killer (NK) cells, and T helper (Th) cells can be influenced by a variety of pathogen-associated molecular patterns (PAMPs) and will lead to enhanced $CD8^+$ effector T cell responses. Specific Pattern Recognition Receptor (PRR) triggering during maturation enables DC to enhance Th1 as well as NK helper cell responses. This effect is correlated with the amount of IL-12p70 released by DC. Activated NK cells are able to amplify the proinflammatory cytokine profile of DC via the release of IFN-γ. The knowledge on how PAMP recognition can modulate the DC is of importance for the design and definition of appropriate therapeutic cancer vaccines. In this review we will discuss the potential role of specific PAMP-matured DC in optimizing therapeutic DC-based vaccines, as some of these DC are efficiently activating Th1, NK cells, and cytotoxic T cells. Moreover, to optimize these vaccines, also the inhibitory effects of tumor-derived suppressive factors, for example, on the NK-DC crosstalk, should be taken into account. Finally, the suppressive role of the tumor microenvironment in vaccination efficacy and some proposals to overcome this by using combination therapies will be described.

1. Introduction

Immunotherapy aims to stimulate or modulate immune response to specifically recognize and attack transformed cells in cancer and infectious diseases. The development of cancer immunotherapy includes various strategies like recombinant protein technologies and cell-based therapies. Clinically applied cellular therapeutic vaccines are currently under development and optimization. The advantage of specific active immunotherapy using Dendritic Cells (DC) is mainly the stimulation of *de novo* antitumor immune responses and the induction of immunological memory to prevent tumor relapse. This requires the coordinated induction of innate and adaptive immune responses including Natural Killer (NK) cells, T helper 1 (Th1), and Cytotoxic T Lymphocytes (CTL). Even though the feasibility of this approach was demonstrated in several clinical studies in cancer patients, there is still need to increase its efficacy. Identifying in general how DC perceive danger signals leading to the generation of *de novo* immune responses against disease-associated antigens and which signals induce and enhance the interaction of DC with different immune effector cells is important to increase the efficacy of cancer vaccination strategies. In this paper, we will therefore briefly discuss the selection of appropriate adjuvants by reviewing the roles of PAMPs and PRRs in vaccination strategies against infectious diseases and focus on the translation of these ideas in the application of cancer vaccines.

2. Adjuvants: Critical Components in Vaccination

Preventing infectious diseases by means of vaccination is considered one of the biggest milestones of modern medicine, saving countless lives. Key components of vaccines are adjuvants, which are added to induce, shape, enhance, accelerate, and prolong the immune responses against a desired antigen (Ag). These immunomodulators can be divided into three classes: nonimmunogenic systems increasing the delivery of Ag to target cells and influencing Ag presentation, immunostimulatory compounds (e.g., ligands of immune receptors), and the combination of both. DC represent a crucial target of most vaccine adjuvants, in both preventive and therapeutic vaccination strategies [1, 2]. Depending on the environmental stimuli DC encounter, they transmit signals to immune effector cells inducing immunogenic or tolerogenic immune responses. Defining optimal adjuvants will lead to (a) reduction of number of immunizations, (b) ensuring a rapid response towards pathogens, (c) reduction of the amount of Ag needed, (d) broadening the induced antibody (Ab) response, and (e) directing and localizing the induced immune responses and ensuring the most effective and suitable response towards a particular Ag [3, 4].

3. PRR-Triggering Agents: What DC Vaccination Can Learn from Prophylactic Vaccines

Over the last decades it has become clear that adjuvants such as oil in water emulsions and alum are required for the effectiveness of vaccines against certain pathogens. However, these most frequently used adjuvants only induce suboptimal cellular immune responses. More recently, the use of selected innate triggers (pathogen-associated molecular patterns or PAMPs), which have been naturally part of live attenuated or inactivated vaccines, has been tested in clinical trials exploring the safety and effectiveness of these innate adjuvants on the promise they induce superior cellular immune responses. This concept can directly be translated to the development of DC vaccines for cancer. Such vaccines are usually generated by differentiating monocytes into immature DC [5], followed by tumor antigen loading and maturation of DC. Many different cocktails of growth factors, cytokines, and PAMPs have been used in the preparation [6–11] indicating the most optimal mixture may not have been identified yet.

One crucial step for vaccination efficacy is the induction of appropriate Th cell subsets from naïve $CD4^+$ T cells. $CD4^+$ T cells are important for helping cellular and humoral arms of the immune response. They are necessary for the induction of $CD8^+$ T cell and B cell memory [12]. Th cell polarization is influenced by antigen presenting cells (APC), like DC. Both the subset of DC being activated and the encountered trigger will influence the fate of Th cells. Even though many promising adjuvants have been revealed in experimental studies, clinical trials with beneficial outcome are scarce (reviewed in [13]). This discrepancy is at least in part explained by crucial differences between the animal models used and the complexity of the human immune system *in vivo*. For instance, Toll Like Receptors (TLR) expression pattern and ligand specificity differ between mice and men [3, 14, 15] and within subsets of DC in men [6]. Therefore, it is important to study the polarization kinetics of naive $CD4^+$ T cells by differently matured DC in an autologous human system *in vitro* [16].

Another very important parameter to consider during the selection process of an appropriate adjuvant is the promotion of NK-DC crosstalk [17]. NK-DC crosstalk amplifies Th1 responses by providing an early source of IFN-γ [7, 18, 19]. Vaccine injection induces upregulation of TLR on NK cells, increases activation, and enhances IFN-γ levels [20]. NK cells play a crucial role as amplifiers of DC-induced responses. If potent cellular responses are desired, the choice of adjuvant should have direct NK cell activating properties as well as the indirect capacity via maturation of DC and NK-DC crosstalk.

The key to determine the optimal use of TLR triggers lies most probably *in vivo*. During a pathogenic insult, the invader is able to trigger several PRRs (on various cell types) leading to the induction of multiple signaling pathways and an optimal cooperation between different immune cells. As such, several experimental studies revealed additive or synergistic activation of DC and a resulting enhanced interaction with immune cells when multiple PRR pathways were stimulated [21–25]. Therefore, there is rational to investigate which PRR triggers can be combined to activate synergistic signaling pathways. It could be plausible that several combinations will also be of inhibitory nature, an aspect that has to be prevented. Moreover, recent evidence suggests that targeting nonimmune cells, like stromal cells, influences Th1 and $CD8^+$ T cell responses [26]. This complex expression pattern of TLR/PRR on various immune and nonimmune cells offers new combination strategies to maximize adjuvant capacities. As such, modern adjuvant selection could benefit from identifying potential synergic combinations of PRR triggers to enhance the induced immune responses against a particular Ag.

4. PAMPs in DC Vaccination to Improve Anticancer Helper T and NK Cell Responses

Since the first clinical trial with DC vaccination in 1996 [27], much effort on improving the efficacy of this potentially powerful anticancer therapeutic approach has been made. In 2010, the FDA approved the first DC-based vaccine against advanced prostate cancer [28]. This vaccine is prolonging the patient's progression-free survival for several months. Different ongoing clinical trials testing DC-based vaccination clearly exemplify the importance of DC-based vaccines in future standard treatments. Even though a stabilization of disease and prolonged survival was observed in several cases, limited effect on bulky tumors was observed [28–31]. The overall benefit on clinical outcome is around 15–20%, indicating that there is need for further optimizations.

Whereas initial research focused on generating mainly tumor-specific CTL responses, it is becoming increasingly clear that the activation of multiple immune effector cells

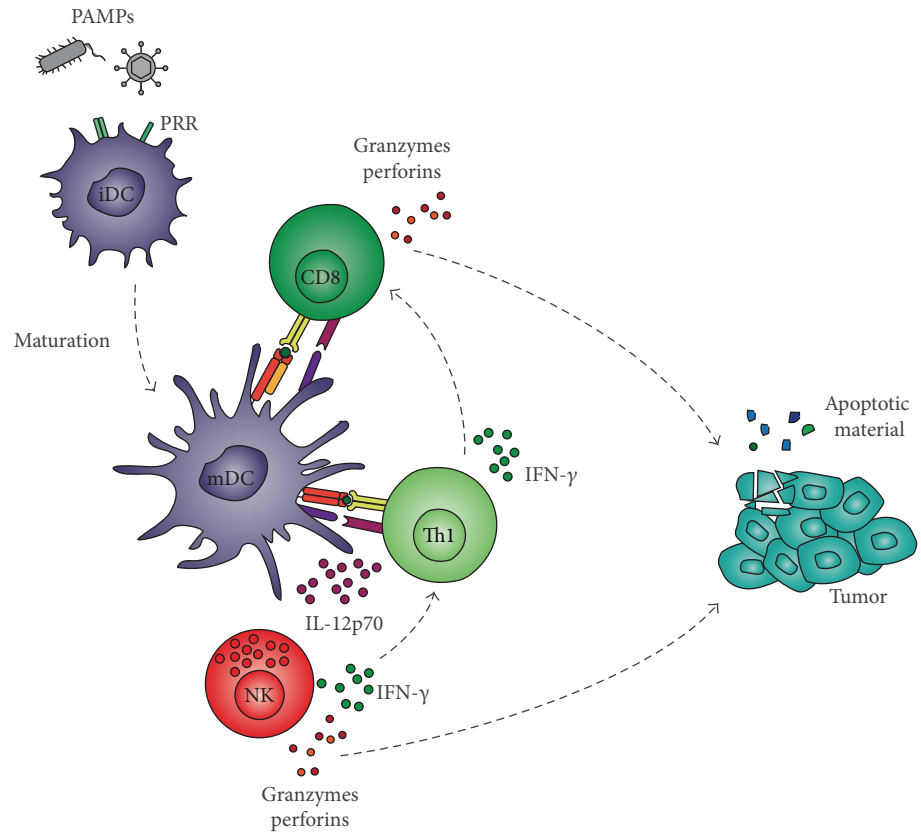

Figure 1: Desired immune interactions upon PAMP stimulation of immature dendritic cells. PAMPs trigger immature MoDC (iDC) to mature DC (mDC) and activate cells involved in antitumor responses (NK cells and $CD4^+$ and $CD8^+$ T cells). The (crucial) cytokine milieu (only IL-12 and IFN-γ are shown) generated by their activation can break the tolerizing effects of the TME resulting in killing of tumor cells by $CD8^+$ T cells and NK cells. Apoptotic material from tumors may be taken up by resident DC and may enhance the immune response.

is the key to success for curative cancer vaccination. *Ex vivo*-matured DC should have the capacity to interact with endogenous immune cells of the patient to induce a potent type-1 immune response enabling the elimination of all tumor cells [32]. The criteria which a potent vaccine should fulfill are challenging and include APC activation, coactivation of $CD4^+$ and $CD8^+$ T cells, priming naive cells and modulating anergic memory $CD8^+$ T cells, crosstalk with DC subsets and NK/NKT cells, and induction of long-lived memory. One way by which DC control and modulate adaptive immune responses is their secretion of cytokines and chemokines. Different from signal 1 (TCR-MHC) and 2 (costimulation) required for proper tumor-antigen-specific T cell activation [33, 34], signal 3 (cytokines) is not only able to polarize T helper cells into a specific lineage but can also recruit and activate other immune cells like NK cells [7, 35–40]. Furthermore, the delivery of signal 4 (homing properties) is important to ensure recruitment of activated T cells [41]. As such, the selection of appropriate PAMPs for priming of DC having capacities to induce type-1 immune responses is desired (Figure 1). We found that combinations of *Klebsiella pneumoniae* membrane fragments (FMKp, confirmed to contain at least TLR2 and TLR4 ligands [42]) and CL075 (TLR7/8 ligand) or poly(I:C) (TLR3 ligand) in combination with IFN-γ are the most powerful combination leading to strong Th and NK helper cell responses [8, 17, 43] (Figure 2), where IL-12 production can be used as a very important read-out marker. In the end, PAMPs can be used as modulator for *ex vivo* DC generation or in combination strategies with radiotherapy, chemotherapy, or targeted therapies in the treatment of cancer [44].

5. Importance of IL-12: Is It All We Need?

IL-12 is long known to be an essential factor driving Th1 responses [34, 45–47] and NK cells responses [34]. We have observed a significant positive correlation between the amount of IL-12 produced by moDC and the resulting polarization of naive $CD4^+$ T cell into Th1 cells and the induction of IFN-γ-producing NK cells [17]. This quantitative requirement needs to be taken into account while screening for new superior maturation cocktails or methods. These findings are strengthened by recent clinical studies indicating a positive correlation between high IL-12p70-producing DC and time to progression [48, 49]. Moreover, older studies tested the systemic application of IL-12 and revealed a positive anticancer effect. However, the implementation of rhIL-12p70 in cancer treatment approaches was hindered by its dose-limiting toxicities [34, 50–55]. Altogether, these findings

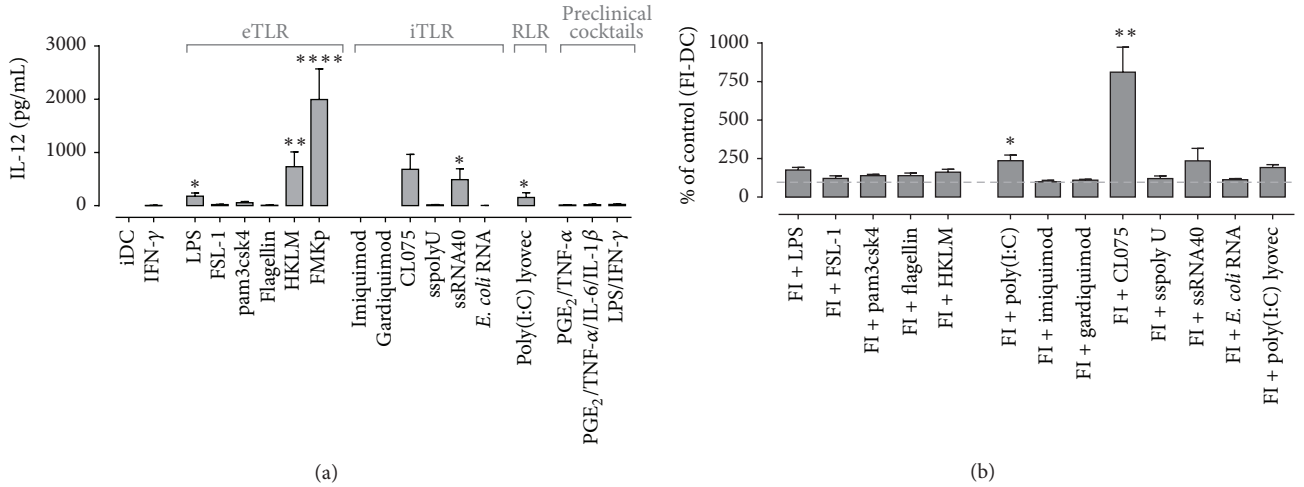

Figure 2: DC-derived IL-12p70 production upon single and multiple PRR triggering. (a) iDC were matured in the presence of IFN-γ and different PRR triggers as indicated on x-axis. Cytokine production was determined in the culture supernatant after 48 h of maturation. Data are represented as mean + SEM and representative of at least 6 independent experiments. Kruskal-Wallis test significance as compared to DC matured with IFN-γ. $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, and $^{****}P \leq 0.0001$. (b) Synergy of FMKp/IFN-γ (FI) maturation with poly(I:C) and CL075 as measured by their IL-12p70 production.

emphasize the use of IL-12p70-producing DC to ensure local production and delivery of this cytokine to come closer to successful vaccination strategies.

The failure of DC to produce ("high enough") IL-12p70 could be one of the factors explaining the limited effects of DC-based vaccination clinical trials (listed in [56]). We tested various DC maturation cocktails used in clinical trials (PGE_2/TNF-α [±IL-6, IL-1β], alpha-DC, and LPS/IFN-γ) and all these DC produced significantly lower levels of IL-12p70 compared to FMKp/IFN-γ-matured DC (Figure 2(a)). FMKp is a membrane fraction of the *Klebsiella pneumoniae* bacterium and contains at least TLR2/4 ligands [12, 30]. FMKp/IFN-γ DC were by far the highest IL-12 producers compared with DC stimulated with other PRR triggers in combination with IFN-γ. Moreover, the importance of IL-12 on *de novo* generation of Th1 responses is underpinned, as only FMKp/IFN-γ DC were able to polarize and induce IFN-γ production in naive $CD4^+$ cells after coculture [16]. Such FMKp/IFN-γ-matured DC also induced the highest NK cell-derived IFN-γ production, followed by LPS/IFN-γ and alpha-DC-activated NK cells. Of note, soluble factors derived from PGE_2/TNF-α (±IL-6 and IL-1β) matured DC did not lead to NK helper activation (unpublished data and [7]).

Besides the finding that PGE_2 exerts a direct inhibiting effect on DC-derived IL-12p70 production [57], another possible explanation for the PGE_2 cocktail not to induce IL-12 producing DC is the absence of IFN-γ in the maturation cocktail. It has been shown that IFN-γ boosts DC cytokine production [58] and additionally prevents DC from exhaustion. We demonstrated that rhIFN-γ dose-dependently determined the magnitude of IL-12p70 production (and production of T cell recruiting CXCL9 and CXCL10) by DC, whereas TNF-α had no effect on the DC-derived cytokine and chemokine production during the priming phase [17]. However, TNF-α was shown to be important for the upregulation of costimulatory markers on DC [59].

Different strategies to maximize the IL-12 production can be applied. One approach is the genetic manipulation of the DC *ex vivo* which was demonstrated to shape key immunological outcome parameters [60]. Another approach is the use of PRR triggers during the *ex vivo* maturation of DC. Several murine and human *in vitro* studies illustrate that the combination of multiple PRR triggers, thus engaging multiple PRR signaling pathways, leads to synergistic effects on DC maturation [8, 11, 61].

We previously showed that the strength of PRR signaling by a single trigger can considerably enhance the IL-12p70 production [16]. Furthermore, cooperative PRR signaling by using the bacterial trigger FMKp with the viral trigger poly(I:C) [17] or CL075 [8] leads to synergistic IL-12p70 production (Figure 2(b)), followed by increased helper cell induction, whereas other combinations did not. This approach requires a thorough search for the most optimal combination of PAMPs of different origins (bacterial, viral, and fungal) or triggering different PRR families (TLR, NOD, CLR, and RLR). The choice of proinflammatory cytokines incorporated into the maturation cocktails can lead to further optimization of cytokine-producing DC [62]. In line, NK cell-derived cytokines do have a decisive influence on DC-derived IL-12p70 production [17]. An increased IL-12p70 production can be achieved by simply adding higher concentrations of rhIFN-γ to a particular PRR-containing cocktail. This provides proof of principle that proinflammatory cytokines can be applied to fine-tune the maturation conditions.

Even though high IL-12-producing DC can be generated *in vitro* by manipulating the composition of the maturation cocktail, one crucial criterion of efficient induction of immune responses is the production of IL-12p70 (and other

cytokines and chemokines) *in vivo* upon DC readministration into the patient. Usually cytokine measurements are performed on *in vitro* 24/48 h-matured DC. Most of the cytokines produced by moDC are released within the first 24 h [63]. In addition, we and others previously showed that in 6 h-matured DC the cytokine induction program is irreversibly primed [7, 64, 65]. Clinical trials employ diverse strategies to mature the DC. Several studies use 24 h/48 h-matured exhausted DC, which are not able anymore to produce IL-12p70 but regain this capacity after T cell encounter and the ligation of CD40. Others employ 6 h maturation protocols, generating semimature DC retaining the capacity to produce IL-12 even before the encounter with T cells *in vivo* [66]. The latter approach is favorable since DC should retain the capacity to produce NK cell-recruiting chemokines as well as NK cell-activating cytokines upon injection.

As diverse polymorphisms affect the IL-12p70 production of DC [67–70], another option is to engineer DC via the usage of mRNA, DNA, or recombinant viruses to constitutively produce IL-12. mRNA electroporation of DC has been shown to be efficient and a clinically safe transfection method has been described [71–73]. Another advantage of engineering DC is the specific selection of "desired" cytokines produced by DC without the production of anti-inflammatory cytokines, for example, IL-10 or TGF-β, or silencing undesired properties. Lipscomb et al. described an IL-12p70-independent mechanism for Th1 polarization when DC expressed ectopic TBX21 (T-bet) via adenoviral infection [74]. These findings were translated into engineering syngeneic TBX21 and IL-12p70 expressing DC. Injection of these DC into mice bearing subcutaneous tumors led to synergistic and robust antigen-specific type 1 immune responses including tumor rejection, crosspriming of Ag, and infiltration of $CD8^+$ T cells [75]. Thus, engineering DC provides a multitude of intervention points [76] and displays a powerful approach to ensure long-lasting provision of cytokines, possibly in combination with other signals (enhancing stimulating or blocking negative modulators) in the tumor microenvironment (TME).

As shown previously [17], the cytokine and chemokine profile of moDC can also be enhanced by soluble factors derived from NK cells. In a similar approach, Berk et al. [77, 78] showed the possibility to use the supernatant of activated lymphocytes to induce maturation of DC including upregulation of phenotype markers, IL-12p70, and CXCL-10 production. These crosstalk features of DC with immune helper cells can be exploited to further boost potential of moDC. Although we showed the importance of high IL-12p70-producing moDC for the induction of Th1 and NK cell responses, also other cytokines were shown in several studies to contribute in an additive or synergic manner to improved helper responses (e.g., IL-15, IL- 18, and IFN-α [79, 80]).

Additionally, plasmacytoid DC (pDC) can also become activated by various PAMPs leading to the polarization of naive $CD4^+$ T cells into Th1 cells [16]. pDC can produce IL-12p70; however, compared to moDC and myeloid DC, their IL-12p70 production level is very limited. Other cytokines have been shown to facilitate IFN-γ production, like IL-18, IFN-α, and IL-27 [79, 80]. Possibly, different DC subsets employ adjusted pathways to activate NK cells or Th1 cells. IFN-α secreted in high amounts by pDC was shown to induce TBX21 expression, although this pathway is less stable compared to IL-12 induction. It remains to be established whether the potency of the different subsets to polarize naive cells into Th1 cells is comparable or whether high IL-12-producing DC subsets favor this induction. Also for NK cell activation, a two-signal activation is much more effective [81, 82]. Likewise, IL-15 can potently enhance proliferation and survival of NK and T cells and enhance NK-DC crosstalk [83, 84]. Arguably, by choosing appropriate maturation stimuli, the DC cytokine profile can be fine-tuned, or DC can be engineered to produce the "optimal" cytokine combinations.

6. Importance of $CD4^+$ T Cells: More Than Helpers?

Numerous lines of evidence indicate the crucial role of $CD4^+$ T cells in the generation of different aspects of adaptive immune responses. They are mainly important for the induction of potent CTL responses and for the generation of long-lived memory responses [85]. Furthermore, they also play an important role in modulating DC maturation by providing diverse cytokines. In mice, $CD4^+$ T cells were shown to be required for improved tumor elimination by $CD8^+$ T cells [86, 87]. $CD4^+$ T cells enhanced the clonal expansion of $CD8^+$ T cells in secondary lymphoid tissue after vaccination and tumor-specific $CD4^+$ also facilitated recruitment, proliferation, and effector function of $CD8^+$ into the TME by secretion of IFN-γ and IL-2. Therefore, it is widely assumed that immunotherapeutic approaches require the involvement of $CD4^+$ T cells. *Ex vivo* maturation of DC should be directed to prime Th1 responses. With a newly developed assay [16], DC-mediated direction, potency, and kinetics of Th cell differentiation can be monitored. Results revealed that PGE_2/TNF-α matured DC, which have been mostly used in clinical studies, induce a Th2-like response. Other differently matured DC promoted significant differences in their Th1 polarization capacity [8, 17].

Recently, the targeting of $CD4^+$ T cells by vaccination with a polytope mRNA vaccine (encoding immunogenic mutant class II epitopes) has been shown to be very efficient in mice by meditating strong antitumor responses [88]. The vaccination led to reversal of suppression by the TME and to induction of CTL. Adding a human leukocyte antigen-(HLA-) class II targeting signal (DC-LAMP) to mRNA encoding tumor antigens will also activate Th1 and CTL responses [70, 73]. These findings highlight the importance of $CD4^+$ T cells in immunotherapy and consist in a very promising approach to become part of the standard therapy in the clinic.

7. Importance of NK Cells

Whereas previous approaches to optimize DC vaccination were mainly based on maximizing intratumoral T cell responses, other players of the immune system may also be important in the process of tumor cell elimination. NK cells are able to exert direct cytotoxic effects on tumor cells

or indirectly modulate the adaptive immunity by cytokine secretion and communication with other immune cells [40, 89–91]. Moreover, low cytolytic activity of NK cells has been associated with 40% increased cancer risk compared to individuals with NK cells having high cytolytic activity [92]. Likewise, levels of intratumoral NK cells and NK cell activity are positively correlated with clinical outcome [93–96]. In patients with cancer, NK cell functions are often impaired displaying reduced cytolytic and cytokine secreting capacities and reduced DC editing [97–100].

As NK cells and DC have a strong mutual interaction, it is plausible to devise strategies combining (actions of) these cells to overcome dysfunction and enhance antitumor responses. For instance, PAMP-stimulated NK cell-derived supernatant can be used in the preparation of DC [101, 102] to maximize their maturation. These findings are strengthened by our recent study describing that soluble factors derived from PAMP-activated NK cells did enhance the cytokine and chemokine profile of *ex vivo* matured moDC (Oth et al., manuscript under revision). We earlier proposed to optimize *ex vivo* maturation of DC in a way where they are able to optimally recruit and activate NK cells [17, 40]. The capacity of DC to efficiently interact with NK cells is influenced by multiple parameters like the differentiation and maturation of DC, as well as the choice and delivery of Ag. TLR agonists are potent and necessary components in the DC maturation process for optimal NK cell activation and recruitment [7, 102]. Also the cytokines used during the differentiation of monocytes (e.g., IFN-α or IL-15) can have an effect on the capacities of DC to recruit and activate NK cells [7, 103–107]. For instance, we have shown that PGE_2 negatively regulates NK-DC crosstalk [57]. Of note, a study by Jensen et al. [62] investigating the effect of different combinations of recombinant human cytokines with PRR triggers revealed that PGE_2 production by moDC is induced upon selected maturation stimuli. The DC maturation cocktails used in clinical trials often contain PGE_2 to induce a migratory capacity of DC [108, 109] but do not avail as only 3–5% of injected DC reach the draining lymph nodes [110]. A combined NK-DC therapy may be more attractive. Antigenic material released by NK cell-killed tumor cells can be taken up and presented by DC. Moreover, NK cells can remove inappropriately matured or immature DC and mature DC may augment NK cell cytotoxicity.

The combination may also induce development of a tertiary lymphoid structure (TLS). The density of such lymphoid islets adjacent to tumors in combination with mature DC correlates with Th1/CTL tumor infiltrating phenotype and with positive clinical outcome [111, 112]. The administered DC will produce chemokines and, thereby, selectively recruit NK effector cells [8] as well as CTLs and Th1 cells [113–115]. We have previously hypothesized [40] that in the event DC recruit all these effector cells a TLS will be formed and replace the interactions normally taking place in lymph nodes. It remains to be established whether the TME suppresses the effector cell induction by DC *in vivo*.

NK-DC crosstalk, however, exerts not only immunostimulatory effects. In this line, a recent study of Sarhan et al. [116] showed that NK-DC crosstalk is inhibited in the presence of IL-2 affecting NK cell-derived IFN-γ production, cytolytic activity, and proliferation. This effect is indirectly mediated by the negative effect of IL-2 on DC-derived IL-12 and lymphotoxin alpha secretion due to STAT3 phosphorylation. Because NK helper cells will mostly interact with DC in the lymph nodes surrounded by naive T cells and Th1 cells and thus IL-2, this is an important aspect to consider for vaccination strategies.

8. Importance of $CD8^+$ T Cells

$CD8^+$ CTL cells are very important effector cells in clearing tumors. It is thus no surprise that the first DC vaccines that were developed focused on MHC class I peptide loaded DC to activate $CD8^+$ T cells (e.g., [117, 118]), this is a sum-up of three parameters, CTL in *in vitro* models, CTL in *in vivo* models (mice), and CTL in biopsies from cancer patients. Several reasons can be indicated for this, but the fact that helper responses are needed for CTLs to stimulate their maturation and improve their killing capacity [119] is beyond doubt. The required help is traditionally provided by $CD4^+$ T cells [120] but also NK cells can provide help [82, 121]. Solutions for the design of DC vaccines lie in the addition of class II targeting sequences as the invariant chain [122] or including DC-LAMP in the mRNA to be transfected [123], resulting in stronger help for $CD8^+$ T cells.

9. Importance of $CD4^+$ Regulatory T Cells

Chemokines released by tumor cells and immune cells present in microenvironment attract also lymphocytes (tumor infiltrating lymphocytes (TIL)). Low numbers of $CD8^+$ TIL and high number of Treg TIL are associated with poor prognosis [124]. The presence of abundant numbers of Treg in the tumor, tumor-draining lymph nodes, and peripheral blood is one of the interfering components hampering DC-induced activation and expansion of type-1 immune responses [125]. Treg can efficiently suppress innate and adaptive arms of antitumor immune responses on multiple levels. Hence, the depletion or functional modulation of these cells is a possible way to restore the immunosuppression.

Furthermore, it is important to check whether DC vaccination does not induce Tregs. It is likely that, by proper stimulation of DC, polarisation of naive T cells into Tregs will not occur. In the polarization assays with FMKp/IFN-γ matured DC and naive $CD4^+$ T cells, we did not detect them (unpublished data).

10. Combination Therapies: Necessity of Multileveled Therapies

Even though in a majority of patients an increased immune response was observed after DC vaccine administration, this effect was not yet reflected in the overall outcome. Many clinical trials applying optimized DC-based vaccines are currently ongoing. However, the direct effect of the TME on DC and the indirect effect on the DC-activated immune effector cells remain a major hurdle in therapeutic DC

vaccine anticancer strategies and cancer immunotherapy in general. There is growing evidence that the host's immune systems play a crucial role in tumor progression [112, 126–128] and that the clinical outcome of treatment is dependent on the patients' TME acting as rheostat on induced immune responses. In this line, patients at the same stage of disease do display different clinical outcomes after intervention [129]. Different approaches are being explored to turn the immunosuppressive environment into an immunosupportive milieu, but they should limit a chronic inflammatory state and thus avoid production of high amounts of TNF-α, IL-1, IL-6, and IL-8.

Another escape phenomenon that should be considered is immune editing. On each time an anticancer treatment induces a potent antitumor response by inducing diverse immune effector cells the pressure on the tumor cells to adapt and to survive is increased. Thus, each treatment will induce partial resistance of the tumor due to its heterogeneity and lead to selective outgrowth of surviving cells (less immunogenic cells). As such, the tumor adapts its phenotype over time [130–134].

The key to success of immunotherapy will most probably be to circumvent the inhibition and escape mechanisms of the tumor. Whereas various single targeted approaches have shown partial success in tumor remission or increase in overall survival, the solution may be not only a multileveled treatment approach combining nonspecific (like adjuvants, cytokines, and checkpoint inhibitors) and specific treatment regimens (antibodies and vaccination) but also including conventional anticancer therapies (radiotherapy and chemotherapy). In particular checkpoint inhibitors have gained great attention. Monoclonal antibodies against inhibitory molecules expressed on T cells like CTLA-4 and PD-1 block the brake of the immune system, resulting in longer lasting immune responses. Although very encouraging clinical results have been obtained as recently reviewed by Mahoney et al. [135], the treatment is still accompanied by toxicity issues [136] that remain to be solved.

Depletion or functional modulation of Treg is a possible way to restore the immunosuppression. Treg are characterized as $CD4^+CD25^+CCR4^+GITR^+$. The depletion can be achieved with mAb against CD25. However, this also affects other (effector) T cell populations which upregulate CD25 as a consequence of their activation [137, 138]. The chemokine receptor CCR4 is highly expressed on effector Treg cells and displays low expression on naive Treg and non-Treg cells [139], making it an interesting target to deplete Treg by using anti-CCR4 mAb. Application of agonist mAb against GITR and OX40, respectively, led to attenuation of suppressive function of Treg and increased effector antitumor T cell functions in several studies [140–144]. The blocking of Treg by stimulating OX40 or GITR to reverse immunosuppressive milieu in the tumor may be a more safe approach than depleting Treg.

One of the biggest challenges in immunotherapy is the lack of biomarkers predicting when to apply which therapy. If tumor biopsies are available, in addition to histopathology, immunophenotyping should also be performed because of the involvement of the immune host defense in tumor progression. This approach was defined as "immunoscore" [145] and consists in detecting TIL in the center and invasive margin of the tumor (number of CD3/CD8 or CD8/CD45RO). As TIL are heterogeneous between tumors and patients, this analysis of immune contexture will help give a better prognosis and make better clinical decisions [146]. Patients with a low immunoscore, meaning low infiltration of $CD8^+$ T cells in the tumor, would be good targets for adjuvant therapy to increase immunogenicity of the tumor.

Another key factor is the tumor burden at the start of the intervention. Low tumor burden seems to be more sensitive to immunotherapeutic approaches. Likewise, an initial tumor treatment approach with conventional therapies may be necessary to remove the majority of the tumor burden. A possible option for applying DC-based vaccines is considering a basic treatment approach around a DC-based vaccine, which has to be adjusted and complemented with different combination strategies depending on both histopathological features as well as the characterization of the tumor microenvironment of the patient (if possible). The rationale for combination therapies is also illustrated in ongoing clinical trials applying DC vaccine strategies and as such DC vaccine in combination with anti-CTLA-4 is currently evaluated in clinical trials [147]. Targeting PD-1 or PD-L1 may also be an interesting combination [148, 149]. In mice, complete eradication of tumors by $CD8^+$ CTLs has been reported after DC vaccine was combined with checkpoint inhibitors [150].

A general prerequisite for the application of checkpoint inhibitors is the immunogenicity of tumors. Presumably this immunogenicity can and should be enhanced locally, for example, by the (intratumoral) administration of certain PRR triggers inducing direct toxicity on tumor cells and generating an immunosupportive environment. Also chemotherapy agents as well as radiotherapy will continue to have a crucial role in the preconditioning of the tumor. The subsequent administration of DC vaccine would enhance antitumor specific responses. Once initiated, blocking, for example, PD-L1 could retain antitumor specific cells in an active state. However, timing will be a crucial factor in any multileveled approach.

11. Conclusions

The interaction of DC with both Th1 and NK cells revealed that high IL-12p70 secreting DC have the capacity to activate both helper responses, resulting in larger and stronger killing capacity by $CD8^+$ CTL. Additionally, NK cells act as amplifiers to enhance cytokine and chemokine production by DC needed for T and NK cells attraction and activation. Furthermore, one of the mechanisms by which tumor environment inhibits immune responses is the blocking of NK-DC crosstalk. Successful combinations of PAMP triggers to mature DC showing enhanced capacities to interact with NK cells and to induce Th1 polarization *in vitro* have been identified. There are important criteria that should be taken into account when selecting PAMPs as adjuvants

for vaccination. Multiple factors explain the so far overall limited clinical outcome of immunotherapy and specifically of DC-based vaccination. Combinations of immunotherapy, including checkpoint inhibitors, with chemotherapy and/or radiotherapy will yield better results, overcoming the suppressive TME by attacking multiple pathways to initiate and elongate desired antitumor immune responses.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

A Comparative Study of the T Cell Stimulatory and Polarizing Capacity of Human Primary Blood Dendritic Cell Subsets

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Dendritic cells (DCs) are central players of immune responses; they become activated upon infection or inflammation and migrate to lymph nodes, where they can initiate an antigen-specific immune response by activating naive T cells. Two major types of naturally occurring DCs circulate in peripheral blood, namely, myeloid and plasmacytoid DCs (pDCs). Myeloid DCs (mDCs) can be subdivided based on the expression of either CD1c or CD141. These human DC subsets differ in surface marker expression, Toll-like receptor (TLR) repertoire, and transcriptional profile, suggesting functional differences between them. Here, we directly compared the capacity of human blood mDCs and pDCs to activate and polarize $CD4^+$ T cells. $CD141^+$ mDCs show an overall more mature phenotype over $CD1c^+$ mDC and pDCs; they produce less IL-10 and more IL-12 than $CD1c^+$ mDCs. Despite these differences, all subsets can induce the production of IFN-γ in naive $CD4^+$ T cells. $CD1c^+$ and $CD141^+$ mDCs especially induce a strong T helper 1 profile. Importantly, naive $CD4^+$ T cells are not polarized towards regulatory T cells by any subset. These findings further establish all three human blood DCs—despite their differences—as promising candidates for immunostimulatory effectors in cancer immunotherapy.

1. Introduction

Dendritic cells (DCs) are professional antigen-presenting cells that possess the unique capacity to activate and prime naive $CD4^+$ and $CD8^+$ T cells [1]. They form a heterogeneous population consisting of specialized DC subsets that differ in their surface marker expression, molecular phenotype, and antigen-processing and antigen-presentation capacity [2–4]. In peripheral blood, at least two major types of DCs can be distinguished, namely, myeloid DCs (mDCs) and plasmacytoid DCs (pDCs) [5, 6]. Myeloid DCs express high levels of CD11c and can further be subdivided based on the differential expression of either CD1c (blood dendritic cell antigen 1 = BDCA1) or CD141 (BDCA3). Each DC subset has its own repertoire of Toll-like receptors (TLRs), underlining their functional specialization [3, 7]. Plasmacytoid DCs express mainly TLR7 and TLR9. Both mDC subsets express TLR3 and TLR8 among others, although expression levels of TLR3 are much higher in $CD141^+$ mDCs [7]. Plasmacytoid DCs are key effectors of innate immune responses due to their capacity to produce large amounts of type I IFNs in response to bacterial or viral infections; this production can also be induced by TLR agonists such as R848 and oligodeoxynucleotides class C (CpG) [8, 9]. Besides their role in the innate immune system, pDCs also participate in priming T helper (Th) cells, depending on the stimulus they receive (summarized in [9]). Myeloid DCs, on the other hand, have the capacity to produce the Th1 skewing cytokine interleukin- (IL-) 12. For both pDCs and mDCs, it has been shown that they induce proliferation in an allogeneic setting and that they can cross-present exogenous antigens to prime $CD8^+$ T cells [10–16].

As a result of their unique capacity to orchestrate adaptive immune responses, DCs are being exploited for cancer immunotherapy. Recently, more advanced examination of primary blood DCs has come within reach through the availability of efficient isolation techniques. Primary DCs are hypothesized to be stronger inducers of anticancer responses than monocyte derived DCs in cell-based vaccination strategies since they differentiate *in vivo* and require only short *ex vivo* handling. The first clinical studies utilizing primary blood DCs have recently been conducted by our group, demonstrating the safety and efficacy of $CD1c^+$ mDCs and pDCs in cancer immunotherapy [17, 18].

In order for DC-based immunotherapy to elicit potent antitumor T cell responses, the administered DCs need to raise an immune-stimulatory rather than tolerogenic T cell response [19]. Naive T cells will proliferate upon encounter with antigen-presenting cells presenting their specific antigen in the presence of costimulatory signals. The nature of costimulation and cytokines from the DC will influence the polarization of the T cells into different T helper phenotypes such as Th1, Th2, and Th17 or regulatory T cells (Tregs). For example, the presence of IL-12 promotes the induction of Th1 cells, whereas IL-10 inhibits induction of Th1 cells and promotes the differentiation of Tregs [20, 21]. In antiviral responses, Th1 cells and antigen-specific cytotoxic $CD8^+$ T cells are elicited to eradicate cells infected by the virus. This type of immune response is also highly desirable in antitumor strategies, in which the aim is to eradicate tumor cells. Toll-like receptor ligands such as polyinosinic:polycytidylic acid (polyI:C), R848, and CpG have been shown to possess Th1 polarizing capacity when used as adjuvants or maturation agents for DCs [22–26].

To be able to successfully manipulate T cell responses for therapeutic strategies, a better understanding of the functional specialization of human DC subsets is needed. In this study, we compared the $CD4^+$ T cell stimulatory and polarizing capacity of human blood mDCs and pDCs side by side—especially the capacity to induce Th1 responses upon differential stimulation.

2. Material and Methods

2.1. Cells. Human blood DCs were isolated from buffy coats (Sanquin) obtained from healthy volunteers after written informed consent and according to institutional guidelines. PBMCs were purified via Ficoll density gradient centrifugation (Lymphoprep by Axis-Shield). Monocytes were depleted via plastic adherence.

DCs were isolated by fluorescence-activated cell sorting (FACS). For this, lineage positive cells were depleted from PBMCs either with Dynabeads Human DC enrichment kit (Invitrogen by Life Technologies) or with anti-FITC microbeads (Miltenyi Biotec) after incubation with FITC-conjugated anti-Lin1 antibody cocktail ($CD3^+CD14^+CD16^+CD19^+CD20^+CD56^+$) (BD Biosciences). The remaining cells were labeled with FITC-conjugated anti-Lin1 antibody cocktail (BD Biosciences), PE-Cy7-conjugated anti-HLA-DR (BD Biosciences), BV421-conjugated anti-CD1c (Biolegend), APC-conjugated anti-CD141 (Miltenyi Biotec), and PE-conjugated anti-BDCA4 (Miltenyi Biotec). Subsets were sorted to obtain $CD1c^+$ mDCs, $CD141^+$ mDCs, or pDCs, respectively (purity 98–100%) (see Suppl. Fig. 1 in Supplementary Material available online at http://dx.doi.org/10.1155/2016/3605643). In some experiments, $CD1c^+$ mDCs were isolated from PBMCs with a $CD1c^+$ DC isolation kit (Miltenyi Biotec). $CD141^+$ mDCs and pDCs were isolated from PBLs by positive selection with anti-CD141 (CD141) and anti-BDCA4 magnetic microbeads, respectively (Miltenyi Biotec). Purity was assessed by flow cytometry (85–97%). Naive $CD4^+$ T cells were isolated from PBLs by depleting $CD4^-$ cells with MACS MultiSort beads and additional use of PE-conjugated anti-CD45RO (Dako) and anti-PE beads (Miltenyi Biotec) for the depletion of $CD45RO^+$ memory T cells (purity > 95%).

All cells were cultured in X-VIVO 15 medium (Lonza) supplemented with 2% human serum (Sigma-Aldrich). The DCs were stimulated with the following TLR ligands: 4 μg/mL R848 (Axxora), 2 μg/mL polyI:C (Sigma) (Figures 1 and 2) or 20 μg/mL polyI:C (Enzo Life Sciences) (Figures 3 and 4), 450 U/mL GM-CSF (Cellgenix), or 5 μg/mL CpG-C (designated CpG throughout text; Enzo Life Sciences). For the control condition of pDCs, the medium was supplemented with 10 ng/mL recombinant human IL-3 (Cellgenix) to ensure pDC survival.

Cell sorting was performed on a BD Aria and flow cytometry on a BD Calibur or BD Verse. The flow cytometry data was analyzed by FlowJo software.

2.2. Phenotype and Cytokine Production of TLR Activated DCs. The DC subsets were incubated overnight at 37°C with different stimuli in triplicate ($CD1c^+$ mDCs, pDCs) or in duplicate ($CD141^+$ mDCs). The next day, supernatants were taken and cells were labeled with PE-conjugated anti-MHC class I and FITC-conjugated anti-MHC class II (BD), PE-conjugated anti-CD80 (BD Biosciences), and PE-conjugated anti-CD86 (BD Biosciences). Marker expression was determined by flow cytometry (BD Calibur and FlowJo software). Supernatants were analyzed for IL-10 (eBioscience) and IL-12p70 (M122 and M121B by Pierce Endogen, standard by BD Biosciences) by standard sandwich ELISA. Depicted in Figure 2 is the cytokine production by 50,000 DCs in a volume of 100 μL. For $CD141^+$ mDCs, in some instances fewer cells were cultured. In all instances, cytokine production per cell was calculated.

2.3. T Cell Proliferation with Allogeneic Naive $CD4^+$ T Cells. $CD1c^+$ mDCs, $CD141^+$ mDCs, or pDCs (1×10^4 cells) were incubated overnight at 37°C with different stimuli in triplicate. The next day, allogeneic naive $CD4^+$ T cells were added to the DCs at a ratio of 1 : 5 (DC : T cell). Proliferative responses were determined by adding 1 μCi [0.037 MBq]/well of tritiated thymidine (^{3}H) (MP Biomedicals) to the cells after three days of coculture. ^{3}H incorporation over a time course of 16 hours was measured with a scintillation counter.

2.4. Cocultures of DCs with Naive $CD4^+$ T Cells and Analysis of the $CD4^+$ T Cell Phenotype. Dendritic cells (1×10^4) were stimulated overnight with the different stimuli in 100 μL

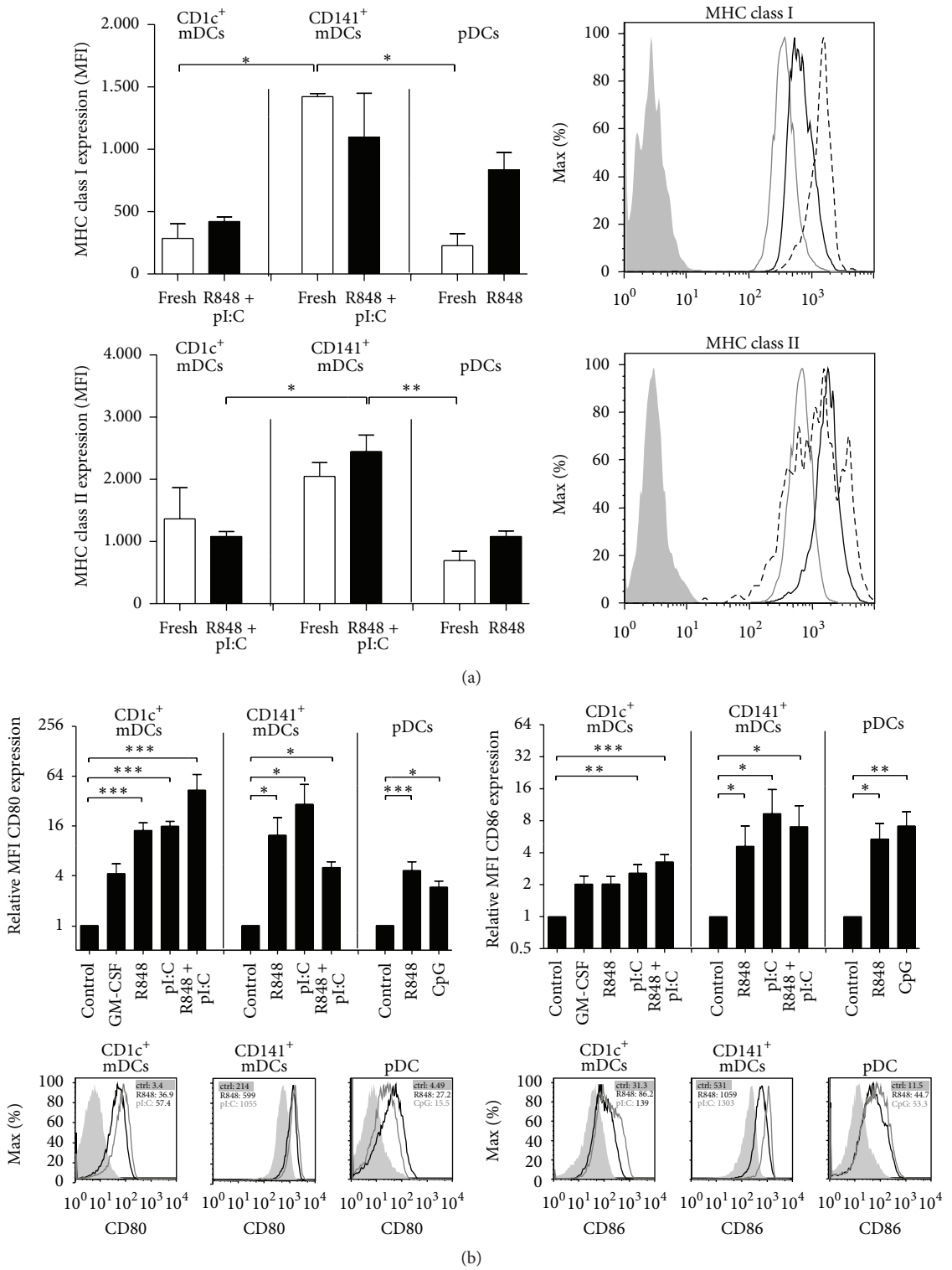

Figure 1: Phenotype of human blood DCs upon TLR stimulation. (a) MHC class I (HLA-ABC) and MHC class II (HLA-DR) expression was analyzed by flow cytometry of DCs kept at 4°C or DCs stimulated and cultured overnight. Myeloid DCs were stimulated with R848 and polyI:C (pI:C), whereas pDCs were stimulated with R848 alone. The bar graphs show the mean ± SEM of the mean fluorescent intensity (MFI) ($n \geq 3$) and the histogram shows expression of freshly isolated DC subsets from a single representative donor (filled histogram: isotype control; grey line: pDCs, black line: $CD1c^+$ mDCs, dashed line: $CD141^+$ mDCs). (b) The bar graphs (upper panel) show the fold change ± SEM of the MFI for surface expression of the costimulatory molecules CD80 and CD86 after overnight stimulation with reference to cells cultured in medium alone (or IL-3 for pDCs) ($n \geq 4$). The histograms (lower panel) show CD80 and CD86 expression from a single representative donor (insets: MFI values). Significance was determined by Kruskal-Wallis test followed by Dunn's testing comparing different conditions of the same subset ($^*P < .05$; $^{**}P < .01$; $^{***}P < .001$).

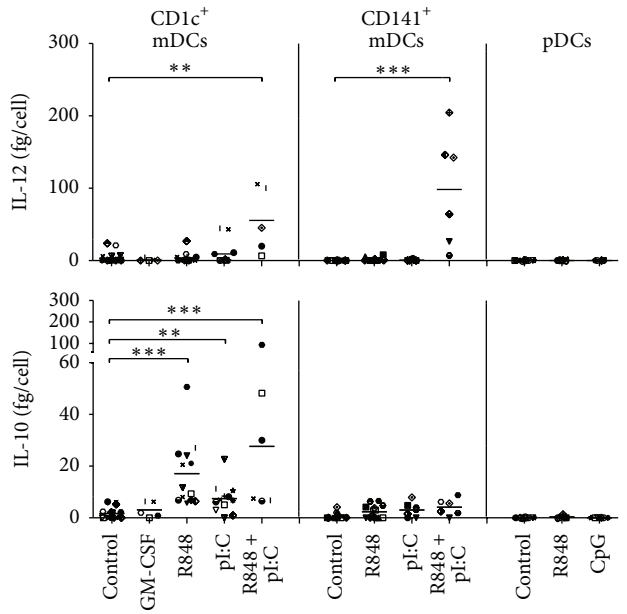

Figure 2: TLR ligation induces differential cytokine production by human blood DCs. The DCs were stimulated as indicated and cultured overnight at 37°C. IL-10 and IL-12p70 production was analyzed in supernatants of overnight cultures by standard sandwich ELISA ($n \geq 6$). Each symbol represents one donor (also across the subsets). Significance was determined by Kruskal-Wallis test followed by Dunn's testing comparing different conditions of the same subset ($^{**}P < .01$; $^{***}P < .001$).

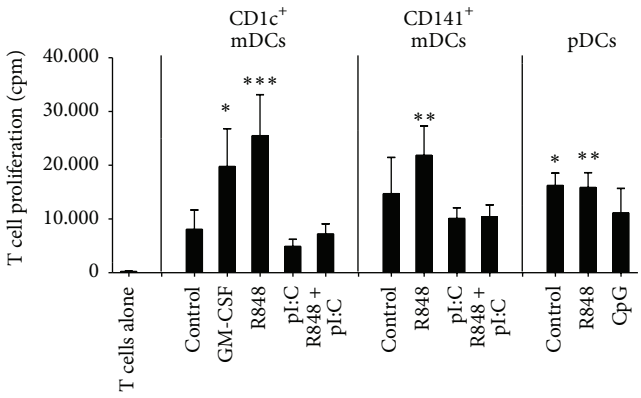

Figure 3: Human blood DCs induce proliferation of naive $CD4^+$ T cells. Human $CD1c^+$ mDCs, $CD141^+$ mDCs, and pDCs were cultured overnight with the stimuli as indicated. The next day, allogeneic naive $CD4^+$ T cells were added to the DCs (ratio 5 : 1). Proliferation was measured at day four of coculture by determining tritiated thymidine incorporation. The graph shows the mean proliferation ± SEM in counts per minute [cpm] ($n \geq 4$). Each experiment was performed in triplicate for $CD1c^+$ mDCs and pDCs and in duplicate for $CD141^+$ mDCs. Significance was tested for each subset in comparison with T cells alone by Kruskal-Wallis test followed by Dunn's testing ($^{*}P < .05$; $^{**}P < .01$; $^{***}P < .001$).

medium. Next, allogeneic naive $CD4^+$ T cells (4×10^4) were added at a ratio of 1 : 4 (DC : T cell) in a final volume of 200 μL medium containing 10 pg/mL superantigen *Staphylococcus aureus* enterotoxin B (SEB) (Sigma). At day 5, human rIL-2 (20 IU/mL, Novartis) was added and the cultures expanded for the next 6–8 days. On days 11–13, resting T cells were counted and analyzed by flow cytometry with three panels. Panel 1 includes anti-CD25-APC (BD Bioscience), anti-CD127-PE (eBioscience), and anti-Foxp3-A488 (eBioscience). Panel 2 includes anti-T-bet-A488, anti-GATA-3-PE, and anti-RORγt-APC (all eBioscience). Panel 3 includes anti-CD45RO-APC (BD Bioscience), anti-CD197 (R&D Systems) with goat-anti-mouse IgG2a-A488 (Life Technologies), and anti-CD62-L (eBioscience) with rat-anti-mouse IgG1-PE (BD Pharmingen). The population of Tregs was determined by selecting $CD25^+$ $CD127^-$ cells and subsequently gating on the $FoxP3^+$ population (Suppl. Fig. 2a). From $CD45RO^+$ cells, T_{CM} were determined by further gating on $CD197^+$/CD62-L^+ and T_{EM} were determined by further gating on $CD197^-$ cells. Both populations are shown as percentage of live cells (forward-sideward scatter) (Suppl. Fig. 2b).

Furthermore, 5×10^4 of the T cells of each condition were restimulated with 5×10^4 anti-CD3/anti-CD28 beads (Dynabeads Gibco by Life Technologies) in triplicate and supernatants from 24-hour cultures were analyzed for levels of IFN-γ (Pierce Endogen), IL-5, and IL-10 (eBioscience) by standard sandwich ELISA.

2.5. Statistical Analysis. Data were analyzed by Kruskal-Wallis test followed by Dunn's testing, by a 1-way ANOVA followed by Tukey testing or with paired Student's t-test using Prism5 (GraphPad Prism5). Statistical significance was defined as <0.05 ($^{*}P < .05$; $^{**}P < .01$; $^{***}P < .001$).

3. Results

3.1. TLR Ligation Increases Expression of Costimulatory Molecules by Human Blood DCs. High expression of MHC molecules is a hallmark of DCs, underlining their antigen-presenting capacities. Accordingly, we found high levels of both MHC class I and MHC class II molecules on all three DC subsets (Figure 1(a)). The levels of both MHC class I and MHC class II molecules were highest for $CD141^+$ mDCs and comparable for $CD1c^+$ mDCs and pDCs, both on freshly isolated cells and after TLR activation.

The expression of costimulatory molecules by DCs is essential to activate T cells and can be induced by TLR ligands. Throughout the study, $CD1c^+$ and $CD141^+$ mDC maturation was achieved by polyI:C, R848, or a combination of both. $CD1c^+$ mDCs were also stimulated with granulocyte-macrophage colony-stimulating factor (GM-CSF). Plasmacytoid DCs were stimulated with R848 or CpG and IL-3 used for the control to secure pDC survival. On $CD1c^+$ mDCs, the costimulatory molecule CD86 was already highly expressed after overnight culture in medium alone; on $CD141^+$ mDCs, this holds true for the expression of both CD80 and CD86 (Figure 1(b)). In comparison, $CD141^+$ mDCs showed the highest expression of CD80 and CD86, both after culturing in medium alone or after TLR ligation (Figure 1(b)). Although CD80 and CD86 molecules were expressed already at high levels on immature DCs, expression of both molecules was

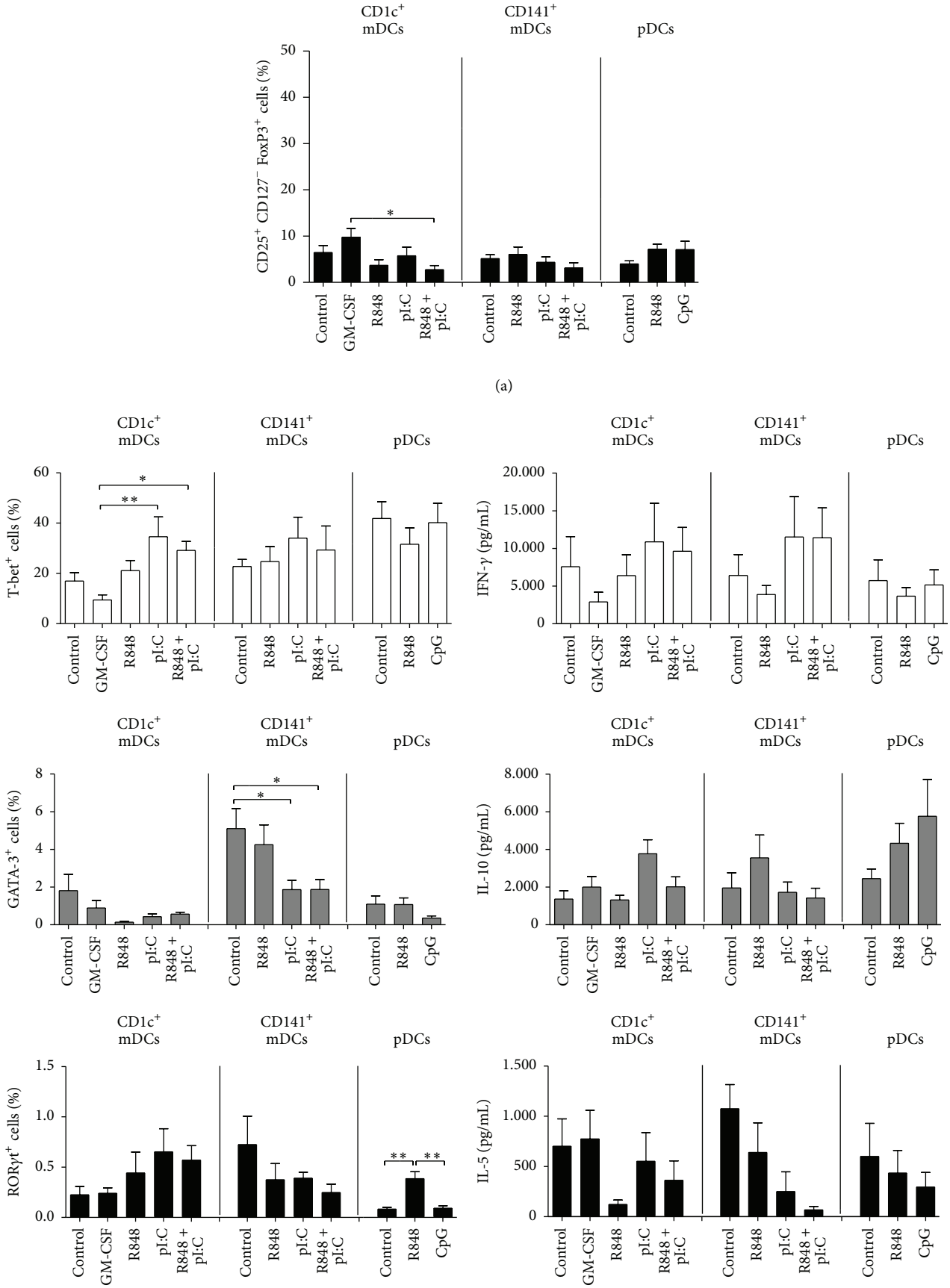

Figure 4: Continued.

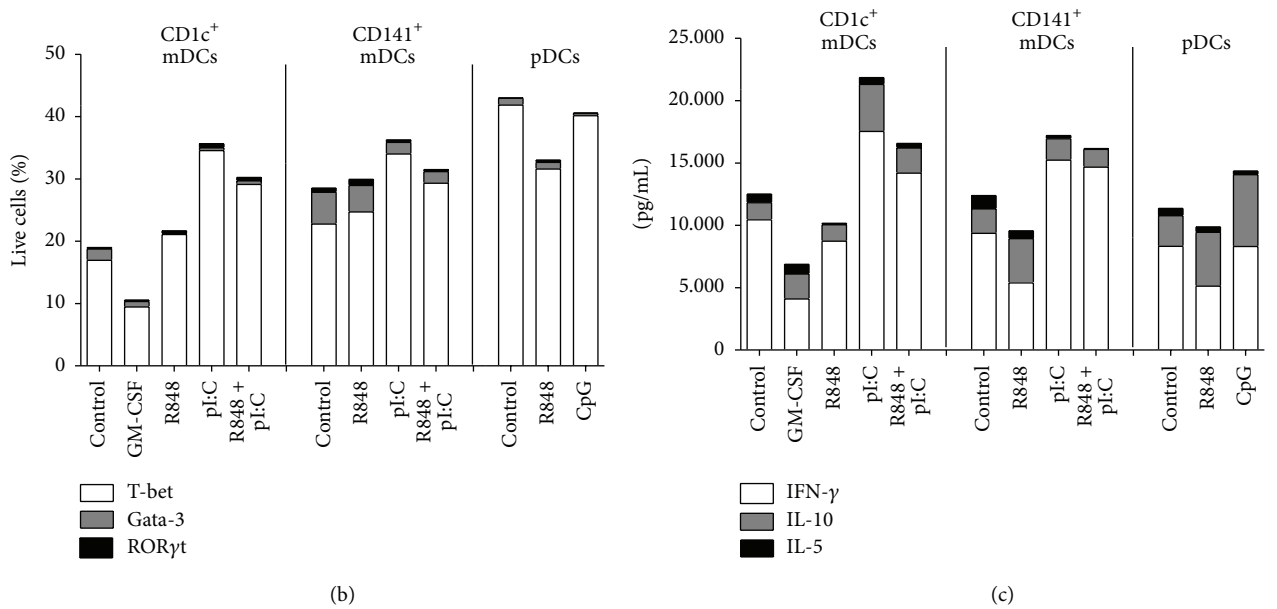

Figure 4: Mature human DC subsets can skew naive $CD4^+$ T cells towards Th1 phenotype and do not induce a big population of Tregs. Human blood DCs were incubated with the indicated stimuli. The next day, allogeneic naive $CD4^+$ T cells were added to the DCs together with a low concentration of the superantigen SEB (10 pg/mL) and cultured until resting (11–13 days). (a) These $CD4^+$ T cells were analyzed by flow cytometry for presence of a Treg population ($CD25^+CD127^-FoxP3^+$ $CD4^+$ T cells) ($n \geq 5$). (b) The cells were also stained for the expression of transcription factors T-bet, Gata-3, and RORγt. In the lower panel, all three transcription factors are depicted in a single bar graph (mean value for each). (c) Furthermore, 5×10^4 of these $CD4^+$ T cells were restimulated for 24 hrs with anti-CD3/anti-CD28 beads. Supernatants were analyzed for IL-5, IL-10, and IFN-γ by sandwich ELISA ($n \geq 4$). The bar graphs show mean cytokine production ± SEM. In the lower panel, all three cytokines are depicted in a single bar graph (mean value for each cytokine). Significance comparing different conditions of the same subset was determined by Kruskal-Wallis test followed by Dunn's testing (a and c), by a 1-way ANOVA followed by Tukey testing or a paired t-test (b) ($^*P < .05$; $^{**}P < .01$).

significantly increased upon culture with TLR ligands on all DCs.

3.2. TLR Ligation Induces Differential Cytokine Production by Human Blood DCs. Dendritic cell-derived IL-10 is known to inhibit Th1 cells and induce type 1 Tregs (Tregs producing IL-10), whereas IL-12 is a Th1-inducing cytokine and therefore desirable in the context of anticancer therapy [20, 21]. We directly compared the secretion of these cytokines by the differentially stimulated DC subsets. Plasmacytoid DCs did not secrete IL-10 or IL-12 at detectable levels, whereas $CD1c^+$ and $CD141^+$ mDCs secreted both IL-10 and IL-12 at differential levels depending on the stimulus (Figure 2). R848 and polyI:C, alone or in combination, induced IL-10 production in $CD1c^+$ mDCs, while only the combination of both TLR ligands induced a significant increase in the secretion of IL-12. $CD141^+$ mDCs secreted low amounts of IL-10, irrespective of the stimulus and at lower levels than $CD1c^+$ mDCs. We observed a significant increase in IL-12 production by $CD141^+$ mDCs after stimulation with both polyI:C and R848, which is higher than the production by $CD1c^+$ mDCs. We can therefore conclude that $CD141^+$ mDCs produce less IL-10 and more IL-12 than $CD1c^+$ mDCs.

3.3. Human Blood DCs Induce Proliferation of Allogeneic Naive $CD4^+$ T Cells. A primary immune response constitutes the activation of naive T cells in response to antigen and their subsequent proliferation and differentiation. Besides recognition of their cognate antigen, naive T cells depend on costimulation by the antigen-presenting cell to start such a primary response. The ability of blood DCs to induce proliferation of naive T cells was directly compared by coculturing overnight stimulated pDCs and $CD1c^+$ and $CD141^+$ mDCs of the same donors with allogeneic naive $CD4^+$ T cells. Proliferation was measured at day four by tritiated thymidine incorporation. All primary blood DC subsets showed the ability to induce proliferation of naive $CD4^+$ T cells (Figure 3). Even so, R848-matured mDCs induced the highest levels of proliferation, while polyI:C maturation did not further increase proliferation as compared to unstimulated mDCs. For pDCs, R848 and IL-3 (control) treatment stimulate similar levels of naive $CD4^+$ T cell proliferation, while the levels for CpG-treated pDCs tend to be lower than for R848 or IL-3.

Besides providing effector T cells, a primary immune response can generate immunological memory in the form of memory T cells. While central memory T cells (T_{CM}) are responsible for rapid clonal expansion after reexposure to antigen and localize in lymphoid organs, effector memory T cells (T_{EM}) localize in mucosal tissue and mediate rapid effector functions there. Although the formation and longevity of such memory cells can only be accurately measured *in vivo*, we wanted to get an idea of the individual

capacities of the different DC subsets to induce them. For this, we cocultured naive, allogeneic $CD4^+$ T cells with the differentially activated blood DC subsets until the T cells had ceased to proliferate [27]. At this resting state (after ~12 days), we analyzed their CD45RO, CCR7 (CD197), and L-selectin (CD62L) expression. The percentages of $CD45RO^+$ $CCR7^-$ (T_{EM}) and $CD45RO^+$ L-$selectin^+$ $CCR7^+$ (T_{CM}) among the T cells did not differ significantly between the subsets or different stimuli (Suppl. Fig. 3 and 2b). At the time point measured, the T cells comprise a larger proportion of T_{EM} (mean 47.14%–71.51%) than T_{CM} (mean 13.47%–24%).

Taken together, all subsets can effectively induce proliferation of naive T cells and are probably able to induce memory T cells. However, mDCs induce significantly higher proliferation when matured with R848 in comparison to polyI:C maturation or culturing alone.

3.4. All Human Blood DCs Can Drive IFN-γ Production by Naive $CD4^+$ T Cells and Do Not Induce Tregs. Dendritic cells play a critical role in the polarization of naive $CD4^+$ T cells into different T helper phenotypes or Tregs. In a Th1 response, cytotoxic $CD8^+$ T cells that are able to kill cells bearing their specific antigen are elicited. Therefore, this type of immune response is highly desirable in antitumor strategies. To compare the differential T cell stimulatory and polarizing capacity—especially the capacity to induce Th1 responses upon differential stimulation with polyI:C, R848, and CpG—and possible Treg induction by human blood mDCs and pDCs, naive $CD4^+$ T cells were cocultured with the DC subsets until they reached resting state. Importantly, analysis of the resting T cells did not show a large fraction of Tregs for any DC subset or condition (mean 3%–10%) (Figure 4(a); Suppl. Fig. 2a). Although the differences are small, it is interesting to note that the percentages of these cells were lowest for polyI:C and R848-matured mDCs and were highest for GM-CSF-stimulated $CD1c^+$ mDCs (mean 3% and 10%, resp.). For the pDC cocultures, there is a tendency of a higher proportion of Tregs being induced after R848 or CpG stimulation compared to the control (IL-3-treated cells) (mean 7%, 7%, and 4%, resp.). Furthermore, we analyzed the induction of Th subset-specifying transcription factors T-bet, GATA-3, and RORγt (Figure 4(b)). We found pronounced populations expressing T-bet across the subsets and stimuli ($CD1c^+$ mDCs: 9%–35%, $CD141^+$ mDCs: 23%–35%, and pDCs: 32%–42%), indicating Th1 polarization by all subsets. GATA-3 expression was overall low, indicating little Th2 polarization. $CD141^+$ mDCs showed the most pronounced GATA-3 expression for control and R848 stimulation (mean 5.1% and 4.25%, resp.), which was significantly reduced with polyI:C or combined R848 and polyI:C stimulation of $CD141^+$ mDCs (mean 1.87% for both). Furthermore, RORγt expression was only detected in a very small population of $CD4^+$ T cells across the subsets (0.08%–0.72%), indicating Th1 rather than Th17 polarization of these cells.

Resting $CD4^+$ T cells were also restimulated with anti-CD3/anti-CD28 beads and their supernatants analyzed for IL-5, IL-10, IL-17, and IFN-γ production to determine the Th1 polarization capacity of the DC subsets. Interleukin-5 is a Th2 cytokine, while IL-10 inhibits Th1 polarization and IFN-γ is a strong Th1 inducer [20, 21, 28]. Notably, coculture with all three blood DC subsets induced T cells with prominent IFN-γ production after restimulation even without TLR maturation (Figure 4(c)). T cells primed by $CD141^+$ or $CD1c^+$ mDCs induced prominent populations of T-bet expressing cells and secreted high levels of IFN-γ, indicating Th1 skewing (Figure 4(c), lower panel). GM-CSF-stimulated $CD1c^+$ mDCs induced smaller populations of T-bet expressing cells and lower levels of IFN-γ and similar levels of both IL-5 and IL-10 as the medium control or TLR-matured DCs; therefore, GM-CSF maturation of $CD1c^+$ mDCs does not induce the most potent Th1 response. Also pDCs induce a prominent population of T-bet expressing cells and IFN-γ release from restimulated $CD4^+$ T cells, although the levels of IFN-γ are lower than for optimally stimulated mDCs. Plasmacytoid DCs induce similar levels of IL-5 in cocultured T cells as mDCs. However, the levels of IL-10 are higher, especially for R848 and CpG stimulated pDCs, which coincides with a tendency for a bigger proportion of Tregs ($CD25^+$ $CD127^-$ $FoxP3^+$) induced in these conditions. We measured no IL-17 for pDCs and modest levels for mDCs stimulated with R848 or $CD1c^+$ mDCs stimulated with the combination of R848 and polyI:C (Suppl. Fig. 4). Together with the RORγt expression data we conclude a Th1 rather than Th17 polarization of the naïve $CD4^+$ T cells. In sum, all subsets polarize naive $CD4^+$ T cells mainly towards Th1 cells with a strong T-bet signature producing mainly IFN-γ after restimulation.

4. Discussion

In order to manipulate T cell responses for DC-based cancer immunotherapy, a better understanding of the functional specialization of human DC subsets is needed. In this study, we compared the capacities of primary human blood mDCs and pDCs to activate and polarize $CD4^+$ T cells side by side. We report that $CD1c^+$ mDCs, $CD141^+$ mDCs, and pDCs all induce proliferation of naive $CD4^+$ T cells. Importantly, naive $CD4^+$ T cells are not skewed towards a regulatory phenotype by coculture with either mature mDCs or pDCs. Despite differences in activation and cytokine profile, both $CD141^+$ and $CD1c^+$ mDCs polarize naive $CD4^+$ T cells towards T cells with a strong IFN-γ signature; also pDCs induce IFN-γ, although at lower levels and accompanied by a higher IL-10 production.

While all DC subsets mature upon TLR ligation, we observed distinct cytokine responses for different subsets and stimuli. $CD1c^+$ mDCs produced only a limited amount of IL-12 after maturation with either R848 or polyI:C alone, but production was significantly increased with a combination of these TLR ligands. Even higher levels of IL-12 are produced by $CD141^+$ mDCs when stimulated with the combination of polyI:C and R848. In contrast to our findings, Nizzoli et al. did not find IL-12 production for $CD141^+$ mDCs after combined polyI:C and R848 stimulation [29]. Other studies have shown that, in order to induce strong IL-12 responses in human and mouse DCs, both an innate trigger such as TLR ligation and a second trigger like ligation of CD40 by CD40L on T cells are needed [7, 30]. More recently, it has been shown for

$CD1c^+$ mDCs that the combination of the TLR ligands R848 and LPS can trigger significant IL-12 production [29]. In the case of $CD141^+$ mDCs, a cocktail of polyI:C together with the cytokines IFN-γ, TNF-α, IFN-α, and IL-1β was shown to induce significant levels of IL-12 [31]. Hémont et al. showed that $CD141^+$ mDCs produced less IL-12 as compared to $CD1c^+$ mDCs for single TLR ligation but that a higher proportion produced IL-12 after TLR1/2 or TLR3 ligation in a whole blood assay [7]. Our data supports the notion that a single stimulus is not sufficient to induce high IL-12 production and with polyI:C and R848 we describe a new combination that can trigger substantial IL-12 secretion by both human mDC subsets.

All DC subsets induced proliferation of naive $CD4^+$ T cells—regardless of the stimulus. The level of T cell proliferation induced by polyI:C-matured mDCs is similar to nonstimulated mDCs. Strikingly, GM-CSF-stimulated $CD1c^+$ mDCs and R848-matured $CD1c^+$ and $CD141^+$ mDCs cause an extra boost in proliferation of naive $CD4^+$ T cells. This is in accordance with an earlier study by Jongbloed et al., which described equally high induction of proliferation of naive $CD4^+$ T cells for nonstimulated or polyI:C stimulated $CD1c^+$ and $CD141^+$ mDCs after six days [31]. Because of the upregulation of the expression of costimulatory molecules with both stimuli compared to untreated DCs, one would expect a higher proliferation rate than with untreated DCs. Certainly, other cytokines and immunostimulatory, but also immunoinhibitory, molecules expressed by cultured DCs are integrated into a single response by the T cells and possible differences in these factors might cause the observed differences in T cell proliferation.

Only a minor percentage of $CD4^+$ T cells that grew out of cocultures with the different DC subsets displayed a Treg phenotype. Earlier studies suggest that pDCs can act as Th1, Th2, Th17, or even Treg inducers in T cell priming, depending on the stimulus they receive (summarized in [9]). One stimulus that can induce this regulatory T cell phenotype is CpG and the proposed mechanism is via the expression of inducible costimulator ligand (ICOS-L) [32]. Ito et al. show in their study that pDCs upregulate ICOS-L upon CpG maturation, which triggers IL-10 production of T cells but no production of IL-4, IL-5, or IL-13. This is in accordance with our findings, where we observed higher levels of IL-10 and a tendency of a higher proportion of Tregs for pDCs matured with CpG or R848 compared to matured mDCs but no elevated levels of IL-5. However, regardless of the stimulus we also found a strong T-bet expression and IFN-γ production by $CD4^+$ T cells that had grown out of cocultures. Plasmacytoid DCs secrete large amounts of type I IFNs in response to bacterial or viral stimuli, including R848 and CpG [8]. Type I IFNs not only are important in innate responses, but can also help to skew T cells towards a Th1 phenotype [33]. Type I IFNs secreted by pDCs might play a role in the observed IFN-γ induction.

Regulatory T cell induction with functional effects on T cells has been described in one study for tissue mDCs of the skin [34]. We show here that primary blood mDCs induce only a low proportion of Tregs and, importantly, the overall $CD4^+$ T cell population displays a Th1 phenotype after coculture (pronounced T-bet expression and high IFN-γ production) and no Th2 or Th17 phenotype. Myeloid DCs induced a strong Th1 phenotype in $CD4^+$ T cells. This is in line with the ability of mDCs to produce IL-12 after combined polyI:C and R848 stimulation. For the other conditions, one can speculate whether the addition of the $CD4^+$ T cells and therefore ligation of CD40 on the DCs give the needed second signal for IL-12 production and help the Th1 skewing. Different groups have shown that blood mDCs can induce IFN-γ production in naive $CD4^+$ and $CD8^+$ T cells [7, 35, 36]. Jongbloed et al. found that $CD141^+$ mDCs were more potent than $CD1c^+$ DCs at inducing IFN-γ responses in total $CD4^+$ T cells, especially after polyI:C stimulation [31]. We found that although $CD1c^+$ mDCs show a less mature phenotype than $CD141^+$ mDCs including higher IL-10 and lower IL-12 production, $CD1c^+$ and $CD141^+$ mDCs induce similar IFN-γ responses after coculture with naive $CD4^+$ T cells. However, there is a tendency for $CD141^+$ mDCs to induce less IL-5 and IL-10 than $CD1c^+$, also arguing for an overall stronger Th1 skewing by this subset. For $CD141^+$ mDCs, high TLR3 expression and the ability to produce IFN-λ and CXCL10, both known to induce antiviral responses, all suggest their capability to induce Th1 skewing in T cells [7, 37, 38]. Certainly, R848 and polyI:C can trigger distinct pathways as TLR3 signals through a TRIF-to-IRF3 pathway, rather than an MyD88-to-IRF7 pathway that is used by TLR8. It is interesting to note that both mDC subsets react strongly and in a similar way to polyI:C, although the expression levels of TLR3 are much higher in $CD141^+$ mDCs than in $CD1c^+$ mDCs [7]. Likely, other receptors for polyI:C contribute to the response in one or both of the mDC subsets. The synthetic dsRNA analog is a ligand for multiple pathogen recognition receptors, and besides TLR3 also triggers cytosolic RIG-I-like receptors that are expressed by mDCs [39, 40]. Perrot et al. suggest in a study on mDCs and NK cells that both TLR3 stimulation and RIG-I-like receptor ligation are needed for IFN-γ induction by mDCs [41].

In addition to their $CD4^+$ T cell activating capacities, all three subsets can cross-present exogenous antigens for cognate restimulation of previously activated $CD8^+$ T cells [10–14], making them promising candidates for DC-based vaccination strategies against cancer. Both $CD1c^+$ mDCs and pDCs have generated promising results in first clinical studies utilizing these primary blood DC subsets as vaccines [17, 18]. These studies support their excellent *in vivo* functioning and mark them as the next generation of cancer vaccines. In this context, we have learned from the current work that GM-CSF is not the optimal stimulus for $CD1c^+$ mDCs, since GM-CSF stimulation showed an overall lower Th1 skewing capacity and induced more Tregs than other stimuli. While maturation with polyI:C or the combination of polyI:C and R848 induces the most pronounced Th1 skewing, the number of T cells that grow out with these stimuli is lower than, for example, with R848 stimulation. Considering the proliferation data and the similar polarization capacity by all subsets and with all stimuli including control DCs, one can only speculate about a recommendation for a suited stimulation of DCs for DC-based

vaccination strategies. However, TLR maturation probably has extra benefits beyond skewing T cell polarization. For example, TLR activation of DCs can lead to the upregulation of otherwise unexpressed TLR ligands in the DCs [42], making them sensitive to a broader range of danger signals. Furthermore, in an *in vivo* situation also other cell types might play a crucial role for the overall outcome of a therapy. Such cells include NK and $CD8^+$ T cells, for which type I IFNs and IL-12—secreted at higher levels upon TLR maturation—are important [43–46]. As discussed above, $CD141^+$ mDCs certainly display promising properties for DC-based anticancer vaccination strategies. Besides their Th1-inducing capacity, human $CD141^+$ mDCs are also excellent cross-presenters of exogenous antigens to $CD8^+$ T cells. While some publications show superior cross-presentation capacity of $CD141^+$ mDCs [31, 47–49] and put them forward as the human counterparts of mouse $CD8\alpha^+$ DCs [31, 47–50], other studies suggest that the different human DCs subsets bear similar cross-presentation capacities at least for soluble antigens delivered through early endosomes [14, 15, 51]. The type and size of the antigen as well as the compartments they are targeted to probably underlie these differing outcomes (reviewed in [52, 53]). In addition to using single subsets for therapeutic approaches, we hypothesize that using a combination of several DC subsets could further increase T cell activating properties, since earlier studies have shown that cell-cell interactions as well as soluble factors can act to cross-activate the different DCs (summarized in [54]). With this comparative study, we have reinforced the establishment of human circulating $CD1c^+$ mDCs, $CD141^+$ mDCs, and pDCs as promising candidates for DC-based immunotherapy in the context of cancer.

Abbreviations

mDC: Myeloid dendritic cell
pDC: Plasmacytoid dendritic cell
BDCA: Blood DC antigen
CpG: Oligodeoxynucleotides class C
polyI:C: Polyinosinic:polycytidylic acid
T_{CM}: Central memory T cell
T_{EM}: Effector memory T cell.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Simone P. Sittig, Jorieke Weiden, Annette E. Sköld, and Jurjen Tel performed experiments. Simone P. Sittig, Ghaith Bakdash, I. Jolanda M. de Vries, and Gerty Schreibelt designed the experiments and directed the research. Simone P. Sittig, Ghaith Bakdash, Carl G. Figdor, I. Jolanda M. de Vries, and Gerty Schreibelt wrote the paper. All authors edited and approved the paper.

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Review Article

Clinical Use of Tolerogenic Dendritic Cells-Harmonization Approach in European Collaborative Effort

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The number of patients with autoimmune diseases and severe allergies and recipients of transplants increases worldwide. Currently, these patients require lifelong administration of immunomodulatory drugs. Often, these drugs are expensive and show immediate or late-occurring severe side effects. Treatment would be greatly improved by targeting the cause of autoimmunity, that is, loss of tolerance to self-antigens. Accumulating knowledge on immune mechanisms has led to the development of tolerogenic dendritic cells (tolDC), with the specific objective to restrain unwanted immune reactions in the long term. The first clinical trials with tolDC have recently been conducted and more tolDC trials are underway. Although the safety trials have been encouraging, many questions relating to tolDC, for example, cell-manufacturing protocols, administration route, amount and frequency, or mechanism of action, remain to be answered. Aiming to join efforts in translating tolDC and other tolerogenic cellular products (e.g., Tregs and macrophages) to the clinic, a European COST (European Cooperation in Science and Technology) network has been initiated—A FACTT (action to focus and accelerate cell-based tolerance-inducing therapies). A FACTT aims to minimize overlap and maximize comparison of tolDC approaches through establishment of minimum information models and consensus monitoring parameters, ensuring that progress will be in an efficient, safe, and cost-effective way.

1. The Case for Cell-Based Therapy in Autoimmunity, Allergy, and Transplantation

The healthy immune system is well balanced to protect against invading harmful pathogens or cancerous cells, whilst maintaining a state of unresponsiveness ("tolerance") to our self-tissues and harmless substances [1]. Breakdown of immunological tolerance can lead to unwanted, detrimental reactions that cause autoimmune diseases (AID) like rheumatoid arthritis (RA), type 1 diabetes (T1D), or multiple sclerosis (MS) and allergies such as allergic asthma and

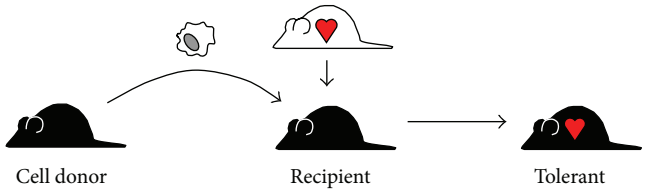

Figure 1: Adoptive transfer of immunoregulatory function. Transplantation of cells with immunoregulatory function to control unwanted immune reactions is not a new proposition. From the earliest discovery that transferring regulatory cells from tolerant to nontolerant animals could establish tolerance in the recipient, it was suggested that the same principle could be applied therapeutically in man. However, while adoptive transfer became a common experimental practice, its translation to the clinic met many obstacles, not least the difficulty of identifying and isolating human regulatory cells.

food allergies. These immune-mediated diseases are a major disease burden. Worldwide, it is estimated that almost 1 in 10 individuals (7.6%–9.4%) [2] suffer from AID, and 1 in 9 have a recorded diagnosis of allergy.

Rejection of allogeneic tissues and graft-versus-host disease (GvHD) are unwanted immune reactions that present major barriers to successful solid organ and bone marrow transplantation. Many factors influence reactivity to foreign transplants, the most fundamental ones of which are graft antigenicity and the contribution of alloreactive effector T cells [3]. Unravelling the rules of donor-recipient histocompatibility has enabled tens of thousands of tissue, organ, and stem cell transplantations to be performed in Europe annually [4]. Nevertheless, as perfect matching of tissue-type is not usually possible, most transplant recipients depend upon lifelong generalised immunosuppression that primarily targets T cells to prevent transplant rejection or GvHD [5].

Existing therapies to treat or prevent AID, allergy, and transplantation reactions mostly include chronic treatment with immunomodulatory drugs. These drugs however are not curative and are inevitably associated with a risk of immediate or late-occurring severe adverse effects (e.g., life-threatening infections, cancer). In addition, general immunosuppressive therapy may become ineffective over time as the physiology of the patient changes (e.g., when neutralising antibodies are induced against a biological agent), low-grade immune reactions ensue, or the pathological mechanisms of disease change under continuous therapeutic pressure. Application and continued monitoring of these lifelong therapies represent an enormous economic burden for society and have a dramatic impact on the quality of patients' lives. Hence, there is an unmet need for more effective and safer therapies aimed at inducing or restoring immune tolerance [6].

The principle of adoptive transfer of immunological function with purified populations of leucocytes has long been known to experimental immunologists (Figure 1). From the very earliest discovery of transferrable suppressor cell populations in animals, it was proposed that cell transplantation could be used as a tolerance-promoting therapy in humans [7]. Recent scientific and technological advances have enabled the identification, isolation, and *ex vivo* manipulation of various types for use as therapeutic agents. The development of cell-based therapies is clinically attractive for many reasons, not least the prospect of low-toxicity and antigen-specific therapies. More remarkably, because immunological tolerance is a self-reinforcing state [8], the therapeutic effects of cell therapy can outlive the therapeutic cells themselves, opening the possibility of curative treatments. Several cell types are now in early-stage clinical trials as adjunct immunosuppressive agents, including various types of regulatory T cells (Tregs) [9] or tolerogenic antigen-presenting cells (including tolerogenic DC (tolDC) and regulatory macrophages (Mregs)) [10–13]. At the present time, it is unclear which of these cell types will prove most suitable as a cell-based therapy; indeed, each has its particular advantages. Here, we describe the collaborative efforts of the A FACTT consortium to tackle the scientific, clinical, and regulatory obstacles to the implementation of therapy with tolAPC.

2. Mononuclear Phagocytes and the Maintenance of Peripheral Tolerance

Precisely to avoid the autoimmune and hypersensitivity reactions described above, immunological responses must be controlled at many levels. During their development, T cells, B cells, and NK cells undergo selective processes that limit their potential for self-reactivity; however, this "central" tolerance alone does not fully account for nonresponsiveness to self and innocuous foreign antigens. Many cooperating mechanisms of "peripheral" tolerance have now been described, including peripheral clonal deletion, anergy, exhaustion, deviation, ignorance, and regulation. In the last 15 years, the preeminent role of active, cell-mediated regulation has emerged from studies of regulatory cell populations, most notably FoxP3$^+$ Tregs. Subsequently, the dependence of T cell-mediated regulation on tolAPC [14] became a subject of intense research. It is now firmly established that specialised subpopulations of mononuclear phagocytes are indispensable for the induction and maintenance of self-tolerance [15], as well as preventing constitutive inflammation in response to nonpathological stimuli [16].

Tolerogenic function is not limited to any particular subset of mononuclear phagocytes; more confusingly, different regulatory DC and macrophages subsets can act through similar cellular and molecular mechanisms. Reflecting on the role of mononuclear phagocytes in the cycle of orderly inflammation may help to explain this apparent redundancy (Figure 2). Macrophages and DC are normal constituents of tissue stroma, serving vital functions in maintaining tissue homeostasis by eliminating necrotic cells and suppressing inflammatory responses against innocuous stimuli. Under steady-state noninflammatory conditions, tissue-resident DC also migrate to lymphoid tissues via afferent lymphatics where they contribute to the maintenance of peripheral T cell tolerance of self and other nonharmful antigens. Macrophages and DC in tissues are exquisitely sensitive to pathogenic signals from their environment, which drive their maturation to an immunogenic state. Activation of mononuclear phagocytes

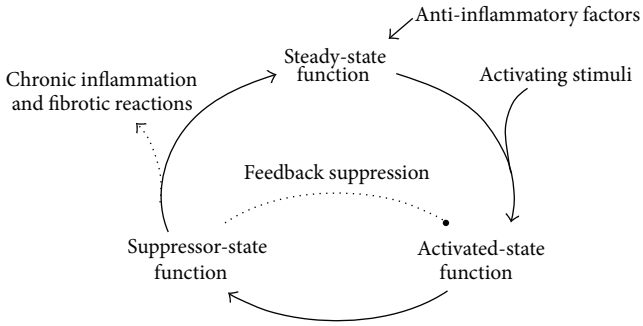

Figure 2: Mononuclear phagocytes are vital for control of inflammatory responses. Mononuclear phagocytes are highly adaptable effector cells that engage in diverse, often antagonistic processes: DC and macrophages are capable of both stimulating or suppressing T cell-mediated responses depending upon their state of activation. Under normal physiological, noninflammatory conditions, immature DC and macrophages present self and innocuous antigens to T cells in a subimmunogenic context. Recognition of cognate antigen in the absence of costimulation causes effector T cells to die, become anergic, or convert into regulatory T cells. Thereby, antigen presentation by nonactivated mononuclear phagocytes contributes to the steady-state maintenance of self-tolerance. A second "class" of myeloid regulatory cell arises as a consequence of persistent stimulation with proinflammatory mediators. Such activation-induced myeloid suppressor cells presumably serve as counterregulators that limit self-injurious inflammatory responses. Activation-induced myeloid regulatory cells are phenotypically diverse and operate through a variety of mechanisms, including production of T cell-suppressive soluble factors, receptor-mediated killing of effector T cells, and the activation-dependent induction of Tregs.

in tissues initiates the acute inflammatory cascade, including further recruitment of inflammatory monocytes from blood, often resulting in secondary tissue injury. Activated DC rapidly migrate into lymphoid tissues to stimulate adaptive immune responses, a key property of inflammatory DC. Importantly, the acute inflammatory reaction is generally self-limiting, which is partly due to repetitively and intensely stimulated mononuclear phagocytes switching to an anti-inflammatory mode. Hence, macrophages and DC can show suppressor functions both as immature cells and as poststimulatory antigen-presenting cells.

3. tolDC and Mregs as Therapeutic Cell Product to Restore Tolerance

The essential role of mononuclear phagocytes in the induction and maintenance of transplant tolerance, especially the many demonstrations that this activity could be adoptively transferred with purified DC or macrophage populations, spurred great interest in the prospect of using tolAPC to suppress pathogenic immune responses [17, 18]. Given the phenotypic plasticity of mononuclear phagocytes, it is perhaps unsurprising that a wide selection of alternative monocyte-derived cell types has been developed as potentially therapeutic cell types [19]. Most attention has focused on treating DC to drive them into a state of arrested immaturity; however, other groups are currently developing therapeutic cell products based on poststimulatory monocyte-derived suppressor cell types or myeloid-derived suppressor cells from early monocyte progenitors (Figure 3).

While the "default" function of DC is to induce tolerance, activated DC have the ability to promote destructive T cell responses. Hence it is clear that maintaining DC in activation-resistant state is an absolute prerequisite for tolDC therapy. tolDC can be defined as a maturation-resistant cell with an immature or semimature phenotype (e.g., low expression of costimulatory molecules) and stable prominent expression of anti-inflammatory molecules and low expression of proinflammatory cytokines. In order to achieve this, several biological and pharmacological agents have been evaluated to generate tolDC *in vitro* [20–25]. Since nuclear translocation of the nuclear factor kappa-light chain-enhancer of activated B cells (NF-κB) is one of the major cellular processes following stimulation with a proinflammatory mediator, several agents that block this pathway and consequently the maturation process of DC have been tested to generate tolDC *in vitro*, including the NF-κB inhibitor, Bay11-7082, vitamin D_3, dexamethasone, or IL-10 [26–28]. In addition vitamin D_3 has also been demonstrated to interfere with cellular metabolism. It counteracts the metabolic shift towards higher glycolysis and progressive loss of mitochondrial oxidative phosphorylation following inflammatory activation [29]. Furthermore, DC can be modified genetically by knocking out immunogenic functions or by inserting tolerogenic characteristics. Overall, tolDC generated *in vitro* using these agents have been demonstrated to reduce symptoms of established AID or to prevent the rejection of transplanted tissues in experimental animal models [30, 31]. These promising outcomes have been instrumental in the development of tolDC therapy for the treatment of human AID and prevention of transplant rejection. Hereunto, a number of methods to generate tolDC *in vitro* have been translated according to Good Manufacturing Practice (GMP) for clinical use in the last 15 years.

Another clinically advanced example of an activation-induced monocyte-derived suppressor cell is Mreg [32]. Through their adherence to plastic surfaces, exposure to serum components, and stimulation with IFN-γ, monocytes are matured to suppressive macrophages that act through indoleamine 2,3-dioxygenase- (IDO-) dependent mechanisms [13]. Mregs express CD86 and HLA-DR, as well as high levels of other maturation-associated markers, like CD274. At least *in vitro*, human Mregs are capable of deleting activated T cells, suppressing T cell proliferation, and driving naïve T cells to become induced Tregs [33].

4. tolAPC-Based Clinical Trials

Several preparations of tolAPC have been tested in phase I clinical trials. Trials with autologous tolDC have been completed for T1D (USA) [12], RA (Australia [10], UK, and South Korea [34]) and Crohn's disease (Spain) [11] (Table 1). So far the results are highly encouraging from a safety standpoint, since none of the trials have found any major concerns that will prevent further testing. tolDC therapy was well tolerated by the patients, and, importantly, autoimmunity in

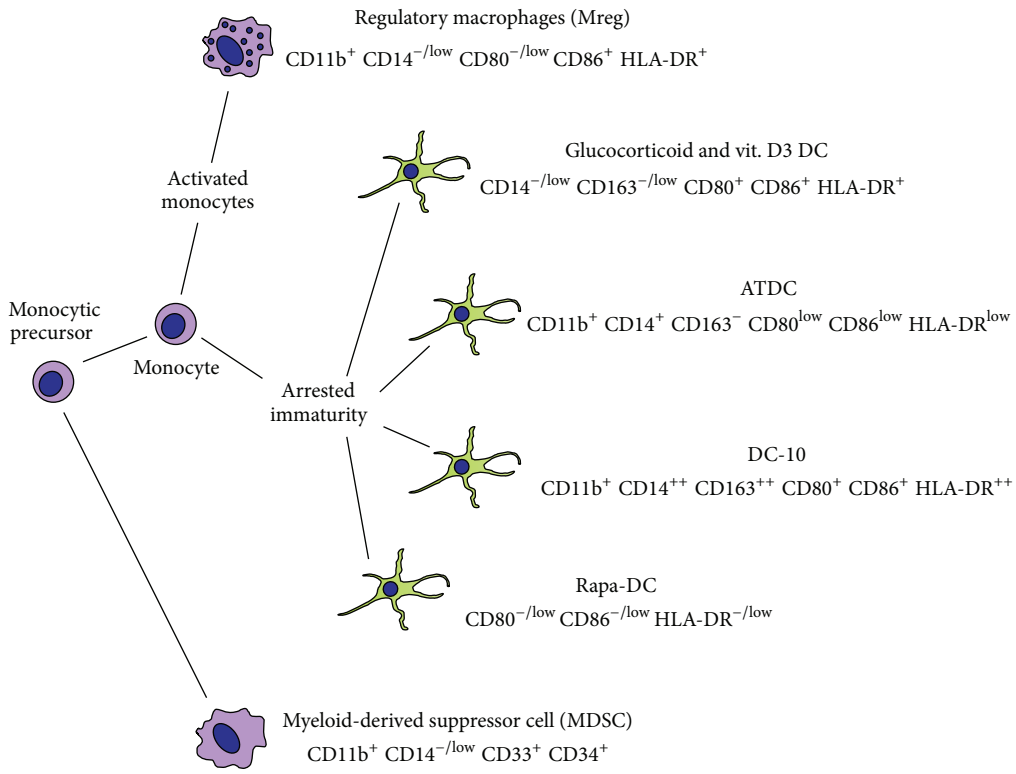

Figure 3: tolAPC types being developed as immunosuppressive cell-based medicinal products. The spectrum of myeloid regulatory cell products currently being developed as medicinal products is diverse, so it is valuable to categorise them as cells in arrested states of immaturity (tolDC), activation-induced suppressor cells, or myeloid-derived suppressor cells. Examples of different tolDC products are depicted.

treated patients was not enhanced. Further phase I tolDC trials are underway in MS (Spain, Belgium, and Russia), neuromyelitis optica (Spain), T1D (The Netherlands), and kidney transplantation (France). Furthermore, phase II trial with tolDC in T1D patients (USA) will start to recruit patients imminently. Mregs containing cell products have now been administered as an adjunct immunosuppressive therapy to more than 20 kidney transplant recipients with promising early results [13, 35, 36]. This therapeutic approach is now being extended in the One Study [37].

5. Collaborative Efforts to Overcome Limitations in tolAPC Therapy

As summarized above, several tolAPC products have been or are being tested in clinical trials. Due to the manufacturing and regulatory complexities associated with initiating a cellular therapy, relatively few groups are preparing or conducting trials with cell-based tolerance-inducing therapies (CTT) in Europe or worldwide. Specific meetings or forums are lacking, since most scientists attend disease-specific meetings or general immunology meetings in which the CTT field, including tolAPC, is underrepresented. Research groups working in kidney transplantation recently initiated a joint initiative in CTT ("One Study" EU consortium), aiming to evaluate CTT in living-donor kidney transplantation; alternatively, many other groups working in other types of organ transplantation or AID are developing their projects independently. Due to this widely distributed and limited action in CTT, joint action is needed to integrate experiences, to share results, and to discuss the strategies to go forward with clinical applications of new clinical trials. To achieve this the EU COST consortium A FACTT (action to focus and accelerate cell-based tolerance-inducing therapies) was initiated in 2014 to accelerate the development and implementation of all CTT, including tolDC and Mreg, by creating a forum for the exchange and integration of knowledge and expertise. This is the first European initiative to bring together different disciplines in the context of human immune tolerance with the main objective to accelerate and advance the clinical application of CTT treatment of AID, allergy, and prevention of graft rejection.

Regarding tolAPC current limitations of this therapy are related to both the production process and evaluation of the clinical trials, which are intended to provide information for the postulated mechanism(s) of action. The first steps to be undertaken by the A FACTT consortium will help move the tolDC field forward by addressing key issues in a collaborative effort between different labs and interests. The most important ones of these issues are discussed below.

5.1. Comparison of tolDC Production Protocols. One outstanding issue within the cellular therapies field is the variation in the methods used for extraction, production, and use of cells for therapeutic purposes. Different methodologies

Table 1: Completed phase 1 safety studies using tolerance-inducing DC.

Group	Indication	Cell culture conditions	Antigen (Ag)	Treatment regimen	Route of administration	Outcomes	Ref.
Giannoukakis, Trucco Pittsburgh, USA	Type 1 diabetes	Use of antisense ODN targeting CD40, CD80, and CD86 in mo-DC	No Ag	4 injections of 1×10^7 cells every two weeks	Intradermal	(i) No AE (ii) No clinical effect (iii) Treatment-associated increase of B220+ CD11c+ B cells (iv) Evidence for reactivation of C-peptide in subjects that were C-peptide negative	[12]
Thomas Brisbane, Australia	RA	Addition of Bay11-7082 to mo-DC cultures	Citrullinated peptides: collagen type $II_{1237-1249}$-Cit1240, fibrinogen α $chain_{717-725}$-Cit720, fibrinogen β $chain_{433-441}$-Cit436, and $vimentin_{447-455}$-Cit450	1 injection of low-dose ($0.5–1 \times 10^6$ cells) or high-dose ($2–4.5 \times 10^6$ cells)	Intradermal	(i) Grade 1 AE (injection site reactions, transient leucopenia, and headache) (ii) No induction of disease flares and reduction of DAS28 in treated patients (iii) Systemic anti-inflammatory effect based on CRP levels, reduced frequency of Teff, and proinflammatory cytokines and chemokines in treated patients	[10]
Panes, Benitez-Ribas, and Ricart Barcelona, Spain	Crohn's disease	Addition of dexamethasone and vitamin A to mo-DC cultures	No Ag	Dose-escalation study: a single or 3 consecutive injections at 2-week intervals of 2×10^6, 5×10^6, and 10×10^6 cells	Intraperitoneal	(i) No AE (3 patients withdrew because of worsening of disease symptoms) (ii) Clinical improvement in 3 out of 13 patients (1 clinical remission, 2 clinical responses) (iii) Increase of circulating Treg and decrease in IFN-γ levels	[11]
Hilkens, Isaacs Newcastle upon Tyne, UK	Inflammatory arthritis	Addition of dexamethasone and vitamin D to mo-DC cultures	Autologous synovial fluid	Dose-escalation study: a single injection of 1×10^6, 3×10^6, and 10×10^6 cells	Intra-articular	(i) No evidence of acute toxicity (ii) Treatment acceptable to patients	
Joo, Bae Seoul, South Korea	RA	CreaVax-RA (autologous tolerogenic DC)	recombinant PAD4, RA33, citrullinated-filaggrin and vimentin	5 injections of low-dose (0.5×10^7 cells) and high-dose (1.5×10^7)	Not indicated	(i) Treatment was well tolerated (ii) Antigen-specific autoantibodies decreased in 5/9 autoantibody-pos. patients	[34]

make it difficult to directly compare different cell products, therefore bringing uncertainty when ultimately comparing final efficacy and safety results. One solution to this problem is to define a set of standard protocols; however, this approach would be difficult since it would require substantial changes to existing methods from many laboratories. As part of the A FACTT project, we are defining a less radical approach of providing a standard reporting framework. We call this MITAP (Minimum Information about Tolerogenic Antigen-Presenting Cells). These guidelines make differences and similarities of approaches immediately clear and transparent. We believe that this approach has a much higher chance of being used by the CTT community as it also provides a checklist for authors when, for example, describing their methods in papers; MITAP makes their jobs easier rather than adding to the burden of scientific publication. We have tested MITAP within the A FACTT community and are ready to release the final version within the immediate future.

5.2. Consensus on Functional Quality Control Parameters. In general, clinically applicable tolDC can be defined as a maturation-resistant cell with MHC II expression and an immature or semimature phenotype (low or limited expression of CD80, CD83, and CD86) and stable prominent expression of anti-inflammatory membrane molecules and/or secreted products and low expression of proinflammatory cytokines, even in presence of environmental proinflammatory signals. Another limitation is that there is no consensus on how to determine the "tolerogenicity" (potency) of the tolDC product, given that tolerance can be achieved by different mechanisms, hereby restricting standardisation of functional quality control (QC) parameter(s), rendering the comparison between products in terms of functionality and safety between different laboratories difficult.

Often functional assays such as a suppression assay or an allostimulatory capacity assay are considered as potency assay. However it has to be taken into consideration that this type of assays is slow and not very precise. Therefore the use of suitable "surrogate" potency markers has to be regarded, for example, the release of inhibitory molecules (IL-10, TGFβ, and IDO), the surface expression of certain surface markers, or even the lack of certain molecules. For this insight into the tolDC products and their mechanism of tolerance induction is important.

Understanding the fundamental biological relationships between alternative tolDC products is a key objective of the A FACTT consortium. Appreciating the similarities and dissimilarities between cell types and how these differences dictate the pharmacological properties of those cells as therapeutic agents is critical to the efficient advancement of the field. Via A FACTT we aim to discuss and share experience to create a consensus and position on a minimal set of functional QC parameters, again documented using the above-mentioned MITAP, hereby making it possible in the future to compare different tolDC approaches.

5.3. Harmonization of Immunomonitoring. Interpretation of the results obtained from immunomonitoring of tolAPC trials is a difficult task due to the variety of methods and protocols available to detect specific T cell responses. The lack of harmonized immunomonitoring protocols for analysis of treated patients makes it difficult to compare outcome of individual trials, decelerating the potential progress of the field.

The capability of tolAPC therapy to suppress pathogenic T cell responses *in vivo* needs to be monitored before and after administration of the tolerance-inducing cell products to determine the effects of tolAPC therapy on the immune system and to correlate these effects with clinical outcomes. Limitations in harmonization of immunomonitoring are due to limited insight into *in vivo* mechanisms of tolerance and lack of proven biomarkers. A FACTT aims to create a consensus and position on relevant immunomonitoring assays and will emphasize the use of minimal information models to describe them. To achieve harmonization for the performance of specific flow-cytometric and functional assays, standardised methods, panels, and sampling conditions will be recommended through publications and focused workshops.

5.4. Regulations. tolAPC are substantially modified cells and therefore must be classified in Europe as somatic cell advanced therapy medicinal products (ATMP). This has been imposed by the Regulation (EC) number 1394/2007 of the European Parliament and of the Council [38]. The most important consequence of this approach is that ATMP are treated similarly to other biological medicinal products and not as cells. Marketing authorisation approval (MAA) for such products is centralized via European Medicinal Agency and the path to offer ATMP to the patients is substantially longer when compared to cells for transplantation or transfusion/blood products, as they must be checked in a series of preclinical tests and in subsequent expensive clinical trials similarly to other classes of drugs. In some cases, this path is difficult to achieve as the cells cannot be defined to the level possible for small-molecule or even biological drugs. Although this is recognized by regulatory bodies, it adds to already very high standards of GMP required to produce cells for clinical use. Since 2007, when regulations were introduced, only five ATMP hold centralized MAA (none of them tolDC) in Europe, which illustrates difficultness of the regulations. Elusive promise of financial reward and very specific expertise necessary to develop ATMP distracts big pharma from investing in this branch of medicine and therefore academic hospitals, universities, and small-sized enterprises (usually academia-based) with limited resources are still the main manufacturers of ATMP. For obvious economic reasons, the regulations create significant hurdles for such organizations and significantly delay the translation of tolDC application.

A FACTT aims to streamline the interaction with the regulatory authorities, in which the opinion and experience of leading groups in CTT are represented, via discussions with authorities and via position papers. Hereby, A FACTT aims to create awareness that therapeutic cells have different mechanisms of action and a different safety profile compared to conventional chemical drugs and thus need unconventional regulatory requirements [39]. Furthermore by sharing

preclinical data necessary for the Investigational Medicinal Product Dossier, A FACTT aims to avoid effort duplication for preclinical studies.

6. Conclusion

Overall, by creating a forum for researchers and clinicians working in the field of CTT therapy, experiences should be shared to enable upcoming trials based on the experience gained in previous trials. This approach saves money in duplicating work and will likely optimize outcomes for future trials. Expertise from ongoing or completed tolDC trials will be shared by our partners with laboratories preparing for new CTT (e.g., through short-term scientific missions). We envisage that the A FACTT collaborative effort will be an important step to accelerate the implementation of CTT in the clinic.

Abbreviations

A FACTT:	Action to focus and accelerate cell-based tolerance-inducing therapies
ATMP:	Advanced therapy medicinal products
AID:	Autoimmune diseases
COST:	European Cooperation in Science and Technology
CTT:	Cell-based tolerance-inducing therapies
DAMP:	Danger associated molecular patterns
GMP:	Good Manufacturing Practice
GvHD:	Graft-versus-host disease
MAA:	Marketing authorisation approval
MITAP:	Minimum Information about Tolerogenic Antigen-Presenting Cells
Mreg:	Regulatory macrophage
MS:	Multiple sclerosis
NF-κB:	Kappa-light chain-enhancer of activated B cells
PAMP:	Pathogen associated molecular patterns
QC:	Quality control
RA:	Rheumatoid arthritis
tolAPC:	Tolerogenic antigen-presenting cells
tolDC:	Tolerogenic dendritic cells
T1D:	Type 1 diabetes.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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