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Chapter 3

Dendritic Cells in Atherogenesis: From Immune Shapers to Therapeutic Targets

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Additional information is available at the end of the chapter

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1. Introduction

Atherosclerosis has been formerly considered as a lipid-mediated disease. It has long been assumed that atherogenesis could be simply explained by lipid accumulation in the vessel wall leading to endothelial dysfunction with adverse vascular wall remodelling. However, over the last decade, a number of studies have clearly demonstrated that lipids are not the whole story in the pathogenesis of atherosclerosis. Accumulating evidence has shown that inflammation and the immune system play a major role in the initiation, progression and destabilization of atheromata [1,2,3,4]. Mainly innate immunity pathways have long been believed to contribute to atherogenesis, and special attention has been given to macrophages, because these effector cells are important for intracellular lipid accumulation and foam cell formation [5]. Yet, although macrophages constitute the largest cell population, other immune cell subsets, namely dendritic cells (DCs) and T cells, can also be found within atherosclerotic plaques and seem to participate in immune responses during atherogenesis.

DCs are the pacemakers of the immune system. These professional antigen-presenting cells play a key role in inducing adaptive immune responses on the one hand, and are critically involved in promoting and maintaining immune tolerance on the other [6]. They originate from hematopoietic stem cells in the bone marrow and circulate as precursors in the blood stream, taking residence in target tissues at sites of potential antigen entry. Within blood vessels [7] and other tissues, they give rise to immature interstitial DCs that act as sentinels, which continuously and efficiently sample the antigenic content of their microenvironment. In the steady state, immature DCs capture harmless self-antigens in the absence of inflammatory signals. They might enter the regional lymph nodes to present the self-antigen to naïve or resting T cells, which will be deleted by apoptosis, silenced by the induction of anergy

or primed to become regulatory T cells [8]. In contrast, when infection and tissue damage occur, immature DCs take up antigens in the presence of inflammatory signals, which evokes activation and functional transformation into mature DCs. Meanwhile, they exit the non-lymphoid tissues to migrate via afferent lymph vessels to lymphoid tissues, where they completely mature. Mature DCs present short peptide fragments, which are bound to the surface molecules CD1 or major histocompatibility complex (MHC)-I or MHC-II. Consequently, they activate (naïve) T and B lymphocytes that recognize the presented antigen [9]. Morphological changes occur as well during the DC life cycle: DC precursors are often small, round-shaped cells that turn into larger cells with an irregular (star-like) shape and cytoplasmic protrusions (dendrites) as the cell matures, while migrating DCs are also called veiled cells, as they possess large cytoplasmic 'veils' rather than dendrites [10].

Following the first observation of DCs in human arteries in 1995 [11], numerous studies suggest that these cells presumably play a crucial role in directing innate or adaptive immunity against altered self-antigens present in atherosclerosis. Localization of DCs nearby vasa vasorum allows monitoring of the major access pathways to the vessel wall and screening of the tissue environment for the appearance of exogenous and endogenous stressors [12]. Once sufficiently activated, DCs in the arterial wall might present the (modified auto-) antigens, such as oxidized epitopes on apoptotic cells, oxidized low density lipoproteins (oxLDL) or heat shock proteins (Hsp) to T cells and initiate inflammatory responses.

2. (Auto-)antigens implicated in atherogenesis and their effects on DCs

Many (auto-)antigens are involved in atherogenesis, both endogenous and exogenous. Here, we summarize some of the best-studied endogenous self-antigens in relation to DC function.

2.1. Oxidized low density lipoprotein (oxLDL)

OxLDL is one of the best-studied antigens in atherogenesis. It is considered as a 'neoantigen', i.e. a self-antigen that has the potential to provoke an auto-immune response upon modification, but that is tolerated by the immune system in its normal (unmodified) form [13]. It has already been shown that oxLDL can induce differentiation of monocytes into phenotypically abnormal cells, when it is added to monocytes during the early stages of differentiation [14]. These cells have functional characteristics of DCs, such as decreased endocytosis capacity, increased ability to stimulate T cell proliferation and secretion of IL-12, but not IL-10. These findings were consistent with our own study (unpublished data), which showed that monocytes differentiated (at least partly) into DCs, when they were incubated with oxLDL. This was evidenced by a pronounced decrease in the expression of CD14, a typical monocyte/macrophage marker, and increased expression of CD1a, which is mainly expressed on cortical thymocytes and DCs, and CCR-6, a receptor for CCL20 that is expressed by resting T cells and DCs (figure 1).

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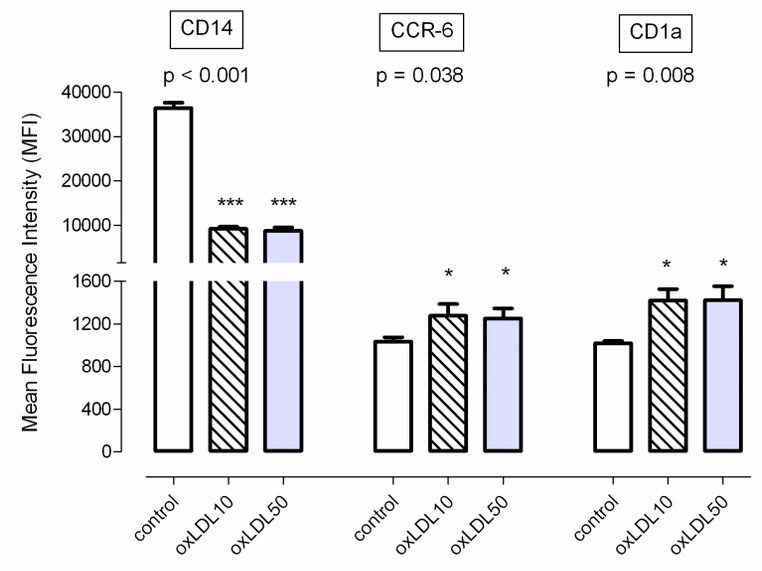

Figure 1. *Effects of oxLDL on monocyte differentiation.* Expression of CD14, CCR-6 and CD1a after 24h incubation of monocytes with 10 µg/mL oxLDL or 50 µg/mL oxLDL points to differentiation to a phenotype with characteristics of DCs (N=3). ***P<0.001, *P<0.05 versus control, Repeated Measures ANOVA and Dunnett's post-hoc test.

Apart from the induction of monocyte differentiation into DCs, oxLDL can also activate DCs, as demonstrated by several *in vitro* studies. After 24h incubation with high concentrations of oxLDL (50 µg/mL), expression of activation markers CD40, CD80 and CD83 was significantly upregulated (figure 2), and endocytotic capacity was significantly reduced (figure 3; own unpublished data).

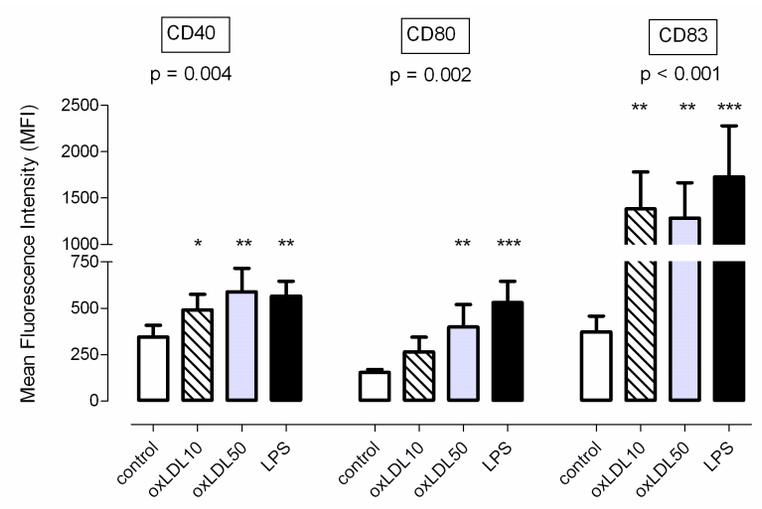

Figure 2. *Effects of oxLDL on maturation of monocyte-derived DCs.* Expression of maturation markers CD40, CD80 and CD83 after 24h incubation of immature monocyte-derived DCs with 10 µg/mL or 50 µg/mL oxLDL (N=4). Black bars represent the positive control for DC maturation, monocyte-derived DCs stimulated with lipopolysaccharide (LPS; 0.1 µg/mL). ***P<0.001, **P<0.01, *P<0.05 versus control, Repeated Measures ANOVA and Dunnett's post-hoc test.

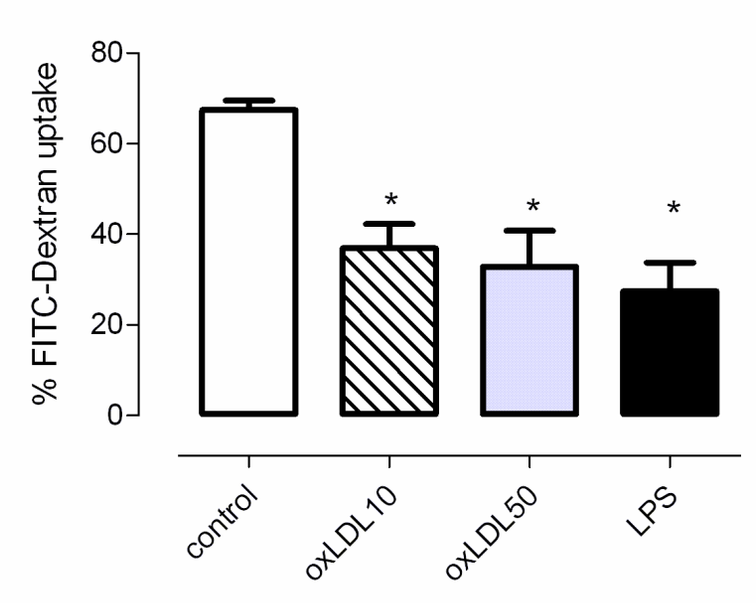

Figure 3. *Effects of oxLDL on endocytotic capacity of monocyte-derived DCs.* Decreased endocytotic capacity of monocyte-derived DCs 24h after stimulation with oxLDL (10 μg/mL or 50 μg/mL) or the positive control LPS (0.1 μg/mL) provides functional evidence of DC maturation (N=5). *P<0.05, Repeated Measures ANOVA, Dunnett's post-hoc test.

Cell morphology pointed to DC maturation as well: oxLDL-stimulated monocyte-derived DCs became more elongated and were arranged in clusters, when compared to unstimulated monocyte-derived DCs. The arrangement in clusters was also more pronounced when cells were stimulated with 50 μg/mL oxLDL as compared to cells stimulated with the lower concentration of oxLDL (10 μg/mL) (figure 4; own unpublished data). Alderman et al. [15] compared the effects of mildly, moderately and highly oxidized LDL and reported a significant upregulation of DC activation markers, including HLA-DR, CD40 and CD86 when cells were incubated with highly oxidized LDL. Furthermore, highly oxidized LDL increased DC-induced T cell proliferation. However, high concentrations of highly oxidized LDL (100 μg/mL) inhibited DC function through increased DC apoptosis [15]. In contrast, another study demonstrated that oxLDL did not trigger maturation of immature DCs [14]. This seems to be a discrepancy, but can easily be explained by a concentration-dependent effect of oxLDL. Perrin-Cocon and colleagues [14] varied the oxLDL concentrations between 2.5-10.0 μg/mL, which could have been insufficient to obtain monocyte-derived DC maturation. Also Zaguri et al. [16] observed no effect of 10 μg/mL oxLDL on CD86, CD83, and CCR-7 expression on DCs, whereas all those activation markers were upregulated with higher concentrations of oxLDL (50-100 μg/mL). Finally, Nickel et al. [17] reported maturation and differentiation of DCs by 10 μg/mL, but he investigated other phenotypic outcomes, such as the expression of scavenger receptors LOX1 and CD36, the mannose receptor CD205 and the activation of the nuclear factor kappa B (NF-κB) pathway.

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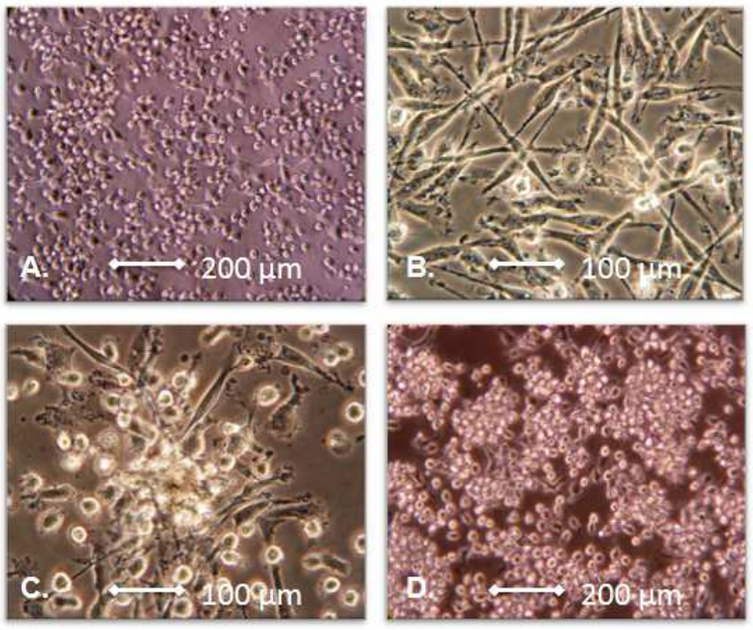

Figure 4. *Effects of oxLDL on morphology of monocyte-derived DCs.* Representative micrographs of immature, monocyte-derived DC cultures after 24h incubation with medium (A), 0.1 µg/mL lipopolysaccharide (LPS; positive control for DC maturation; B), 10 µg/mL oxLDL (C), or 50 µg/mL oxLDL (D). Phase contrast light microscopy, magnification: 10x (A, D), 20x (B, C).

2.2. Beta2-Glycoprotein I

Beta2-glycoprotein I (β2-GPI) is a plasma protein involved in the haemostatic system that has been detected in carotid atherosclerotic lesions [18]. A previous study in mice showed that the transfer of lymphocytes obtained from β2-GPI-immunized LDLr$^{-/-}$ mice into syngeneic mice resulted in larger fatty streaks within the recipients compared with mice that received lymphocytes from control mice [19]. From that study, it appeared that T cells specific for β2-GPI are able to increase atherosclerosis, suggesting that β2-GPI is a target auto-antigen in atherosclerosis [19].

In vitro studies have demonstrated that oxidative modification of β2-GPI, either spontaneously or induced by treatment with hydrogen peroxide, rendered the self-antigen able to induce an autoimmune response. Oxidized β2-GPI caused DC maturation, indicated by increased expression of CD80, CD86, CD83 and HLA-DR [20]. In addition, the interaction between oxidized β2-GPI and DCs led to enhanced secretion of IL-12, IL-1β, IL-6, IL-8, TNFα and IL-10. DCs stimulated with oxidized β2-GPI showed increased allostimulatory ability and induced T-helper (Th)1 polarization [20]. Also, glucose-modified β2-GPI caused phenotypic and functional maturation of iDCs, by activation of the p38 MAPK, ERK and NF-κB pathways. However, DCs stimulated with glucose-modified β2-GPI primed naïve T cells toward a Th2 polarization [21].

2.3. Heat shock proteins

Another category of auto-antigens that have been implicated in atherosclerosis are the stress-induced heat shock proteins (HSPs) [22]. HSPs are responsible for the repair or degradation of denatured proteins and, by maintaining protein conformation, they enhance the cell's ability to survive under conditions of metabolic or oxidative stress [23]. The mRNA expression level of several HSPs, including HSP40 and HSP70, has been shown to be significantly increased in carotid endarterectomy specimens as compared to healthy arteries [24]. HSP70 seems to be homogenously distributed throughout the intima and media in healthy aortas, and a strong increase in its immunostaining intensity is observed in aortic atherosclerotic plaques [25]. They appear to stimulate an immune response leading to the development and progression of atherosclerosis [26]. A number of studies indicate that HSPs are associated with DC function and might trigger DC activation and maturation. DCs seem to overexpress HSP70 in atherosclerotic plaques and the latter protein is presumably an important trigger for DC activation [27]. Gp96 (of the HSP90 family) and HSP70 have indeed been shown to stimulate bone marrow-derived DCs *in vitro* to secrete cytokines [28] and to express antigen-presenting (MHC II) and costimulatory molecules (B7.2) [29]. However, Todryk and colleagues [30] reported that HSP70 targets immature DCs to make them significantly more able to capture antigens. The presence of HSP70 inhibited DC maturation induced by tumour cell lysates from parental B16 cells and maintained the DC precursor population in a more poorly differentiated phenotype. Thus, there is still controversy on whether HSPs activate DCs or keep them in an immature state, and data are lacking to robustly support a conclusion.

3. Survival of DCs in oxidative stress environments

Atherosclerosis is a disease that is associated with strong oxidative stress, and the creation of neo-epitopes is one of the consequences of this situation. As mentioned in section 2, the presence of reactive oxygen species (ROS) in atherosclerotic plaques may lead to the formation of oxLDL and oxidized β2-GPI, which might affect DC phenotype and function. Indeed, oxidative stress has been shown to alter the capacity of antigen-presenting cells to process antigens and to initiate a primary T-cell response. In this respect, it is interesting to unravel whether DCs show phenotypic adaptations in order to function under oxidative stress situations. In a recent study, we demonstrated that DCs appear to be resistant to the detrimental effects of oxidative stress. We showed by confocal live cell imaging that monocyte-derived DCs, which were generated as described earlier [31], were better capable of neutralizing ROS induced by tertiary-butylhydroperoxide (*tert*-BHP) in comparison to their precursor monocytes [31]. *Tert*-BHP was selected to induce ROS because it acutely evokes oxidative stress, resulting in cell toxicity [32]. Decomposition of *tert*-BHP to alkoxyl or peroxyl radicals accelerates lipid peroxidation chain reactions [33]. By means of a neutral red viability assay, we observed that *tert*-BHP induced significant and rapid cell death in both monocytes and DCs. Yet, monocyte-derived DCs were more resistant to *tert*-BHP-induced cell death than their precursor cells [31]. A PCR profiler array specific for oxidative stress and antioxidant-

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related pathways revealed an upregulation of several important antioxidant genes during differentiation of monocytes into DCs, including catalase, peroxiredoxin 2 (PRDX2) and glutathione peroxidase 3 (GPX3). Catalase encodes the enzyme that catalyses the decomposition of hydrogen peroxide to water and oxygen. GPX3 and PRDX2 are genes encoding enzymes that can detoxify hydrogen peroxide and lipid hydroperoxides [34,35]. However, PRDX2 is more efficient in neutralizing hydrogen peroxide than catalase or GPXs [36,37]. Immunoblotting or immunohistochemistry showed that the upregulated transcription of PRDX2 and GPX3 was translated in a significant increase at the protein level. Especially PRDX2 appears to be an important factor in the neutralization of ROS induced by *tert*-BHP [31]. Previously, and in accordance with our recent findings, two studies that used different detection methods reported high expression of antioxidant enzymes in monocyte-derived DCs. A functional study indicated indirectly that monocyte-derived DCs might show enhanced activity of catalase [38]. A proteomic analysis showed higher expression of superoxide dismutase (SOD)2, PRDX1 and PRDX2 in monocyte-derived DCs when compared to precursor monocytes [39]. The latter study also stated that DCs were more resistant than monocytes to apoptosis induced by high amounts of oxLDL [39]. It is conceivable that the good survival skills of monocyte-derived DCs in oxidative stress environments are crucial in atherosclerotic plaques, enabling these professional antigen-presenting cells to exert their function(s).

4. DC subtypes in mice and men

As discussed above, DCs process and present self and foreign antigens to T cells and are therefore important inducers of adaptive immune responses. However, 'the' DC does not exist, as DCs comprise a network of subsets that are phenotypically, functionally, and developmentally distinct [40,41]. It is essential to understand the diversity in DC subtypes to target DCs for immunomodulating therapies. Most studies on DC subsets have been performed in mice, because lymphoid tissue is easier to obtain from mice than from humans. Mature mouse DCs are identified based on their expression of the integrin alpha X chain CD11c, the costimulatory molecules CD40, CD80 and CD86, and high surface levels of the antigen-presenting molecule MHC II [42,43,40]. The T cell markers CD4 and CD8 (in the form of a $\alpha\alpha$-homodimer) are also expressed on mouse DCs, and can be used to distinguish different subtypes [44]. In general, three DC subsets can be characterized in mouse lymphoid tissue (table 1): 1) CD8α^+ CD4$^-$ DCs; 2) CD8α^- CD4$^+$ DCs; and 3) CD8α^- CD4$^-$ DCs [44]. The CD8α^+ CD4$^-$ DCs are mainly localized in the T cell areas of lymphoid organs, whereas the CD8α^- CD4$^+$ DCs are found in the marginal zones. Yet, upon stimulation by microbial products, such as lipopolysaccharide, the latter can also migrate to the T cell zones [45,46]. Other markers that can be used to further subdivide mouse DC subsets include the integrin alpha M chain CD11b and the endocytosis receptor CD205 (DEC205). The CD8α^+ CD4$^-$ DCs are also CD205$^+$ CD11b$^-$ and they are mainly present in the thymus, and at moderate levels in lymph nodes [40]. Lymph nodes further contain, in contrast to spleen, CD8α^- CD4$^-$ CD11b$^+$ CD205^{mid} DCs which are considered as the mature

form of tissue interstitial DCs [40,42,43] (table 1). Another DC subtype, which is langerin^{high} CD11b^{+} CD8α^{low} CD205^{high}, is only found in skin-draining lymph nodes and considered as the mature form of epidermal Langerhans cells. These cells are also positive for MHC II and CD40, CD80 and CD86, suggesting that they are fully activated [42].

The numerous DC subtypes in mouse lymphoid organs are all able to present antigens to T cells, however, they differ in other aspects of DC-T cell communication [40]. CD8α^{+} DCs mainly induce Th1/Th17-polarizing cytokine responses in CD4^{+} effector T cells, whereas CD8α^{-} DCs are able to induce Th2-biased cytokine responses [47,48,49,50]. CD8α^{+} DCs also seem to be specialized for the uptake and cross-presentation of exogenous antigens on MHC I and consequently stimulate CD8^{+} cytotoxic T cells, whereas CD8α^{-} DCs mainly stimulate CD4^{+} T helper cells [51,52].

DC subtype	Subdivision according to localization	Phenotype DC subsets	
		MOUSE	HUMAN
cDCs	lymphoid organ-resident cDC	CD8α^{+} CD4^{-} CD205^{+} CD11b^{-}	lineage^{-} HLA-DR^{+} CD11c^{+} CD1b/c^{+}
		CD8α^{-} CD4^{+}	lineage^{-} HLA-DR^{+} CD11c^{+} CD141^{+}
		CD8α^{-} CD4^{-} CD205^{mid} CD11b^{+}	lineage^{-} HLA-DR^{+} CD11c^{+} CD16^{+}
	circulating cDC	CD8α^{-} CD11b^{+} CD11c^{high}	CD1c^{+} CD11c^{+}
		CD8α^{+} CD205^{+} CD11c^{+}	CD141^{+} CD11c^{+} XCR1^{+}
pDCs	lymphoid organ-resident pDC		CD11c^{-} CD304^{+}
	circulating pDC	PDCA-1^{+} CD11c^{+} CD11b^{-}	CD303^{+} CD304^{+} CD123^{+}
	DC activation status	Markers	
Activated (mature) DCs	Costimulatory molecules	CD40 CD80 CD86	
	Activation molecules	CD83	

cDC = conventional dendritic cell, pDC = plasmacytoid dendritic cell

lineage = cocktail of CD3, CD14, CD16, CD19, CD20, CD56; CD1c = BDCA-1; CD303 = BDCA-2; CD141 = BDCA-3; CD304 = BDCA-4

BDCA = blood dendritic cell antigen

Table 1. Markers used for characterization of DC subtypes in mice and men

It has to be noticed that the association between mouse and human DC subsets remains elusive, making translation of the above-mentioned findings difficult. One of the major barriers in comparing mouse and human DC subsets is the lack of CD8α expression on human DCs

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[53]. As a result, it remains unclear which subtype represents the human equivalent of mature mouse CD8α^+ DCs. Another important barrier is that most human studies are performed on blood, due to the limited availability of human spleen tissue. Moreover, human blood DCs are mainly immature and heterogeneous in their expression of a range of markers. It might be that part of the heterogeneity reflects differences in the maturation or activation state of DCs, rather than that they all represent separate sub-lineages. Yet, one subtype that is similar to its mouse counterpart is the human Langerhans cell, which expresses CD1a and langerin and is characterized by the presence of Birbeck granules [40].

In human blood, the first made classification is often the distinction between plasmacytoid (p)DCs, and myeloid or conventional DCs (cDCs) (table 1). Freshly isolated pDCs resemble plasma cells and have a morphology typical of that of large, round cells with a diffuse nucleus and few dendrites. These type I IFN-producing cells (IPCs) are specialized in innate antiviral immune responses by producing copious amounts of type I interferons. pDCs express CD303 (blood dendritic cell antigen (BDCA) 2), CD304 (BDCA 4) and CD123 (IL 3Rα), whereas cDCs are characterized by their expression of CD1c (BDCA 1) and CD11c [54] (table 1). In addition, pDCs and cDCs also express different sets of Toll-like receptors (TLRs). In brief, pDCs express mainly TLR7 and TLR9, whereas cDCs exhibit strong expression of TLR1, TLR2, TLR3, TLR4, and TLR8. Accordingly, pDCs mainly recognize viral components with subsequent production of a large amount of IFN-α. In contrast cDCs recognize bacterial components and produce pro-inflammatory cytokines such as IL-12p70, TNF-α, and IL-6 [54,7].

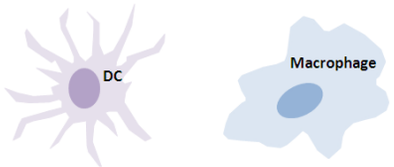
Furthermore, cDCs and pDCs also differ in migration behaviour. Generally it is assumed that myeloid (m)DCs are the conventional DCs that infiltrate peripheral tissues, while pDCs migrate directly from the blood into lymphoid organs [54]. Finally, a small third population of blood DCs expressing CD11c and BDCA-3 (CD141) but not BDCA-1, CD123 or BDCA-2 can be distinguished (table 1). Of particular importance is their superior antigen cross-presentation capacity and expression of the XC chemokine receptor 1 (XCR1), suggesting that they represent the human counterpart of mouse CD8α^+ DCs. They emerge as a distinctive myeloid DC subset that is characterized by high expression of TLR3, production of IL-12 and IFN-β, and a superior capacity to induce T helper-1 cell responses, when compared to BDCA-1$^+$ mDCs [54,7].

Only in a few recent studies, human DCs have been isolated from lymphoid tissues, which allow direct comparison with mouse DC subtypes. Mittag and colleagues [41] identified four DC subsets in human spleen that resemble DCs found in human blood. These include three cDC subtypes and one pDC subtype (table 1). The cDCs are all negative for lineage markers and positive for HLA-DR and CD11c, and they differ in their expression of CD1b/c (= BDCA-1), CD141 (= BDCA-3) and CD16. The pDCs express high levels of CD304 (= BDCA-4), but not CD11c [41]. Moreover, the hallmark functions of mouse CD8α^+ DC subsets, which include IL-12p70 secretion and cross-presentation, appeared to be not restricted to the equivalent human CD141$^+$ cDCs as thought earlier, but shared by CD1b/c$^+$ and CD16$^+$ DC subsets [41].

5. Discriminating between DCs and macrophages

It has become clear that DCs, especially DCs from myeloid origin, are very heterogeneous, representing several subtypes with a common origin, but different anatomical locations (lymphoid organs vs. non-lymphoid organs), function and phenotype. Moreover, there is also a very close relationship between myeloid DCs and macrophages (figure 5).

The distinction of the differences between macrophages and the heterogeneous family of DCs is notoriously difficult and complicated by the plasticity of both cell types [55]. Monocytes that exit the blood and enter tissues under inflammatory conditions can differentiate to macrophages, but also to DCs that share several phenotypic features and functions, making it difficult to unambiguously define macrophages and DCs as individual entities [56]. In addition, resting peripheral monocytes, obtained from mouse peritoneal cavity lavage, represent an immature population, capable of further differentiation along either the dendritic or the macrophage pathway, depending on the type of stimuli (cytokines, growth factors) they receive [10]. Furthermore, many DC subsets are not clearly defined and it is absolutely necessary to bear in mind that different groups use different methods to identify and characterize DCs [57]. Often, the starting populations are preselected based on randomly defined expression levels of markers that were believed to be specific for either DCs or macrophages, but are in fact expressed by both [58] (figure 5).



	Dendritic cells	Macrophages	
Surface markers			**Species**
CD11c	x	x	mouse, human
F4/80	x	xx	mouse
CD11b	x	x	mouse, human
MHC II	x	x	mouse, human
BDCA-1	xx		human
CD68	x	xx	mouse, human
DC-SIGN	xx		human
Functional characteristics			
T cell stimulation	xx	x	
Naïve T cell stimulation	xx		
Antigen presentation	xx	x	
Phagocytosis	x	xx	
Cytotoxicity	x	x	
Tissue sentinel role	x	x	
Migration	xx	x	

Figure 5. Functional characteristics and surface markers of DCs and macrophages. Increasing evidence demonstrates an enormous overlap between what is considered a 'macrophage' and a 'DC'. *Abbreviations: MHC II, major histocompatibility complex class II; BDCA-1, blood dendritic-cell antigen-1; DC-SIGN, dendritic cell-specific ICAM-3-grabbing non-integrin.*

Consequently, confusion in distinguishing between macrophages and DCs has been – at least in part – caused by the use of nonspecific cell surface markers, such as CD11c. In addi-

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tion, the number of DC and macrophage subpopulations that can be defined is an exponential function of the number of markers that has been examined [59]. Moreover, since each gene/protein has its own intrinsic expression level, the heterogeneity is really unlimited [60]. CD11c, a commonly used DC marker, was already known to be expressed by most tissue macrophages before the use of CD11c-reporter transgenes as markers of DCs, and of CD11c-DTR mice to 'selectively' deplete them [59,61]. Other markers that have been used to track macrophages and DCs in mice include F4/80, CD11b and MHC II, but they have also turned out to be nonspecific [57]. Too little attention has been paid to the expression of antimicrobial effector molecules, such as lysozyme, which is highly secreted by monocytes and macrophages, but only weakly expressed, if at all, by DCs [62]. Part of the confusion may also result from the flexibility and plasticity of macrophages and from the presence of resident and migratory activated DCs in the same organ [63].

The confusion could be possibly resolved if the appropriate reflections are considered [57]. For example, the correctness of CD11c to identify DCs depends on the anatomical site in question. In the spleen and lymph nodes, mononuclear phagocytes with high expression levels of CD11c – though not those with low or intermediate CD11c – appear to be DCs rather than macrophages. Accumulating evidence confirms that spleen and lymph node DCs are functionally different from macrophages, do not originate from differentiating monocytes, and share fewer characteristics with monocytes than macrophages [64,65,66]. However, in the lung, high levels of CD11c are expressed on macrophages [67,68], and there are many other anatomical locations apart from the lymphoid organs where macrophages are CD11c-positive. It has been proposed many times that the same set of markers that allows us to discriminate between DCs and macrophages in lymphoid organs, can also be used in non-lymphoid organs, but it has become clear that this assumption is not correct.

Recent *in vivo* experiments in mice have increased our understanding of the development and functions of DC and macrophage subsets [69,70,71]. However, despite this progress in mice, corresponding human subsets are yet to be characterized. Until now, there is no morphologic or protein marker of macrophages or DCs which is unambiguous. Moreover, a single set of markers cannot be assumed to apply to all stages of cell differentiation and activation. In conclusion, there is insufficient knowledge to make definitive claims about any marker combination, particularly in non-lymphoid compartments.

If the distinction between DCs and macrophages cannot be made based on morphological features, can it be based on function? Several criteria to define DCs include the property of DCs to localize in the T cell zone of lymphoid organs where they can stimulate T cells, as well as their ability to migrate and carry antigen [72,73]. In contrast, macrophages are best defined by their phagocytic activity and are generally considered as tissue-resident cells. However, recent studies show that macrophages can also migrate and that Langerhans cells (i.e. DCs from the skin and mucosa that carry large Birbeck granules) are not important for T cell priming [74]. In addition, some macrophage subtypes, such as microglia, show only poor phagocytic capacity [57]. Taken together, there is no good functional criterion to define macrophages and monocyte-derived DCs (figure 5), since they represent not just two differ-

ent cell populations, but various cell subtypes. As they are derived from a common precursor, it is really hard to fully identify macrophages and DCs as two separate entities.

6. Pro-and anti-atherogenic properties of various DC subtypes

We and others discovered a profoundly altered circulating DC compartment in patients with coronary artery disease (CAD), the clinical manifestation of atherosclerosis, as compared to healthy donors [75,76,77,78,79,80]. In 2006, we reported for the first time a decrease in circulating DC precursors (BDCA-1$^+$ mDCs, BDCA-2$^+$ pDCs) in CAD patients by flow cytometry. CAD was determined by angiography and defined as more than 50% stenosis in one or more coronary arteries [77]. In parallel, Yilmaz et al. [79] found a marked reduction in mDC precursors in CAD patients, though the decline in pDCs was less pronounced. Next, we studied whether the lower blood DC counts in CAD patients were related to the extent of atherosclerosis (one- versus three-vessel disease) or type (stable versus unstable angina pectoris) of CAD. Again, we observed significantly lower relative and absolute numbers of pDCs and mDCs in patients with coronary atherosclerosis [78]. Interestingly, the overall lineage-negative HLA-DR-positive blood DCs, which also include other blood DCs (such as BDCA-3$^+$) or more mature blood DCs, confirmed the decline of BDCA$^+$ DC precursors. However, the counts of circulating DCs dropped to the same extent in three groups of CAD patients, irrespective of the number (one or three) of affected arteries or the type (stable or unstable) of angina [78]. Consistent with our results, Yilmaz and colleagues [79] reported no differences between clinically stable or unstable CAD. Yet, in a later and more extended study with a cohort of 290 patients, in which a more refined 'CAD score' was used to classify patients, they found that the numbers of pDCs, mDCs, and total DCs decreased when the extent of coronary atherosclerosis increased [80].

Besides flow cytometric studies, we performed immunohistochemical analyses demonstrating increased intimal DC counts with evolving plaque stages, in close relationship with lesional T cells [81]. These findings strongly suggest that blood DCs migrate from the circulation to the atherosclerotic lesion, possibly attracted by chemokines produced by the inflammatory infiltrate in the plaque, and subsequently stimulate T cell proliferation [7]. However, it is unlikely that accumulation of DC into a single tissue site is responsible for the major changes in the number of circulating DCs in CAD [12]. Possibly, DCs may leave the blood to migrate into lymphoid tissues in response to systemic inflammatory activation, which redirects trafficking and compartmentalization of antigen-presenting DCs as well as lymphocytes. Indeed, it has been mentioned that DC numbers of lymph nodes attached to atherosclerotic wall segments exceed those in lymph nodes attached to non-atherosclerotic arteries [7]. The declined circulating DC numbers in atherosclerosis might also be the result of impaired differentiation from bone marrow progenitors. Interestingly, we recently showed that plasma Flt3 ligand (Flt3L) concentrations were reduced in CAD patients [75]. Flt3L is a major cytokine involved in both pDC and mDC development from haematopoietic stem cells and their release from the bone marrow [82,83,84]. As plasma Flt3L correlated with blood DC counts, the reduced blood DCs in CAD might

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be caused by impaired DC differentiation from bone marrow progenitors. Until now, it remains unclear why plasma Flt3L levels are lowered in CAD. Other possible explanations for the decrease of circulating DC subsets in CAD patients include DC activation resulting in enhanced migration or in loss of subset markers, drug-induced changes, or increased DC turnover, and are reviewed elsewhere [7].

The finding that blood DCs are decreased in CAD patients and that atherosclerotic arteries display a marked increase in the number of DCs suggest the involvement of DCs in the pathogenesis of atherosclerosis. Yet, the exact role of DCs in atherogenesis has not been fully clarified. Moreover, increasing evidence points to different behaviour of DC subsets in the initiation and progression of the disease. We have recently demonstrated *in vitro* that mDCs in CAD operate in a normal way, whereas pDCs from CAD patients are not only reduced in number, but also seem to be functionally impaired [75].

Most evidence points to a proatherogenic role for mDCs. Apolipoprotein E (ApoE)/IL-12 double knockout mice develop smaller atherosclerotic lesions than ApoE deficient ($ApoE^{-/-}$) mice, illustrating the proatherogenic effect of IL-12, which is the main cytokine secreted by mDCs [85]. Moreover, daily IL-12 administration promotes atherosclerosis in $ApoE^{-/-}$ mice [86]. Because mDCs from CAD patients are still able to mature [75], it is plausible that the blood mDCs that are activated by atherosclerosis-favouring factors in the circulation migrate to the atherosclerotic plaque or the lymph nodes attached to the atherosclerotic wall segments. Once arrived, they might initiate and maintain the inflammatory response by continuous T-cell stimulation. Nevertheless, DCs are not only implicated in the immune response in atherosclerosis, they are also involved in cholesterol homeostasis. A recent study using a mouse model in which the receptor for diphtheria toxin was expressed under the CD11c promoter (CD11c-DTR) showed that (transient) depletion of $CD11c^{+}$ cDCs resulted in enhanced cholesterolaemia [87]. The latter indicates that DCs are important in regulating the accumulation of lipids during the earliest stages of plaque formation. In contrast, enhancement of the life span and immunogenicity of DCs by specific overexpression of the anti-apoptotic gene hBcl-2 under the control of the CD11c promoter was associated with an atheroprotective decrease in plasma cholesterol levels, neutralizing the proatherogenic signature of enhanced T cell activation, a Th1 and Th17 cytokine expression profile, and elevated production of T-helper 1–driven IgG2c autoantibodies directed against oxidation-specific epitopes. -As a net result, there was no acceleration of atherosclerotic plaque progression [87].

It is not yet clear whether pDCs are proatherogenic or atheroprotective. PDCs might be involved in plaque destabilization, as they have the unique ability of producing large amounts of type I IFNs. This cytokine exerts strong antiviral effects, but more importantly, it induces marked upregulation of tumour necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) on $CD4^{+}$ T cells, which might lead to killing of plaque-resident cells, potentially weakening the scaffold of the lesion and rendering the plaque vulnerable [88]. In addition, nucleotides released from necrotic or apoptotic cells can induce IFN-α production by pDCs in the presence of antimicrobial peptides released from inflammatory cells [89]. Plaque-residing pDCs have also been shown to respond to CpGs (containing motifs typically found in microbi-

al DNA) leading to enhanced IFN-α expression. This process amplifies inflammatory TLR-4, TNF-α, and IL-12 expression by mDCs, and correlates with plaque instability [90]. A recent study in ApoE$^{-/-}$ mice reported that administration of a plasmacytoid dendritic cell antigen-1 (PDCA-1) antibody to deplete pDCs protected from lesion formation [91], demonstrating that pDCs indeed exert proatherogenic functions during early lesion formation. In contrast, pDC depletion by administration of the 120G8 monoclonal antibody promoted plaque T-cell accumulation and exacerbated lesion development and progression in LDLr$^{-/-}$ mice [92]. PDC depletion was accompanied by increased CD4^{+} T-cell proliferation, IFN-γ expression by splenic T cells, and plasma IFN-γ levels, pointing to a protective role for pDCs in atherosclerosis. Thus, the exact role of pDCs in atherosclerosis remains to be further unravelled.

7. DCs as therapeutic targets

Until now, it is impossible to fully inhibit the formation or progression of atherosclerotic lesions in the clinic. Current therapies for atherosclerosis (e.g. statins, stent placement) focus on relieving symptoms, and consequently many patients remain at high risk for future acute coronary events. A very effective strategy in other immune-related pathologies is vaccination, where the culprit protein or the weakened/dead version of the micro-organism is injected to the body in order to create a highly specific primary humoral immune-response [93]. New vaccines have recently been developed that deliver relevant antigens and adjuvants to redirect the immune system for the individual's benefit [94]. Because DCs are the most effective antigen presenting cells that initiate and regulate the immune response, they seem extremely suitable as vaccine basis. On the one hand, they can activate T cells, on the other hand, they can specifically silence unwanted immune reactions by inducing tolerance [95]. They might function as natural adjuvants for the induction of antigen-specific T-cell responses. Approaches using DCs in atherosclerosis immunotherapy may be comparable to those already used for cancer immunotherapy [96,97,98], although a different immune response is required. One approach that is already intensively studied is the immunization with autologous, monocyte-derived DCs from the patient that are loaded with appropriate antigens *ex vivo* [96]. Such *ex vivo* generated and antigen-loaded DCs have nowadays been used as vaccines to improve immunity in patients with cancer [99] and chronic human immunodeficiency virus (HIV) infection [100,101], providing a "proof of principle" that DC vaccines can work.

In the context of atherosclerosis, immunization of hypercholesterolemic animals with oxLDL or specific epitopes of ApoB100 has already been shown to inhibit atherosclerosis [102,103,104,105,106]. When LDL receptor-deficient (LDLr$^{-/-}$) rabbits were immunized with malondialdehyde modified LDL (MDA-LDL), a reduction in the extent of atherosclerotic lesions was observed in the aortic tree [102]. These observations were confirmed in LDLr$^{-/-}$ and apolipoprotein E deficient (ApoE$^{-/-}$) mice [103,104]. Also hypercholesterolemic rabbits that were immunized with oxLDL showed reduced atherosclerotic lesions in the proximal aorta [107]. Possibly, oxLDL-pulsed DCs or DCs pulsed with immunogenic components of oxLDL

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could be used for vaccination as well, thereby avoiding the side effects of direct vaccination with oxLDL [108]. A series of studies have already used pulsed DCs as an immunotherapy for atherosclerosis in mice, however, results were not always consistent. Repeated injection of $LDLr^{-/-}$ mice with oxLDL-pulsed mature DCs resulted in attenuation of lesion development with a decreased amount of macrophages and increased collagen content, contributing to a more stable plaque phenotype [109]. Moreover, a similar approach was carried out using mice expressing the full-length human ApoB100 in the liver and humanized lipoprotein profiles [110]. Those mice were repeatedly injected with mature DCs that were incubated with IL-10 and ApoB100, prior to the initiation of a Western diet. The immunosuppressive cytokine IL-10 was used to induce tolerogenic DCs [110]. This approach resulted in attenuation of atherosclerotic lesion development in the aorta, which was associated with decreased cellular immunity to ApoB100. Also, decreased Th1 and Th2 responses most likely due to enhanced regulatory T cell (Treg) expansion were observed [110]. In contrast, subcutaneous injection of DCs that were simultaneously pulsed with LPS and MDA-LDL into $ApoE^{-/-}$ mice at frequent intervals during lesion formation caused a significant increase in lesion size in the aortic root [111]. These differential effects may be due to different forms of antigen presentation leading to qualitatively different immune responses. Apart from oxLDL, DCs might also be pulsed *ex vivo* by cultivating them with a total extract or suspension of atherosclerotic plaque tissue, for example, from patients undergoing carotid endarterectomy [95,108] (figure 6). A major advantage of such a therapy, where a patient is vaccinated with its own DCs pulsed by its own antigens is the efficiency, because it would imitate events as they occur in plaques *in situ* in the patient.

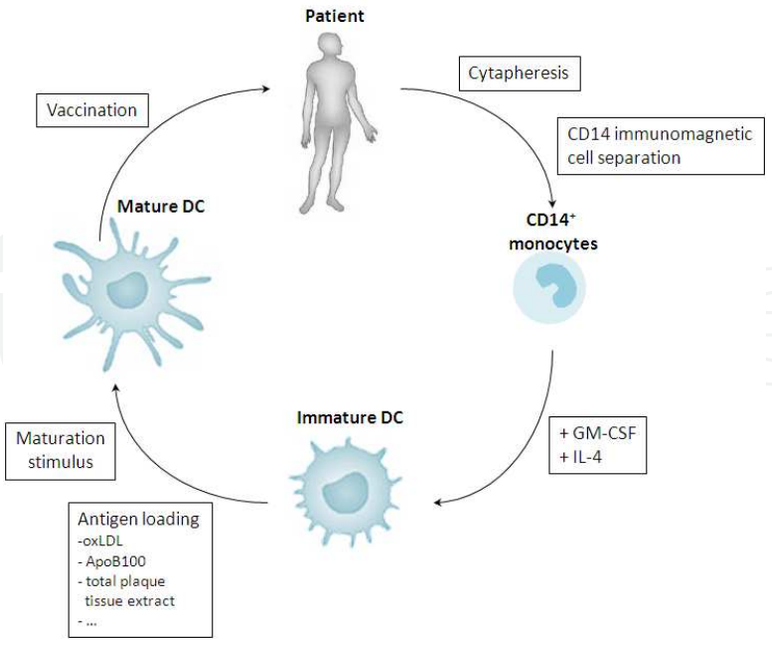

Figure 6. Promising areas for further research to treat immune-mediated diseases, such as atherosclerosis. Immunization of patients with autologous, monocyte-derived DCs that are loaded with appropriate antigens *ex vivo*. This approach has already been proven successful in cancer and HIV patients.

Another promising area for further research is the development of tolerogenic vaccines for immune-mediated diseases (figure 7). Both foreign and self-antigens can be targets of tolerogenic processes. DCs can be converted to 'tolerogenic DCs' by addition of various immunomodulating agents, including IL-10, transforming growth factor-beta (TGF-β) and 1,25-dihydroxyvitamin D3 [8], or they can be generated by using small interfering RNA (siRNA) that specifically targets IL-12p35 gene [112] (figure 7). Tolerogenic DC-based immunotherapy has recently been tested in mice as a possible novel approach to induce immunological tolerance for prevention or treatment of atherosclerosis [110]. Hermansson et al. [110] used IL-10 to induce tolerogenic DCs. Another group showed that oral administration of calcitriol, the active form of vitamin D3, induced the generation of tolerogenic DCs as well as a significant increase in $Foxp3^+$ Tregs in the lymph nodes, spleen, and atherosclerotic lesions of $ApoE^{-/-}$ mice, which resulted in an inhibition of atherosclerosis [113]. This was associated with increased IL-10 and decreased IL-12 mRNA expression. Furthermore, DCs from the calcitriol group showed reduced CD80 and CD86 expression and decreased proliferative activity of T lymphocytes, indicating that tolerogenic or maturation-resistant DCs show some similarities with immature DCs [113]. Hussain and colleagues [114] hypothesized that aspirin may also induce tolerogenic DCs and $CD4^+$ $CD25^+$ $FoxP3^+$ Treg cells activity/augmentation in experimental models of autoimmune atherosclerosis. Aspirin-induced tolerogenic DCs initiated regulatory activity in responder T cells as they showed a decreased expression of costimulatory molecules and an increased expression of immunoglobulin-like transcript 3 (ILT-3), which is a co-inhibitor of T cell activation required to induce Tregs [114,115,116]. Indeed, the presentation of antigen complexes to T cells in the absence of costimulatory signals could lead to anergy or apoptosis of T cells, or the induction of Treg. Therefore, it might also be useful to adjust the expression of costimulatory molecules on pulsed DCs *ex vivo* prior to the vaccination [96,98,97,94,117,118].

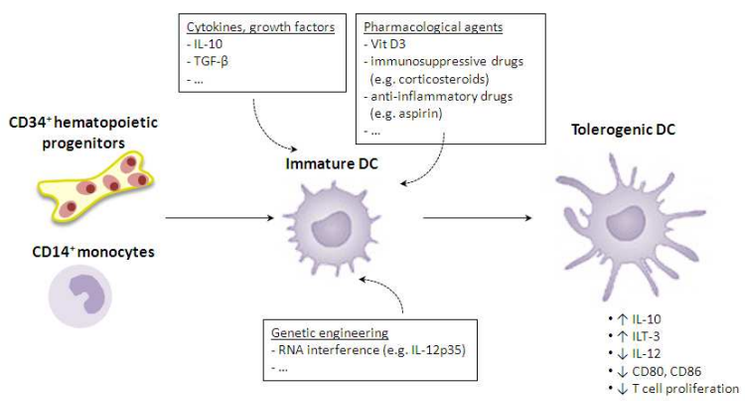

Figure 7. Generation of tolerogenic DCs to develop tolerogenic vaccines. Tolerogenic DC-based immunotherapy has recently been successfully tested in mice as a possible novel approach to induce immunological tolerance for prevention or treatment of atherosclerosis.

A completely different strategy that might be used in therapeutic intervention implicates the use of DCs to deplete specific immune cells, such as the detrimental Th1 or Th17 cells, in atherosclerosis. The opposite approach has been shown to work in a mouse model of athero-

sclerosis. Van Es et al. [119] used DCs to deplete atheroprotective Tregs by vaccinating $LDLr^{-/-}$ mice with DCs which were transfected with Foxp3 encoding mRNA. This approach resulted in a cytotoxic T lymphocyte (CTL) response against Foxp3 and a subsequent depletion of $Foxp3^+$ Tregs. Vaccination against Foxp3 aggravated atherosclerosis, it resulted in a reduction of $Foxp3^+$ regulatory T cells in spleen, lymph nodes and circulation, and in an increase in initial atherosclerotic lesion formation. Besides an increase in lesion size, vaccination against Foxp3 also induced a 30% increase in cellularity of the initial lesions, which may indicate an increase in inflammation within the lesions [119].

Another approach for therapeutic intervention against atherosclerosis might involve the direct targeting of DCs *in vivo* by manipulating the functions of different DC subsets [95]. Based on the hypothesis that cDCs act rather proatherogenic, whereas pDCs might be atheroprotective (see section 4), suppression of the myeloid DC subset and activation of the lymphoid subset might enable immune reactions in atherosclerosis to be regulated [95]. For future studies, it would be very useful to isolate DCs resident in plaques to be able to identify a unique antigen(s) on their surface. That would possibly lead to new strategies where plaque DCs can be targeted to deliver biologically active substances to atherosclerotic lesions. The challenge is to selectively identify regulatory molecules and novel therapies in order to inhibit DC migration and function during atherogenesis without affecting normal DC function under physiological conditions.

8. Conclusion

As it is now well accepted that atherosclerosis is an immune-mediated disease, the targeting of its cellular components might open possibilities for new therapeutic strategies to attenuate the progression of the disease. DCs seem to initiate and regulate immune responses in atherosclerosis and they are also involved in controlling cholesterol homeostasis by yet unknown mechanisms. It would be important to identify the pathway(s) through which $CD11c^+$ cells may modulate the levels of plasma cholesterol. One should take into account that DCs represent a very heterogeneous population, with many subsets that have different phenotypes, functions, origin and anatomical distribution. So far, it is unclear if all DCs have equal antigen-presenting capacities, and very little is known about a preferential DC subset that is responsible for T cell-induced inflammation in the vessel wall. Moreover, there is a close relationship between DCs and macrophages, and the distinction between both cell types is even further complicated by their plasticity. Future studies are essential to determine which DC subtypes exert pro- or anti-atherogenic effects. It is crucial to understand the diversity in DC subsets to target DCs for immunomodulation therapies. Furthermore, functional differences between phenotypically similar mouse and human DC subtypes should also be studied. Nevertheless, DC-based vaccination strategies have been proven successful and animal studies provide some promising data for the treatment of atherosclerosis as well. Yet, several issues, such as the most appropriate antigen(s) for loading DCs and the optimal type of DC used for vaccination remain to be further investigated.

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