Dendritic cells in hyperlipidemia-associated atherosclerosis

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Dendritic cells in hyperlipidemia-associated atherosclerosis

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1

General Introduction

1 | General Introduction

As a consequence of the worldwide improvement of living conditions, the average life-span of elderly persons has significantly increased in the last 100 years. This change is accompanied by a dramatic increase in diseases associated with aging, including neurodegenerative disorders, cancer, infections, as well as cardiovascular diseases (CVDs). Moreover, life style changes, such as sedentary life, diet, smoking as well as stress, are risk factors contributing to the development of the above mentioned diseases. Atherosclerosis, a chronic inflammatory disease of the arteries, is the underlying pathological process of most CVDs, which are nowadays the leading cause of mortality in the Western Society.⁽¹⁾ In the last decade(s) researchers tried to unravel, which immune cell subsets/ mechanisms are crucially contributing to atherosclerosis development. The subsequent introduction will give a short overview on the immune system, how different cell subsets influence immune responses – focusing on dendritic cells- and how the immune system is involved in atherosclerosis, which serves as a guideline on the following chapters.

1. The immune system

The immune system is the body's defense against infectious organisms, build up by a network of specialized cells -called white blood cells or leukocytes- tissues, and organs that work together. The immune system is divided into the innate (non-specific) and the adaptive (also known as acquired) immune system. The innate immune system is the first line of defense against pathogens. Activation of the innate immune system relies on a set of specific pattern recognition receptors (PRRs)⁽²⁾ that recognize so-called pathogen-associated molecular patterns (PAMPs). These receptors function as a switch to trigger the innate immune system, and to regulate adaptive immune responses to microbial infections. In mammals, Toll-like receptors (TLRs) are the best characterized innate immune receptors.^(3, 4) They are expressed throughout the body and are mainly found on professional innate immune cells, including macrophages, dendritic cells (DCs) and mast cells. The activation of the innate immune system induces a rapid inflammatory response to eliminate pathogens.⁽⁵⁾ However if the innate immune system fails to remove dangerous invaders, the activation of the adaptive immune system becomes crucial. Specialized antigen-presenting cells (APCs), most importantly DCs, trigger adaptive immune responses through presentation of pathogenic

antigens to T and B cells, which subsequently leads to their activation. T and B cells have the unique ability to form receptors, which are not germline-encoded, but are *de novo* generated from rearranged gene segments. This leads to a great variability and adaptive capacity in antigen receptors, important for eliciting specific immune responses.⁽⁶⁾ Activated T cells induce cell-mediated immune responses, whereas B cells excite humoral immune responses.⁽⁷⁾

2. Dendritic cells- orchestrators of innate and adaptive immune responses

DCs constitute a heterogeneous family of cells with distinct phenotype and function. As specialized antigen-presenting cells they are central mediators of adaptive immune responses and, depending on the local environment, of the development of immunological memory and tolerance. DC development, as well as phenotype and function of the distinct subsets will be described in the following sections.

2.1. Dendritic cell development

Both innate and adaptive immunity are built on various leukocyte subsets, which are derived from hematopoietic stem cells (HSC) in the bone marrow. HSCs further differentiate into common lymphoid precursors (CLPs) and common myeloid precursors (CMPs).⁽⁸⁾ The latter gives rise to monocytes/macrophages, granulocytes, DCs, and megakaryocytes, which are all belonging to the innate immune system. CLPs give rise to T and B cells, but can also differentiate into DCs, emphasizing a key role for DCs as orchestrators of innate and adaptive immunity.⁽⁹⁾ Monocytes and DCs share a common progenitor called the monocyte dendritic cell precursor (MDP; $\text{lin}^- \text{ckit}^{\text{high}} \text{CD115}^+ \text{CX}_3\text{CR}_1^+ \text{Flt3L}^+$),^(10, 11) whereas a common DC progenitor (CDP; $\text{lin}^- \text{ckit}^{\text{low}} \text{CD115}^+ \text{Flt3L}^+$) is restricted to generate classical DCs and plasmacytoid DCs (pDCs).⁽¹²⁾ MDPs and CDPs are confined to the bone marrow (**Figure 1**).⁽⁹⁾ Macrophage colony-stimulating factor (M-CSF) and FMS-related tyrosine kinase 3 (Flt3L) are essential growth factors for the differentiation of the myeloid cell lineage, where M-CSF is critical for the development of monocytes, and Flt3L for DC lineage development.⁽¹³⁾

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adapted from G. T. Beil & S. L. Nutt, *Nature Reviews Immunology* 2012, 101-113

Figure 1. The developmental pathways of lymphoid and myeloid progenitors to lymphocytes (T and B cells), DC subsets and macrophages, and key transcription/ growth factors essentially required for DC development (indicated by vertical lines). MDP, CDP, and pre-DC development are confined to the bone marrow. Monocyte and DC development depends on the transcription factor PU.1, which regulates the expression of the cytokine receptors FLT3R, M-CSFR and GM-CSFR. CD11b⁺ cDCs require a set of transcription factors for their development, including RELB, interferon-regulatory factor 2 (IRF2), IRF4 and Ikaros, as well as M-CSF and GM-CSF. CD8α⁺ and CD103⁺ cDCs are dependent on IRF8, inhibitor of DNA binding 2 (ID2), E4 promoter-binding protein 4 (E4BP4) and basic leucine zipper transcription factor, ATF-like 3 (BATF3), as well as on FLT3 signalling. pDCs require E2-2, Ikaros and IRF8 for their development. CDP: common DC progenitor; CLP: common lymphoid progenitor; CMP: common myeloid progenitor; LMPP: lymphoid-primed multipotent progenitor; MDP: monocyte and DC progenitor; FLT3: FMS-related tyrosine kinase 3 receptor; M-CSFR: macrophage colony-stimulating factor receptor; GM-CSF: granulocyte– macrophage colony-stimulating factor receptor.

2.2. Conventional dendritic cells

Conventional dendritic cell (cDC) precursors (pre-DCs) leave the bone marrow and migrate via the blood stream to secondary lymphoid organs (lymph nodes, spleen and thymus), and peripheral tissues such as skin, lung, the intestinal tract, kidney and liver, where they convert into lymphoid resident and migratory cDCs, respectively.⁽¹⁴⁾ The phenotypic characterization of cDCs is quite complex. In human, three major CD11c⁺ circulating cDC subpopulations are described: (1) CD16⁺, (2) blood dendritic cell antigen

(BDCA)-1⁺ and (3) BDCA-3⁺ cDCs, which can be distinguished in their immune functions, including cytokine release and T cell stimulatory capacity.⁽¹⁵⁾ In mouse, cDCs can be categorized into different subsets. The (1) CD11b⁺ DCs include the CD4⁺ and CD4⁻ CD8⁻ lymphoid-tissue resident cDCs, the dermal CD11b⁺ DCs and their counterparts in lung and other tissues, the Langerhans cells, and the CD103⁺ CD11b⁺ DCs of the gut. The (2) CD11b⁻ cDC subset includes lymphoid-resident CD8 α ⁺ cDCs, as well as the CD103⁺ langerin⁺ DCs of skin, lungs and other tissues.⁽¹⁶⁾ Newly generated cDCs have an immature phenotype, characterized by the low surface expression of major histocompatibility complex (MHC) molecules I and II, and T cell co-stimulatory molecules (CD40, CD80, CD86).⁽¹⁷⁾ While monitoring the environment for the presence of bacterial and viral pathogens, cDCs maintain an immature state. Without an overt presence of stimuli, steady-state immature cDCs induce and foster peripheral tolerance.⁽¹⁸⁾ Upon inflammation, cDCs capture pathogenic antigens, but also self-antigens from damaged or apoptotic cells through PRRs, such as TLRs, C-type lectin receptors (CLRs) and CD1 receptors, which in turn induces DC maturation.⁽¹⁹⁾ This is accompanied by characteristic functional changes of DCs, such as loss of endocytic capacity, up-regulation of MHC molecules, T cell co-stimulatory molecules and the production of cytokines such as tumor necrosis factor α (TNF- α), interleukin 12 (IL-12), IL-23 and IL-10.⁽¹⁹⁾ Concomitant with their maturation process, migratory and lymphoid-resident cDCs traffic to the T cell zones in the secondary lymphoid organs to prime and stimulate naïve, memory and effector T cells, and natural killer T (NKT) cells by antigen presentation.⁽²⁰⁾ CD11b⁻ cDCs are dedicated to prime cytotoxic T cell immunity and responses to cell-associated antigens, whereas CD11b⁺ cDCs are principally involved in CD4⁺ T cell stimulation, as well as humoral immunity and responses to extracellular pathogens.⁽¹⁶⁾ DC-T cell crosstalk predominantly occurs in secondary lymphoid organs, but it is well accepted that under certain pathological conditions the maturation of cDCs can also take place in the periphery, where DCs cluster with T lymphocytes at sites of infection.⁽¹⁷⁾

2.3. Plasmacytoid dendritic cells

pDCs are an apart DC subset, first described as plasmacytoid T cells or plasmacytoid monocytes in human.⁽²¹⁻²³⁾ They represent a rare leukocyte population, originating in the bone marrow from a common DC progenitor,^{(13,}

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¹⁴⁾ and are specialized in the secretion of type I interferons (IFNs), i.e. IFN- α , IFN- β , IFN- ω , IFN- λ .^(24, 25) Human pDCs have a unique cell surface phenotype, expressing blood dendritic cell antigen 2 (BDCA-2) and BDCA-4, CD123 (IL-3R α), immunoglobulin-like transcript 7 (ILT7), CD4 and MHC class II, and being negative for lineage markers (CD3, CD19, CD14, CD16) and CD11c.⁽²⁶⁻²⁸⁾ The mouse pDC counterpart is characterized by the expression of bone marrow stromal antigen 2 (BST-2), Siglec-H, B220 and Ly6C. They can also express variable amounts of CD8 α .⁽²⁹⁾ Under healthy steady-state conditions, pDCs are present in low numbers, mainly in T cell areas of lymph nodes and spleen, mucosal-associated tissues, thymus and liver, while accumulating in lymphoid and non-lymphoid tissues under pathological conditions.⁽²⁹⁾ pDCs are characterized by their ability to release high amounts of type I IFN upon viral infection, thus inducing a strong antiviral state.^(25, 30-32) Viruses and pathogens are sensed through TLR7 and TLR9.^(24, 33) Besides the production of type I IFNs, pDCs also secrete IL-12, IL-6, TNF- α , as well as pro-inflammatory chemokines such as CXCL9, CXCL10, CCL3, CCL4 and CCL5. Thereby, pDCs can attract CD4⁺ and CD8⁺ T cells to sites of infection, inducing long-term T cell survival and memory,⁽³⁴⁾ T helper 1 (Th1) polarization, cytolytic T cell responses,⁽³⁵⁾ DC maturation⁽³⁶⁾ and differentiation of B lymphocytes into immunoglobulin-secreting plasma cells.⁽³⁷⁾ Upon maturation, pDCs undergo important phenotypic changes, most notably being the acquisition of a dendritic morphology and concurrent up-regulation of MHC and T cell co-stimulatory molecules (CD40, CD80, CD86). This enables pDCs to present antigens to CD4⁺ T cells, although less efficiently than cDCs.^(35, 38) Thus pDCs have the unique capacity to promote anti-viral innate as well as adaptive immune responses.

Figure 2. Simplified scheme representing the phenotypic characterization of immature and mature conventional and plasmacytoid dendritic cells. Uptake of epitopes, derived from invading pathogens or released from injured tissue, by certain receptors (mainly Toll-like receptors), leads to major phenotypic changes and maturation of different DC subsets. Activated pDCs are characterized by their ability to release huge amounts of type I IFNs, f.e. IFN- α , whereas cDCs mainly produce IL-12, TNF- α and IL-23. Both matured cDCs as well as pDCs can either act immunogenic (induction of Th1, Th2 or Th17 CD4⁺ T cells; activation of cytotoxic CD8⁺ T cells) or tolerogenic (induction of Treg's) upon stimulation. DAMP: danger-associated molecular patterns; HLA-DR: human leukocyte antigen DR; IFN: interferon; IL: interleukin; PAMPs: pathogen-derived molecular patterns; Th: T helper; TNF: tumor necrosis factor.

2.4. Monocyte-derived inflammatory dendritic cells

During pathogenic inflammation an additional DC subset appears in human, which is termed the 'inflammatory DC'. Inflammatory DCs are the equivalents of murine Ly6C^{high} monocyte-derived inflammatory DCs.⁽³⁹⁾ Monocytes are short-lived myeloid cells that originate from the bone marrow, and upon release they are circulating patrolling sentinels.⁽⁹⁾ In mouse and human, several monocyte subsets have been described. Mouse Ly6C^{high} CCR2⁺ CX3CR1^{low} monocytes (equivalents of human CD14^{high} CD16^{low}) are referred to as (1) 'inflammatory' monocytes as they preferentially recruit to inflamed tissues, whereas (2) Ly6C^{low} CCR2⁻ CX3CR1^{high} monocytes (equivalents of

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human CD14^{low} CD16^{high}) patrol healthy tissues through crawling on the resting endothelium, which enables them to rapidly invade tissues at sites of infection.⁽⁴⁰⁾ Under homeostatic conditions monocytes might be involved in scavenging dead cells and toxic molecules.⁽⁴¹⁾ During inflammation, circulating monocytes migrate to the inflamed tissue where they can differentiate into inflammatory macrophages or DCs.⁽⁴²⁾ Human and mouse inflammatory DCs share a similar phenotype, both expressing CD11b, CD206, CD172a, and FcεRI.⁽⁴³⁻⁴⁶⁾ Inflammatory DCs migrate to secondary lymphoid organs, where they present antigens to both CD4⁺ and CD8⁺ T cells,^(44, 45) or transfer them to resident DC subsets.⁽⁴⁷⁾ Moreover, peripheral memory T cells can get directly activated by inflammatory DCs.⁽⁴⁸⁾

2.5. T cells

As discussed above, immune responses are very much dependent on the interplay of different immune cell types, and the local context. Thus, DCs are being influenced by others, but also induce specific immune responses by interfering cell subsets, among which T cells are briefly described.

T cells differentiate from CLPs and are an essential part of the adaptive immunity. After their complete maturation in the thymus, they consist of two principal groups, based on the expression of CD4 and CD8 on their surface. Upon stimulation via APCs, mainly DCs, naïve CD4⁺ T helper (Th) cells can differentiate into various Th cell subsets, based on their cytokine profile and the immune responses they elicit. Th1 cells are characterized by an interferon- γ (IFN- γ) signature and mediate cellular immune responses, whereas Th2 cells are involved in allergic responses and asthma by secretion of IL-4, IL-5, IL-10 and IL-13, and the activation of B cells to produce antibodies.^(49, 50) Instead, Th17 cells are involved in host responses to fungi and extracellular bacteria, such as *Candida albicans* and *Staphylococcus aureus*,^(51, 52) but also in the development of certain autoimmune diseases such as inflammatory bowel disease, rheumatoid arthritis and experimental autoimmune encephalomyelitis.⁽⁵³⁾ Interestingly, IL-6, IL-23 and low levels of TGF- β were found to be required for the differentiation of IL-17/ IL-10 producing Th17 cells, whereas IL-1 β is essential for the induction of IL-17/ IFN- γ double producing Th17 cells (cells with a hybrid Th17/ Th1 phenotype).⁽⁵⁴⁾ Th1, Th2 and Th17 cells have been shown to counterbalance each other. Regulatory T cells (Treg's) are of protective nature, specialized in maintaining central and

peripheral immune tolerance by suppressing auto-reactive and pathogenic T cell responses. Natural Treg cells develop in the thymus and are important for the recognition of self-antigens, whereas induced Treg cells develop from naïve peripheral CD4⁺ T cells at foci of sustained inflammation under the induction of either IL-10 (Tr1 cells) or tumor growth factor- β (TGF- β) (Th3 cells) during ongoing inflammatory processes.⁽⁵⁵⁾ CD8⁺ T cells have the ability to kill virus-infected and tumor-cells, due to their release of perforins and granzymes. Hence, they are also called cytotoxic T cells, and recognize their targets via MHC I molecules. CD4⁺ T cells recognize antigen-MHC II complexes presented by APC.⁽⁵⁶⁾

3. Atherosclerosis

CVD is one of the leading causes of death and morbidity worldwide.^(57,58) Several systemic risk factors, such as hypertension, type II diabetes mellitus, obesity, and hypercholesterolemia,^(59, 60, 61, 62, 63) as well as physical inactivity and smoking^(64, 60) can disturb the cardiovascular system. Coronary artery disease, peripheral arterial disease, and cerebrovascular disease are the most common forms of CVD. The main underlying pathological process is atherosclerosis, a slowly progressing disorder of large and medium-sized arteries that becomes apparent when it causes thrombus formation and vessel occlusion.⁽⁶⁵⁻⁶⁷⁾ Our understanding of the pathophysiology of atherogenesis has evolved fundamentally. Atherosclerosis was first identified as a lipid-storage disease, but is now additionally recognized as a chronic inflammatory disease of the vessel wall, characterized by the infiltration of immune cells, which interact with each other and with cells of the arterial wall.⁽⁶⁸⁻⁷⁰⁾ The first signs of atherosclerosis are characterized by the formation of fatty streaks, which occur already early in life, with children showing lipid-rich lesions in the aortic arch and the abdominal aorta.^(71, 72) In the last years it has been evidenced that plaque formation occurs particularly at sites with disturbed laminar flow, such as arterial branch points.^(73, 74) This phenomenon implies endothelial cell (EC) activation, leading to an increase in endothelial permeability, and to a pathological elevation of endothelial adhesion molecules, such as vascular cell adhesion molecule-1 (VCAM-1) and intracellular adhesion molecule-1 (ICAM-1). Finally, EC dysfunction supports low-density lipoprotein (LDL) influx, and the migration of inflammatory cells into the subendothelial space.^(75, 76) LDL particles, enriched beneath the

endothelium in the intimal space, are sensitive to oxidative modifications by reactive oxygen species (ROS) or enzymes such as myeloperoxidase and lipoxygenase, released from resident vascular cells. Accumulated LDL and oxidized LDL (oxLDL) further increase the expression of chemokines and adhesion molecules on ECs, such as E- and P-selectin, thereby promoting immune cell invasion to the site of injury.^(77, 78) Fatty streak lesions, enriched in lipid-laden foam cell macrophages,^(79, 80) progress into mature atherosclerotic plaques, due to the continued recruitment of leukocyte subsets and the accumulation of extracellular lipids.⁽⁸¹⁾ Additionally, vascular smooth muscle cells (vSMC) start to proliferate and to migrate from the media into the intima in response to growth factors such as fibroblast growth factor (FGF), platelet-derived growth factor (PDGF) and TGF- β .^(82, 83) This leads to the formation of a core region that is surrounded by a cap consisting of vSMCs and a collagen-rich matrix. Advanced stable plaques are relatively quiescent in nature, although they can provoke blood vessel narrowing (stable angina). Over time, a more complex lesion develops, enriched in inflammatory cells as well as necrotic and apoptotic cells, cell debris and cholesterol crystals, leading to the formation of a necrotic core that is covered by a fibrous cap. The rupture-prone shoulder region is particularly enriched in activated macrophages, mast cells, DCs and T cells, which are sustaining ongoing inflammatory processes in the lesion due to the release of pro-inflammatory cyto- and chemokines.⁽⁸⁴⁾ The secretion of matrix-degrading proteases, such as matrix-metalloproteinases (MMPs) can cause fibrous cap thinning and plaque rupture, which exposes the thrombogenic material of the necrotic core to the blood. This leads to the formation of an atherothrombus, which can elicit myocardial infarction or ischemic stroke due to vessel occlusion.^(70, 84)

4. The contribution of the immune system to atherosclerosis

The concept that the immune system plays an indispensable role in atherosclerosis has gained widespread acceptance. Both innate and adaptive immune responses influence disease onset and progression.^(67, 85) In the next paragraphs the role of innate immune signals, the contribution of different innate immune cell subsets, as well as the adaptive immunity in atherosclerosis will be described.

4.1. Innate immune signals in atherosclerosis

It has been shown that TLRs are expressed in the non-diseased human vessel wall,⁽⁸⁶⁾ and the expression of certain TLRs is increased in mouse and human atherosclerotic plaques, mainly recognized on macrophages, ECs and vSMCs.⁽⁸⁷⁻⁹⁰⁾ Studies, using mouse models for atherosclerosis, in which specific TLRs and their downstream signaling intermediates were genetically deleted, demonstrated a significant pro-atherogenic contribution for TLR2 and TLR4.⁽⁹¹⁻⁹⁴⁾ Apart from pathogenic agents, TLRs are able to recognize endogenous ligands, present at sites of inflammation. These ligands are products of tissue damage, so-called danger-associated molecular patterns, which alert the innate immune system of tissue injury in the absence of infections.⁽⁹⁵⁾ During the last years, considerable evidence has supported the involvement of endogenous ligands, such as heat shock proteins (HSP), high-mobility group box 1 (HMGB-1), and RNA/ DNA immune complexes, which are released from necrotic and dying cells, as well as degradation products of extracellular matrix macromolecules, such as fibronectin and hyaluronan, to be involved in innate immune activation in atherogenesis. Additionally, cholesterol crystals and (modified) LDL particles were shown to activate immune cells, thereby driving atherogenesis.⁽⁹⁶⁻¹⁰²⁾

4.2. Cells of the innate immune system in atherosclerosis

Over the past two decades it has been evidenced that immune cells are critically involved in the pathogenesis of atherosclerosis. The continued recruitment and accumulation of various leukocyte subsets within the plaque is associated with lesion progression and plaque rupture.

Monocytes and macrophages were the first inflammatory cells to be associated with atherosclerosis, and are the predominant cell type within the plaque.⁽⁸⁰⁾ Blood monocyte depletion in rabbits was shown to significantly reduce lesion size, thus emphasizing their importance in atherosclerosis.⁽¹⁰³⁾ During atherogenesis, Ly6C^{high} monocytes are continuously recruited to the plaque, and monocyte accumulation was found to correlate with lesion size.⁽¹⁰⁴⁾ In hyperlipidemic mice, Ly6C^{high} monocytes make use of the chemokine receptors CCR1, CCR2 and CCR8 to enter the plaque and to differentiate into macrophages, whereas Ly6C^{low} monocytes employ CCR5 and CX₃CR₁.⁽¹⁰⁵⁾ Combined genetic deletion of CCR2, CCR5 or CX₃CR₁ almost abolished atherosclerosis in apolipoprotein E deficient (*ApoE*^{-/-}) mice, stressing the

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importance of chemokine receptors in monocyte recruitment to the atherosclerotic plaque.⁽¹⁰⁶⁾ Monocyte-derived macrophages play a pivotal role during all stages of atherosclerosis. Advanced plaques contain various macrophage subsets, including M1, M2, M4 and Mox macrophages. However the phenotype of lesional macrophage subtypes is incompletely understood.⁽¹⁰⁷⁾ Lipoprotein uptake by macrophages is mediated via certain scavenger receptors (CD36, CD68, SR-A, MARCO, SR-B and LOX-1) and leads to macrophage activation and the formation of lipid-laden foam cells.⁽¹⁰⁸⁾ Macrophages metabolize and eliminate lipoproteins through their ATP-binding cassette transporters (ABCs), such as ABCA1 and ABCG1. An imbalance in lipoprotein uptake and efflux capacity (defective efferocytosis) leads to macrophage apoptosis, a process that contributes to necrotic core formation and subsequently to plaque progression and destabilization. Additionally, release of pro-inflammatory cytokines and matrix-degrading enzymes by macrophages further provoke fibrous cap thinning.⁽¹⁰⁹⁾

In the recent years it has been shown that resident vascular DCs are present within the healthy aorta and may play a role in the maintenance of vascular homeostasis.⁽¹¹⁰⁾ Local inflammatory processes in the vascular wall promote the recruitment of further DCs to the aorta. Mature DCs are described to be located in inflammatory spots in the plaque-shoulder area in close proximity with activated T cells.⁽¹¹¹⁾ However, there is still limited information about DC (subset) phenotype and functionality within the atherosclerotic plaque. The current knowledge of the various DC subsets, and their interplay with certain immune cell subsets will be described in-depth in the **following chapters** of my thesis.

Next to DCs, neutrophils and their mediators have been identified in mouse and human early lesions as well as in rupture-prone atherosclerotic plaques within regions of high inflammatory activity.^(112, 113) Neutrophils are classically described as short-lived phagocytes characterized by the release of vast amounts of soluble anti-microbials, including azurocidin, LL-37, myeloperoxidase (MPO), MMPs, and cathepsins, as well as neutrophil extracellular traps (NETs = fibers composed of chromatin and serine proteases), important during bacterial infections.⁽¹¹⁴⁾ It has been shown that hyperlipidemia induces neutrophilia by stimulating the egress from the bone

marrow, and circulating neutrophil numbers correlate with plaque size and cardiovascular events.⁽¹¹²⁾ A recent study demonstrated that disruption of the CXCR4-CXCL12 axis caused neutrophilia and aggravated atherosclerosis, whereas neutrophil depletion markedly diminished early lesion size,⁽¹¹⁵⁾ thus pointing towards a functional role for neutrophils in murine atherosclerosis. Several recent studies suggested an important role for neutrophils in the onset of atherosclerosis by triggering monocyte and T cell recruitment to the lesion.⁽¹¹⁶⁾ Besides, experimental data encourage a contributory role of neutrophils to plaque destabilization by release of MPOs, and MMP-2 and MMP-9.^(117, 118)

Another important cell type of the innate immune system is the mast cell. Mast cells are large granular cells, best known for their functions in allergy and anaphylaxis. Recent studies confirmed the presence of mast cells in coronary and carotid artery plaques at sites of plaque erosion, rupture or hemorrhage, and in mouse mast cell presence was associated with plaque progression and lipid accumulation, as they provoke high-density lipoprotein (HDL) degradation and impair cholesterol efflux.^(119, 120) In addition, mast cells have been shown to promote murine plaque instability and rupture through release of pro-inflammatory cytokines (IL-6, IFN- γ) and serine proteases, such as chymase and tryptase.^(121, 122)

4.3. Adaptive immunity in atherosclerosis

In the last years it has been evidenced that the adaptive immune system is deeply involved in chronic inflammatory processes that drive the development of atherosclerosis.⁽¹²³⁾ Initial studies have ascribed a pro-atherogenic role for T cells in atherosclerosis.⁽¹²⁴⁾ Remarkable numbers of T cells, mainly CD4⁺ Th cells, were characterized in all lesion stages in mouse and human, and were correlated with disease severity.⁽¹²⁴⁾ CD4⁺ T cells are attracted to the atherosclerotic plaque through DC-derived chemokines via CCR7, such as CCL19 and CCL21, and they are co-localized with mature DCs within the plaque.⁽¹²⁵⁾ It is believed that T cell activation is elicited in the lesion in response to LDL/oxLDL and endogenous ligands in a MHC-dependent manner.^(126, 127) The importance of antigen presentation through MHC molecules was underlined by results of a study demonstrating that deficiency in the MHC class II invariant chain (CD74) significantly attenuated T cell

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activation and plaque development in low-density lipoprotein receptor deficient (*Ldlr*^{-/-}) mice.⁽¹²⁸⁾ Besides, the interplay between APCs and T cells in the atherosclerotic plaque has been evidenced by several studies, in which co-stimulatory receptor-ligand pairs, known to be indispensable for T cell activation, were interfered. For instance, inhibition of the CD40-CD40L signaling pathway, a co-stimulatory dyad that promotes inflammatory processes through T cell stimulation, led to reduced lesion development and a more stable plaque phenotype.⁽¹²⁹⁻¹³¹⁾

During early lesion formation Th1 cells predominate over other Th cell subsets and IFN- γ release by Th1 cells can exert potent pro-atherogenic actions by activating macrophages and DCs. It has been shown that deficiency in IFN- γ or its receptor significantly reduced lesion size in *ApoE*^{-/-} mice, and led to a more stable plaque phenotype.⁽¹³²⁾ The contribution of Th1 cells to lesion development and plaque destabilization was further supported by studies in which the T-box transcription factor TBX21 (T-bet), a key transcription factor for Th1 differentiation, was genetically deleted in *Ldlr*^{-/-} mice. TBX21-deficiency reduced the atherosclerotic plaque burden, and caused a switch towards Th2 cells.⁽¹³³⁾ In summary, these data corroborate the pro-atherogenic nature of Th1 responses.

In the context of atherosclerosis, Th2 cells have been denoted to function anti-atherogenic, and they are regarded as counterplayers of Th1 cells.⁽¹³⁴⁾ In the mouse plaque severe and persistent hyperlipidemic conditions seem to shift the balance from a Th1 towards a Th2 cell type, accompanied by a loss of IFN- γ producing Th1 cells and the formation of anti-oxLDL IgG1 autoantibodies, further supporting the anti-atherogenic effect of Th2 cells. However, the role of Th2 cells in atherosclerosis remains controversial, and seems to depend on the stage of lesion, the site of disease, and the experimental animal model used. For instance, the development of atherosclerosis in IL-4 deficient *Ldlr*^{-/-} mice was reduced, specifically in the aortic arch and the thoracic aorta, whereas results of another study did not show effects of IL-4 deficiency on lesion development.^(135, 136) In contrast, IL-10 deficiency in *ApoE*^{-/-} mice resulted in more advanced plaques that were prone to rupture and enriched in T cells and IFN- γ .⁽¹³⁷⁾ Nevertheless, IL-10 deficiency can also compromise Treg as well as tolerogenic Th17 cells.

A functional role for Th17 cells in atherosclerosis remains subject for discussion, and results are divergent. Hematopoietic deficiency in the IL-17

receptor in *Ldlr*^{-/-} mice resulted in derogated atherosclerotic plaques.⁽¹³⁸⁾ In line, blockade of the IL-17 signaling in *ApoE*^{-/-} mice by administration of IL-17 neutralizing antibodies was shown to decrease atherosclerosis development.⁽¹³⁹⁾ In addition, IL-17 mRNA expression in human carotid plaques was correlated with complicated, unstable, and lipid-rich lesions.⁽¹⁴⁰⁾ In contrast, elevated levels of IL-17 in *Ldlr*^{-/-} mice were seen to decrease the expression of VCAM-1 on ECs, thereby reducing vascular inflammation, and attenuating atherosclerotic lesion size.⁽¹⁴¹⁾ To summarize, defining a functional role for Th17 cells in chronic inflammatory diseases, such as atherosclerosis, needs further examinations.

Several studies have shown anti-atherogenic activities of Treg cells, either by direct effects on T cells or through effects on APCs.⁽¹⁴²⁾ For instance, loss of natural Treg cells by use of CD25 neutralizing antibodies deteriorated atherosclerosis.^(143, 144) A recent study identified DC-derived CCL17 as a central regulator of Treg homeostasis in atherosclerosis. Deficiency or antibody blockade of CCL17 in hyperlipidemic mice reduced athero-progression by expanding Treg cells, whereas CCL17 expression in DCs restricted Treg cell maintenance to promote atherosclerosis.⁽¹⁴⁵⁾

B cells, the second cell type of the adaptive immune system, are implicated in atherosclerosis as well. Their main function is to produce antibodies of various isotypes. Besides, they can also function as APCs. In recent years, the contribution of B cells to atherosclerosis has been confirmed. Several studies suggested a protective role for B cells, mainly through production of IgG and IgM anti-oxLDL autoantibodies.^(146, 147) B cells have been detected in mouse and human atherosclerotic plaques, preferentially localized in the adventitia.^(148, 149) Chimeric *Ldlr*^{-/-} mice, deficient in B cells, revealed aggravated atherosclerotic plaques,^(150, 151) supporting a protective role for B cells in atherosclerosis. However, a more recent study argued B cell deficiency to be athero-protective.⁽¹⁵²⁾ B cell depletion by use of a monoclonal anti-CD20 antibody in *Ldlr*^{-/-} and *ApoE*^{-/-} mice resulted in significantly reduced atherosclerosis, and was accompanied by reduced DC and CD4⁺ T cell activation.

Hypothesis and study aims of this thesis

Noticeable progress has been made in the recent years to understand the contribution of innate and adaptive immune responses to the onset and progression of atherosclerosis, as can be appreciated from the above sections. Specialized immune cells are involved in the pathophysiology of hyperlipidemia-associated atherosclerosis. In recent years different DC subsets were shown to be present in the healthy vasculature and the heart. In this regard, a possible role for DCs as contributors to inflammatory processes in atherosclerosis is gaining more and more attention, as they are important players in bridging innate and adaptive immune responses. The functionality of certain DC subsets under hyperlipidemic conditions and in the atherosclerotic plaque remains poorly understood, and studies addressing these issues are rare.

Here, we hypothesize that different DC subsets are crucially involved in the pathophysiology of atherosclerosis by triggering immune responses.

Therefore, one of the processing goals of this thesis was to explore their participation, with special emphasis on pDCs, in inflammatory processes in hyperlipidemia-associated atherosclerosis.

In particular I will address the following research questions:

- Do pDCs influence plaque development and progression?
- Does a cholesterol-rich environment influence homeostasis and activation status of different peripheral (p)DC subsets?
- Do pDCs have a specific gene signature in the human atherosclerotic plaque?

In **Chapter 2**, a potential role for various DC subsets in certain CVDs, with a focus on the current knowledge in atherosclerosis, and their prospective use as therapeutic target in CVDs is reviewed. In **Chapter 3**, we studied effects of pDC depletion on atherosclerosis onset and progression in *Ldlr*^{-/-} mice by use of a specific depletion antibody (120G8). We were the first to identify an athero-protective role for pDCs by tuning peripheral T cell proliferation and activity. In **Chapter 4**, we made use of a second conditional pDC ablation model - the BDCA-2 diphtheria toxin receptor (DTR) model- to study pDC

influence on atherosclerosis. A subgroup of mice additionally received a CD4⁺ T cell depleting antibody to address the contribution of CD4⁺ T cells to pDC immunity in atherosclerosis. In **Chapter 5** we hypothesized that a dyslipidemic microenvironment inhibits TLR9-induced activation of pDCs, skewing T cell-mediated immunity. Therefore, we investigated how lipoprotein exposure affects human pDC maturation, activation and T cell stimulating capability. In addition, we investigated in **Chapter 6**, whether a hyperlipidemic environment is influencing DC homeostasis, their maturation and T cell priming ability in peripheral organs. In **Chapter 7** we examined the impact of human plaque pDCs on its local context. Besides comparing the gene expression patterns between pDC-enriched and pDC-deficient regions, we employed co-expression cluster analyses to pinpoint specific pDC-associated gene modules that correlate with the presence of T cells and macrophages in the pDC-enriched plaque regions. **Chapter 8** affords a discussion of the most relevant results of this thesis and debates future perspectives.

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Cardiovascular diseases (CVD) are the number one cause of death in the Western World. It has been so for decades, notwithstanding a wide array of – mostly preventive - treatment modalities targeting known risk factors, such as hyperlipidemia, type 2 diabetes mellitus, hypertension or obesity. Recent technical and conceptual advances have unveiled important contributions of the immune system in the pathophysiology of CVD. The pronounced presence of several subsets of dendritic cells (DC), central orchestrators of both innate and adaptive immune responses, in vasculature, heart and brain during disease onset as well as progression already alludes to such a role. In support, recent studies point to their likely involvement in immune responses in atherogenesis, as well as after myocardial infarction and ischemic stroke. DCs might thus represent a real opportunity to treat CVD not by a preventive strategy, but by interfering with the pathophysiological processes themselves: they hold promising potential as therapeutic tools in cardiac transplantation or as part of a future atherosclerosis vaccination strategy. In recent years, several research groups have addressed different aspects of DC biology in CVDs. Nevertheless, the full scope of DC-driven effects in CVD is still unclear. Here, we present an overview of the current knowledge on DC's impact on CVDs. We shall discuss pitfalls and shortcomings, and define questions required to enable a thorough appreciation of the validity and potential of DCs as therapeutic targets in CVDs.

Contribution to the manuscript:

This review was written and adapted by Anette Christ. Dr. Temmerman, Prof. Daemen and Prof. Biessen contributed essentially to the final version by critically revising the written draft of Anette Christ. Bart Legein was involved in making the figure.

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2

Dendritic cells in cardiovascular diseases: epiphenomenon, contributor or therapeutic opportunity?

- manuscript in revision in Circulation -

1. Introduction

Cardiovascular diseases (CVDs) are one of the leading causes of mortality worldwide.⁽¹⁾ A growing body of evidence points to a pivotal role of the immune system in the pathophysiology of a variety of CVDs such as atherosclerosis, ischemic stroke, chronic heart failure, and other myocardial conditions like myocardial ischemia and reperfusion, viral myocarditis and cardiac transplantation.⁽²⁻⁴⁾ In many of these disorders so-called danger-associated molecular patterns (DAMP), released from necrotic tissue and dying cells, can lead to the activation of certain immune cell populations such as monocytes/ macrophages, granulocytes and T cells, thus aggravating ongoing inflammatory processes at the lesion site. Dendritic cells (DCs) are key modulators of immunity, pivotal in directing innate and adaptive immune responses against microbial, but also modified self-antigens present at sites of injury.⁽⁵⁾ Given the tissue trauma underlying various CVDs, it is not surprising that recent observations have allocated a regulatory role for DCs in CVD-associated immune responses. Interestingly, non-diseased arteries of young individuals were seen to host a network of resident vascular DC⁽⁶⁾, a finding that was confirmed for the atherosclerotic-prone lesser curvature and sinus of aorta in mouse models of atherosclerosis.⁽⁷⁾ DCs, exhibiting an immature phenotype with low expression of co-stimulatory molecules, are present in the subendothelial space with occasional probing into the vascular lumen. DCs have also been observed in human heart and in cardiac valves of healthy C57BL/6 mice.⁽⁸⁾ It is assumed that these immature, resident DCs contribute to the maintenance of vascular homeostasis and tolerance by scanning their microenvironment for self and non-self antigens. Indeed, Choi *et al.* were able to show that resident DCs, isolated from the aorta and the valves of wild-type mice, have the capacity to present antigens to CD8⁺ T cells *in vitro* and *in vivo*, indicating that they are fully functional in eliciting a T cell response.⁽⁹⁾ In diseased vessels, heart and brain of human CVD patients, but also in the circulation, DC subset numbers were reported to be modified, associating DCs with CVD onset and progression.⁽¹⁰⁻¹²⁾ This notion is substantiated by a wealth of experimental animal studies addressing the involvement of DCs in CVDs. However, it remains mainly unsettled whether actions of different DC subsets are either detrimental or beneficial for lesion formation. Then again, DCs might function both-way, dependent on the lesion stage. This review thus aims to

provide an in depth overview of the role of DC subsets in several cardiovascular conditions in human and experimental animal models to reveal underlying patterns, expose lacunae in our current understanding of DC's function in CVDs, and explore possible therapeutic opportunities, exerted by modified DCs.

2. Dendritic cells: conductors of innate and adaptive immune responses

DC are professional antigen-presenting cells (APC) that originate from hematopoietic precursors in the bone marrow and are distributed throughout the whole body. DCs have the unique ability to induce T cell responses by capturing, processing and presenting antigens to naïve T cells. As such, they are central mediators of adaptive immune responses, and, depending on subset and activation status, of the development of immunological memory and tolerance.⁽⁵⁾ Since the discovery by Steinmann and Cohn,⁽¹³⁾ DCs were seen to represent a heterogeneous family of cells, differing in terms of development, migratory cues, compartmentalization, phenotype and immunological functions. DCs are categorized into conventional DCs(cDCs) and non-conventional DCs, the latter comprising plasmacytoid DCs (pDCs) and monocyte-derived inflammatory DCs,⁽⁵⁾ characterized by the expression of a panel of specific surface markers. For further details we refer the reader to a variety of excellent reviews on DC development, phenotyping and/or DC functions.^(14, 15)

3. Circulating DC subsets in patients with coronary and peripheral arterial disease

As an indirect measure of DC's association with CVDs, DC (precursor) numbers and functionality have been evaluated in blood of patients with CVD, such as coronary and peripheral artery disease.^(16, 17) In 2006, van Vre *et al.* were the first to describe a marked decrease in circulating DC precursors (circulating cDC and pDC) in patients with coronary artery disease (CAD), defined by angiography as > 50% stenosis in one or more coronary arteries.⁽¹⁸⁾ Until now, several studies confirmed a significant decrease in blood DCs (cDC and pDC) in CAD patients, irrespective of CAD grade (stable vs unstable angina pectoris, AMI), number of diseased vessels, or subset markers used for DC

enumeration.^(16, 19-25) In sharp contrast, Shi *et al.* reported increased circulating cDC and unaltered pDC numbers in patients with stable CAD.⁽²⁶⁾ By investigating the distribution of circulating DCs in patients with different stages of peripheral arterial disease, including patients with intermittent claudication and critical limb ischemia, Dopheide and coworkers showed that blood cDC numbers were increased, whereas pDC numbers were reduced in peripheral arterial disease patients compared to healthy controls.⁽¹⁷⁾ Of note, both cDCs and pDCs from critical limb ischemia patients revealed an immature phenotype, suggesting that severe ischemia and/or prolonged inflammation in this ailment might lead to an attenuation in the pro-inflammatory membrane patterns of circulating DC subsets.⁽¹⁷⁾

Generally, most patient studies show declined blood DC numbers in CAD patients. Inconsistent results may be explained by differences in the extent and localization of disease, the timing of blood sampling (before/ after a surgical intervention; lesion onset versus progression), the prevalence of risk factors across the patients included in these studies, and the cohort sizes consulted (**Table 1**).

Nevertheless, the actual cause for reduced circulating DC numbers remains unaddressed. One possibility for the decrease in circulating DCs might be their enhanced recruitment to the disease site (hence plaque or the ischemic heart).⁽¹⁰⁾ Alterations in circulating DCs have been described in other autoimmune diseases as well, such as systemic lupus erythematosus (SLE),⁽²⁷⁾ where markedly lowered blood DC numbers correlated with an accumulation of activated DC in the inflamed tissue. In analogy, DCs could well be recruited to secondary lymphoid organs to prime naïve T cells.⁽²⁸⁾ Circulating oxidized low-density lipoprotein (oxLDL) or circulating immunocomplexes,⁽²⁹⁾ but also ischemic tissue derived DAMPs were seen to induce DC activation,⁽³⁰⁾ thus promoting their extravasation to spleen or lymph nodes. Actually, several studies have shown in CAD patients a more mature phenotype on a minor subset of circulating CD11c^{high} (BDCA-1⁺) cDCs, BDCA-2⁺ pDCs, as well as on monocyte-derived DCs, represented by the up-regulation of CD83, CD80, CD86 and/ or CCR7.^(31, 32) Second, the apparent blood DC depletion could however also be explained by increased DC turnover, due to increased circulating cholesterol levels. Indeed, Alderman *et al.* have demonstrated *in vitro* that high concentrations of oxLDL provoke DC apoptosis.⁽³³⁾ Otherwise, declines in DC numbers could be a temporary response to acute ischemia. Third, reduced

blood DC numbers might also result from decreased release from bone marrow. Interestingly, van Brussel *et al.* have shown diminished plasma levels of FMS-like tyrosine kinase 3 ligand (Flt3L) in CAD patients, a growth factor that is responsible for DC differentiation and release from bone marrow.⁽²²⁾ Fourth, the altered blood DC abundance, phenotype and function could be owing to CAD patient's medication, including aspirin, statins, beta-blockers and angiotensin-converting enzyme (ACE-) inhibitors. While supported by several *in vitro* studies,⁽³⁴⁻³⁷⁾ the validity of this notion needs further investigation.

Recapitulating, circulating DC decline cannot be exclusively linked to the compartmentalization of this subset, be it to atherosclerotic plaque or lymphoid organs, as other covariates may as well modify blood DC numbers and functionality. This needs to be further addressed in future studies. Moreover, these observational studies leave unaddressed whether DCs are active contributors or just casual bystanders.

2 | Dendritic cells in cardiovascular diseases

Cohort size	Total DCs	cDCs	pDCs	Reference
CORONARY ARTERY DISEASE				
CTR: 19 SAP: 20 UAP: 19 AMI: 17		↓ All CAD groups	=	Yilmaz ¹⁹
CTR: 18 CAD: 18		↓ (BDCA-1 ⁺ mDCs)	↓ (BDCA-2 ⁺ pDCs)	Van Vré ¹⁸
CTR: 11 Stable CAD: 21	↑	↑ (CD11c ⁺ cDCs)	= (CD123 ⁺ pDCs)	Shi ²⁶
CAD excluded: 57 Early CAD: 63 Moderate CAD: 85 Advanced CAD: 85	↓ correlates with severity of CAD	↓ correlates with severity of CAD	↓ correlates with severity of CAD	Yilmaz ²⁰
CTR: 15 SAP (1 vessel): 15 SAP (3 vessels): 15 UAP (1 vessel): 16	↓ All CAD groups	↓ (BDCA-1 ⁺ mDCs) All CAD groups	↓ (BDCA-2 ⁺ pDCs) All CAD groups	Van Vré ¹⁶
CTR: 10 SAP: 10	↓ (irrespective of marker used)	↓ (BDCA-1 ⁺ or CD11chi mDCs)	↓ (BDCA-2 ⁺ or CD123+ pDCs)	Van Brussel ²¹
CTR: 12 CAD: 15		↓ (CD11c ⁺ cDCs) less CD86 ⁺ , CCR7 ⁺ cDCs no functional differences	↓ (CD123 ⁺ pDCs) Less functional	Van Brussel ²²

Cohort size	Total DCs	cDCs	pDCs	Reference
CTR: 29 SAP: 30 UAP: 56 AMI: 50		↓ UAP and AMI	=	Wen ²³
PERIPHERAL ARTERY DISEASE				
CTR: 30 IC:30 CLI:30		↑ lower HLA-DR, CD86, CD40 in CLI compared to IC	↓ lower HLA-DR, CD86, CD40 in CLI compared to IC	Dopheide ¹⁷
ACUTE MYOCARDIAL INFARCTION				
CTR: 19 SAP: 19 AMI: 26		↓ More activated in AMI	↓ Less functional	Fukui ²⁵
CTR: 45 NSTEMI: 44 STEMI: 34	↓ ↓↓ in STEMI	↓ ↓↓ in STEMI	↓ ↓↓ in STEMI	Kretzschmar ²⁴
ACUTE STROKE				
CTR: 29 ACI-S: 46 TIA: 39 AIS: 73 AHS: 31	↓ (AIS and AHS) correlates with stroke severity	↓ (AIS and AHS) correlates with stroke severity	↓ (AIS and AHS) correlates with stroke severity	Yilmaz ¹¹
TYPE 2 DIABETES				
CTR: 44 (no atherosclerotic complications) EXP: 11 (atherosclerotic	↓	↓ (CD33 ⁺) less functional	↓ (CD33 ⁺) less functional	Corrales ⁶⁴

2 | Dendritic cells in cardiovascular diseases

Cohort size	Total DCs	cDCs	pDCs	Reference
complications)				
CTR: 15 UAP: 18 T2D: 18 T2D + UAP: 21	↓ (in T2D + UAP)	↓ (in T2D + UAP) But more mature/active	= (in T2D + UAP)	Yao ⁶³
CTR: 21 T2D: 21		=	↓ (subgroup "poor glycemic control" within T2D) Less functional (all T2D)	Blank ⁶⁷
HEART FAILURE				
CTR: 14 HF: 16 (pre-transplant)	↑	↑ More CD83 ⁺ CCR7 ⁺	=	Athanassopoulos ⁷³
CTR: 18 NYHA Class II: 12 NYHA Class III-IV: 28	↑ Increase in end-stage (III-IV)	↑↑ More CD83 ⁺ CCR7 ⁺	↑ No difference in CD83/CCR7	Athanassopoulos ⁷⁴
CTR: 21 NYHA Class III-IV: 27		↓ (During acute decompensated phase) but more activated	↓ (During acute decompensated phase) but more activated	Sugi ¹²

Table 1. Circulating DC populations in human CVDs. Overview of the most important studies describing DC numbers in blood of patients suffering from various cardiovascular conditions. Publications are grouped per condition, and results on total DC, cDC and pDC numbers are summarized. In addition, information about the build-up of the cohort size is given as well as the article citation. CAD: coronary artery disease, PAD: peripheral artery disease, T2D: type 2 diabetes, HF: heart failure, CTR: control, EXP: experimental group, SAP: stable angina pectoris, UAP: unstable angina pectoris, AMI: acute myocardial infarction, mDC: myeloid DCs, STEMI: ST-segment elevation myocardial infarction, NSTEMI: non-STEMI, IC: intermittent claudication, CLI:

critical limb ischemia, ACI-S: asymptomatic cerebral infarction stenosis, TIA: transient ischemic attack, AIS: acute ischemic stroke, AHS: acute haemorrhagic stroke, NYHA: New York Heart Association (classification for heart failure).

4. DC involvement in vascular inflammatory processes

Beside their presence in atherosclerosis, DC attendance has been described in other chronic inflammatory vasculopathies, such as giant cell arteritis, Takayasu's arteritis and Kawasaki disease.⁽³⁸⁻⁴⁰⁾ It is hypothesized that they contribute to the first critical steps in disease pathogenesis through breakdown of vascular tolerance. In these vasculopathies resident DCs are located in the adventitia and adventitia-media border, and (c)DC numbers are seen to increase with disease progression.⁽⁴⁰⁾ Dense infiltrates of mature cDCs and T cells have been described at later stages as well, reflective of DC-initiated, antigen-specific immune responses. Considering that DC networks are present in healthy arteries and that they function as professional APCs, they might well be involved in disease onset and progression through presentation of modified (self-) antigens to T cells. The actual triggers to activate vascular DCs are yet unknown, as is their relative contribution to immune priming in comparison to other vascular resident APCs such as macrophages. Altogether, a functional role for DCs in the pathogenesis of these diverse inflammatory vasculopathies remains to be established, as studies are of rather descriptive nature.

A functional role for dendritic cells in atherosclerosis

In human atherosclerotic plaques, fully mature DCs (CD83⁺ CD86⁺) accumulate within the rupture-prone atherosclerotic plaque shoulder where they produce T cell chemotactic (CCL19 and CCL21), and pro-inflammatory cytokines (interleukin-12 (IL-12), IL-23 and tumor necrosis factor α (TNF- α)).⁽⁴¹⁾ Mapping of plaque-residing DCs revealed a close contact between DCs and activated T and NKT cells, suggesting that DCs tune or even orchestrate immune responses relevant to atherosclerosis.⁽⁴¹⁾ Of note, many of the histology studies are thwarted by the moderate/poor specificity of most DC markers, rendering DC's immunohistochemical detection a delicate issue. The additional use of other techniques, such as flow cytometric cell isolation, could

help here to better appreciate DC presence and phenotype in different stages of atherosclerosis.

Mouse models have been very insightful in elucidating DC functions in atherosclerosis. For instance, lipid accumulation in the initial stages of atherosclerosis was recently shown to be directed and regulated by intimal CD11c⁺ DCs, residing in the atherosclerotic-prone lesser curvature of the aortic arch.⁽⁴²⁾ The impact of lipid uptake by resident vascular DCs on their functionality remains a controversial subject. Dyslipidemia was seen to lead to a retention of DCs and macrophages in the atherosclerotic plaque.^(43, 44) However, as reported in several studies antigen-presentation and T cell priming ability by DC remains unaffected under hyperlipidemic conditions, even after excessive lipid-loading.^(45, 46) In a transgenic mouse model with cDC specific overexpression of the anti-apoptotic gene *hBcl-2*, the induced enhanced lifespan and immunogenicity of circulating cDCs was associated with enhanced T cell activation, elevated levels of T helper 1 (Th1) and Th17 cytokines and increased production of Th1-driven IgG2c antibodies under hyperlipidemic conditions.⁽⁴⁵⁾ This major functional DC expansion did not aggravate lesion formation, as it was compensated for by decreased plasma cholesterol levels. Further support for a link between DC function and lipid metabolism was derived from the augmented plasma cholesterol levels after cDC depletion in hyperlipidemic *ApoE*^{-/-} mice crossed to CD11c diphtheria toxin receptor (CD11c-DTR) transgenic mice.⁽⁴⁵⁾ Precise mechanisms that underlie cDC involvement in cholesterol homeostasis have only scarcely been explored. Cholesterol lowering by DCs is suggested to occur in the periphery where DCs are amongst others exposed to lipoproteins. The behavior of circulating cDCs in patients with familial hypercholesterolemia has not yet been analyzed. Such data will allow to elucidate whether DCs are involved in lipoprotein clearance and how this is influencing DC functionality. Several lines of evidence suggest that oxLDL, present in the plaque, can impact DC maturation already in the initial stages of atherosclerosis. *In vitro* exposure to oxLDL during differentiation of human monocytes resulted in phenotypically mature DCs with upregulated HLA-DR, CD40 and CD86 and induced capacity to T cell activation.^(33, 47) However, incubation with high concentrations of oxLDL attenuated DC function and induced apoptosis.⁽³³⁾ A strong inhibition of oxLDL on expression of CCR7 and its ligand CCL21 by monocyte-derived DCs was recently reported by Nickel *et al.*, which likely will impact their migratory

capacity in plaque.⁽⁴⁸⁾ As shown by Kopf and coworkers, dyslipidemia also affects CD8 α^+ cDC function, impairing their response to Toll-like receptor (TLR) stimulation *in vitro* and *in vivo* during *Leishmania major* infection. OxLDL appeared to be directly responsible for this effect, as it uncoupled TLR-mediated signaling in DCs, leading to decreased DC activation and dampened Th1 responses.⁽⁴⁹⁾ Similarly, Bluemel *et al.* have shown that oxidized phospholipids impair DC maturation by blocking TLR3 and TLR4 mediated up-regulation of co-stimulatory molecules and induction of pro-inflammatory cytokines in human DCs.⁽⁵⁰⁾ These data are in line with previous studies, showing an anti-inflammatory effect of oxLDL in LPS-stimulated macrophages.^(51, 52)

Taken together, considerable controversy exists on DC function(s) in hyperlipidemia-associated atherosclerosis. The still ongoing characterization of classical DC subsets within the vasculature^(30, 53) makes it even more complex to comprehend DC (subset) contribution to plaque burden. It will be of importance to uncover how early and more advanced stages of hyperlipidemia impair DC (precursor) homeostasis, including their development in the bone marrow, DC mobilization into the circulation, peripheral phenotype, and migratory routes. In addition, extensive knowledge about DC actions within a lipid-rich environment such as the atherosclerotic plaque is lacking. How lipid uptake and prolonged intracellular storage interfere with signaling pathways responsible for DC activation is still controversial and will require further study.

A functional role for plasmacytoid dendritic cells in atherosclerosis

The group of Weyand and coworkers has recently shown the presence of CD123 $^+$ pDCs in human carotid atherosclerotic plaques, mainly located in the shoulder region that was also enriched in interferon- α (IFN- α) positive cells, thus associating pDC presence with IFN- α production.⁽⁵⁴⁾ Furthermore, pDC numbers were significantly increased in unstable compared to stable human lesions. *In vitro* CpG-induced IFN- α release by pDCs induced a 10-fold up-regulation of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) expression on CD4 $^+$ T cells, thus promoting apoptosis of vascular smooth muscle cells (vSMC) and endothelial cells (ECs), processes that tremendously contribute to plaque destabilization.⁽⁵⁴⁾ However, these data leave unaddressed whether pDCs are functional in the atherosclerotic plaque

in vivo. We and others have recently shown that CD123 displays only moderate specificity for human pDCs, being co-localized also with CD68⁺ macrophages and with ASMA⁺ vSMC.^(10, 55) Use of the human pDC specific marker blood dendritic cell antigen 4 (BDCA-4) by our group revealed the scanty and equivalent presence of pDCs in human stable and unstable plaques. Moreover, we demonstrated that expression of IFN- α in human unstable versus stable carotid endarterectomy tissue specimens did not differ, suggesting that in chronic low grade inflammatory processes, such as atherosclerosis, pDC activation may not be a prominent feature.⁽⁵⁵⁾

Our group has recently shown that selective depletion of pDCs by 120G8 monoclonal antibody administration in *Ldlr*^{-/-} mice fed a high fat diet (HFD) exacerbated lesion size in the carotid artery and the aortic roots, and promoted plaque T cell accumulation as well as peripheral CD4⁺ T cell activation. pDCs isolated from atherosclerotic mice suppressed CD4⁺ T cell proliferation in an indoleamine-2,3-dioxygenase (IDO)-dependent manner, pleading for an athero-protective role for pDCs in atherosclerosis.⁽⁵⁵⁾ In contrast to our study, Doering *et al.* and MacRitchie *et al.* reported significantly decreased diet-induced lesion formation in the aortic root and the aorta of pDC-depleted *ApoE*^{-/-} mice, while plaques showed a more stable phenotype. Both groups investigated the impact of pDC depletion by use of the PDCA-1 depletion antibody on early lesion development (4 weeks of HFD feeding).^(29, 56) These controversial findings are intriguing. A seeming paradox was in the presence of pDCs in the atherosclerotic plaque. While pDCs could barely be detected in mouse atherosclerotic lesions in *Ldlr*^{-/-} mice⁽⁵⁵⁾, Doering *et al.* showed the presence of pDCs in lesions of *ApoE*^{-/-} mice, mainly in the plaque shoulder, at which pDC abundance was increased with HFD feeding and lesion progression.⁽²⁹⁾ Conversely, MacRitchie *et al.* described the constitutive presence of mostly immature pDCs in non-inflamed aortic tissue of normolipidemic mice, at numbers similar to those seen in atherosclerotic *ApoE*^{-/-} mice. Nevertheless, antigen presentation capacity of aortic pDCs from *ApoE*^{-/-} mice was enhanced.⁽⁵⁶⁾ In line, Doering *et al.* showed that sorted aortic pDCs from hyperlipidemic mice, *ex vivo* primed, were capable to trigger T cell stimulation *in vivo*.⁽²⁹⁾ Finally, baseline IFN- α levels were below detection levels or not affected by pDCs in our study and the study of MacRitchie *et al.*,^(55, 56) whereas Doering *et al.* demonstrated elevated IFN- α levels in plaque (mRNA) and serum in HFD fed *ApoE*^{-/-} mice, being reduced after pDC depletion.⁽²⁹⁾ It has

to be noted that the groups employed different methodologies, such as the use of the depletion antibody, administration regimen and mouse models. As instance, *ApoE^{-/-}*, the model used by Doering *et al.* and MacRitchie *et al.*, displays more aggressive atherosclerosis than the *Ldlr^{-/-}*, which may favor pDC switching to an immunogenic mode. Regarding the complex pathophysiology of atherosclerosis, pDCs could well exert dual functions in early and advanced stages of disease, dependent on their microenvironmental context. During episodes of fulminant plaque inflammation pDCs acquire pro-atherogenic functions by rapid secretion of type I IFNs and pro-inflammatory cytokines, while during low grade chronic inflammatory stages, pDCs may act tolerogenic by inhibiting proliferation of CD4⁺ T cells. Indeed, pDCs are involved in the pathogenesis of a range of autoimmune diseases characterized by a type I IFN-signature,⁽⁵⁷⁾ whereas alternatively activated pDCs are considered to contribute to tolerance induction.⁽⁵⁷⁾ Further studies are warranted to elucidate the actual pathways that are activated in pDCs during different stages of atherosclerosis by using more advanced animal models, such as conditional E2-2 knockout.^(57, 58) Nevertheless, the above findings clearly identify this cell type as an interesting new target for future therapeutic intervention studies in the treatment of atherosclerosis.

5. CVD risk factors: contribution of DCs?

Type 2 diabetes mellitus (T2D) and hypertension are major risk factors for the development of atherosclerosis and its cardiovascular complications. Chronic inflammation is thought to accelerate the progression of these pathological conditions.^(59, 60) DCs are likely to contribute here by triggering cell-mediated immune responses. The following two sections outline the current knowledge on potential DC functions in T2D and hypertension.

A role for DCs in type 2 diabetic patients with atherosclerotic complications

Insulin resistance and hyperglycemia in T2D are associated with a systemic pro-inflammatory state (increase in pro-inflammatory cytokines such as IL-6, activation of immune cells) that facilitate the development of atherosclerosis.⁽⁵²⁾ *In vitro* studies have shown that advanced glycosylation end products (AGEs)⁽⁶¹⁾ and hyperinsulinemia⁽⁶²⁾ enhance DC maturation and induce

an antigen-specific T cell activation, thus supporting a contributory role of DC (subsets) on the immune reactions in diabetic atherosclerosis. Yao *et al.* recently reported a significant decline in circulating cDCs in T2D patients with unstable angina pectoris (UAP) vs. healthy controls and T2D patients, while pDC numbers remained mainly unaltered. cDCs showed a more mature and activated phenotype, evidenced by the up-regulation of CD86 and the enhanced capability to stimulate T cell proliferation *in vitro*.⁽⁶³⁾ Reduced circulating DC numbers in T2D patients with atherosclerotic complications was attributed to an increased trafficking into the inflamed vulnerable plaque or to neighbouring lymph nodes as patients had significantly increased levels of fractalkine, an important chemokine for DC attraction to sites of inflammation.⁽⁶³⁾ In contrast to these findings, Orfao and coworkers showed both quantitatively and functionally impaired pro-inflammatory cytokine response by circulating DCs from T2D patients with atherosclerotic complications.⁽⁶⁴⁾ Conceivably, the increased plasma TNF- α levels observed in patients with diabetic atherosclerosis may underlie this impairment, as it can tone blood DC differentiation.⁽⁶⁵⁾ This notion is encouraged by studies, describing an inverse correlation between blood DC numbers and plasma TNF- α concentrations in T2D.^(66, 67) Of note, medication employed for glycemic control and/or for the treatment of diabetes-related comorbidities (ACE antagonists, angiotensin receptor blockers, or statins) could be partly causal in the altered blood DC abundance.⁽⁶⁰⁾ Altogether, although DC function is clearly perturbed in T2D, the present state of knowledge does not allow to segregate atherosclerosis from T2D intrinsic DC effects.

A role for DCs in hypertension

T cells have been described to contribute to hypertension,⁽⁶⁸⁾ a process that likely involves their priming by APCs, such as DCs, with the capacity to present neoepitopes, generated by necrotic and apoptotic cells.⁽⁵⁹⁾ However, less is known regarding the role of DCs in hypertension. DC accumulation in alveolar lesions of human and experimental pulmonary arterial hypertension has been described.⁽⁶⁹⁾ Recently, Vinh *et al.* have shown that the CD28 blocking agent Abatacept prevents angiotensin II (Ang II)-induced hypertension in mice, supporting a contributory role for DCs as APCs in hypertension.⁽⁷⁰⁾ Additionally, they observed increased activated DC numbers in spleen and lymph nodes of

hypertensive mice.⁽⁷⁰⁾ However, these data leave unaddressed whether DCs are the primary cell type responsible for antigen presentation. The more abundant vascular macrophages in the vessel wall might as well function as APCs. Interestingly, the renin-angiotensin-aldosterone system can by itself initiate/ modulate innate and adaptive immune responses and inflict target-organ damage as shown by the group of Mueller and coworkers in a compound transgenic rat model harboring human renin/angiotensin genes.⁽⁵⁹⁾ Activation of the AT-1A receptor, amongst others expressed on DCs, promoted DC migration to the kidneys and their activation, potentially inducing renal damage.⁽⁵⁹⁾ Moreover, *Id2*^{-/-} mice – lacking Langerhans cells (LC) and CD8⁺ DCs - infused with Ang II remained normotensive and failed to develop albuminuria and renal damage, firmly establishing a role for LCs and CD8⁺ DCs in Ang II-induced hypertension.⁽⁵⁹⁾ These data support the idea that Ang II itself can influence T cell ‘fate’ both directly or indirectly.

6. DC contribution to ischemic stroke: friend, foe or bystander?

Only recently immune mechanisms were recognized to contribute to the pathophysiology of ischemic stroke, and involve both the innate and adaptive immune system.⁽⁴⁾ A potential role for DCs as potent mediators of inflammation in stroke has not been investigated extensively. Yilmaz *et al.* have shown that circulating cDC as well as pDC numbers were transiently reduced in patients with acute stroke.⁽¹¹⁾ They postulated that circulating DCs are recruited into the infarcted brain to elicit antigen-specific immune responses through T cell activation. Indeed, HLA-DR expressing cDCs co-localized with T cells in dense infiltrates around cerebral vessels in the stroke area.⁽⁶⁰⁾ The pro-oxidant conditions of ischemia-reperfusion may give rise to the formation of neoepitopes, which can well be presented by APCs, such as HLA-DR expressing residential microglia or recruited DCs. It cannot be excluded that other factors, such as increased cell apoptosis may contribute to the declined circulating DC numbers, as it has been shown for lymphocytes after stroke.⁽⁷¹⁾ Interestingly, Gelderblom *et al.* revealed in a rodent stroke model (temporary middle cerebral artery occlusion) the early accumulation of DCs, peaking on day 3 after reperfusion.⁽⁷²⁾ DCs showed strong and sustained up-regulation of MHC class II, but an absent concomitant up-regulation of co-stimulatory molecules, possibly leading to disrupted T cell activation. While

such a phenotype is conceivable, this warrants further study on whether DCs are active contributors to local immune responses after stroke, either in an immunogenic or immunosuppressive way.

7. DC involvement in the diseased heart

Circulating DCs in heart failure patients

The association between circulating DCs and heart failure has only been subject of a few scattered studies and study outcomes are rather divergent. Athanassopoulos *et al.* have revealed an increase in total blood DCs in patients with chronic heart failure (non-ischemic dilated cardiomyopathy) compared to controls due to an increase in the mature fraction of the cDC subset (CD83⁺ CCR7⁺), suggesting a possible Th1 response in end-stage heart failure.^(73, 74) In seeming contrast, Sugi *et al.* showed that total circulating DC numbers were transiently declined, but residual DCs appeared more activated during the acute decompensated phase of heart failure.⁽¹²⁾ Comparable to the decline in circulating DC counts in CAD patients, the authors associate diminished DC numbers in heart failure with DC recruitment from systemic circulation into the damaged tissue in response to released DAMPs. Clearly, other confounders with the potential to modify circulating DC counts (e.g. development in the bone marrow and release into the circulation, cell apoptosis, trafficking routes) have to be taken into account. To conclude, a direct involvement of DCs in the pathophysiology of heart failure, while plausible, remains elusive. Moreover, further studies are warranted to precisely define the cause of systemic DC modifications, taking different disease stages (acute versus chronic) into account.

Myocardial Ischemia and Reperfusion

A role for DCs in the pathogenesis of cardiac ischemia/ reperfusion injury (IRI) is not well-established. In 1993, Zhang *et al.* demonstrated the rapid accumulation of interstitial DCs in the border zones 7 days post myocardial infarction (left coronary artery ligation) in the rat heart.⁽⁷⁵⁾ DCs tended to be assembled in small clusters with CD4⁺ T cells, which disappeared 21 days after coronary ligation. It is assumed that these DCs are involved in post-ischemic short-term cytoprotective responses through TLR2 and TLR4 stimulation by

tissue released DAMPs.⁽⁷⁶⁾ Maekawa *et al.* on the contrary demonstrated in a murine myocardial infarction model (left coronary artery ligation) that deletion of interleukin-1 receptor-associated kinase-4 (IRAK-4), a downstream effector of the TLR/MyD88 axis, had beneficial effects on survival and function after myocardial infarction by blunting DC mobilization into the heart and attenuating local inflammatory processes.⁽⁷⁷⁾ Of note, cardiomyocytes and residential macrophages also express TLR2 and TLR4, and are able to respond to endogenous DAMPs, thereby contributing to inflammatory processes after myocardial infarction.⁽³⁾

Altogether, the presence of DCs in the heart and their post-ischemic accumulation has been demonstrated. Concerning their role as professional APCs in parallel to phagocytes and cardiomyocytes, DCs likely exert functions in acute myocarditis by steering cell-mediated immune responses.

Viral myocarditis

Even less is known about a contribution of DCs to the pathogenesis of viral myocarditis. Virus infections can inflict significant damage on cardiomyocytes by (1) immune-response mediated, (2) autoimmune-mediated, or (3) direct virus-induced myocardial injury, which can cause myocarditis and dilated cardiomyopathy. Many animal studies have been performed in this field using models of myocarditis caused by coxsackievirus B3 (CVB3).⁽⁷⁸⁾ In response to viral infections, immune cells, such as cardiomyocytes, endothelial cells, fibroblasts, and DCs were reported to infiltrate up to five days postinfection, supposed to limit viral replication by the release of pro-inflammatory cytokines (IL-1 α , IL-1 β , IL-6, IL-18, TNF- α , TNF- β , and IFN- γ).^(79, 80) In the subacute phase (within two weeks after viral infection) the release of progeny virus into the interstitium stimulates the infiltration of pro-inflammatory natural killer (NK) cells and macrophages, followed by a considerable influx of antigen-specific CD4⁺ Th and cytotoxic CD8⁺ T cells, which recognize viral antigens presented via MHC molecules on the surface of infected cardiomyocytes.^(80, 81) Interestingly, in a mouse model of Coxsackievirus myocarditis it has been shown that CD8⁺ DCs, next to cardiomyocytes, are implicated in CD8⁺ T cell priming, thereby curtailing viral infection in the acute phase (within 8 days postinfection).⁽⁸²⁾ Whether or not DCs are implicated in low-grade inflammation during chronic myocarditis (days

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15-90 postinfection) remains unclear. In particular pDCs, the major type I IFN producers in response to viral infection, have not received much attention so far, but may be likely mediators in anti-viral defense in the heart, although endogenous type I IFN release by infected cardiomyocytes could as well limit viral replication in the heart.

Figure 1. Schematic overview representing the possible impact of DCs in CVDs. (A) Risk factors critically contributing to ischemic stroke and heart failure can directly interfere with the activation state of DCs. **(B)** Epitopes released from necrotic tissue or dying cells of the damaged organs could additionally induce phenotypic changes in DCs (indirect effect), thus finally leading to **(C)** DC immunogenicity or tolerance. **(D)** Chronic DC activation potentially constitutes detrimental effects due to release of pro-inflammatory cytokines and the activation of CD4⁺ and CD8⁺ T cells, which in turn causes further organ damage. Otherwise, **(E)** induction of tolerance by DCs may thwart immunogenic responses by eliciting T cell anergy/ apoptosis and the generation of regulatory T cells. **(F)** Pathogen-derived signals, and **(G)** the local environment most likely impair DC functionality sustained through **(a)** or **(b)**, thereby boosting or abating DC-mediated immune responses.

8. DC targeted therapeutic opportunities in cardiovascular disease

Targeting DCs in cardiac transplantation: contributors to graft rejection or useful tools in tolerance induction

The role of DCs as modulators of alloreactive and autoreactive T cell responses after transplantation has been extensively studied.⁽⁸³⁾ DCs hold promise as therapeutic tools to ameliorate or prevent graft rejection or graft-versus-host disease (GVHD), and to treat autoimmune diseases.^(84, 85) DCs are implicated in the recognition of allo-antigens by the host's immune system. As Larsen *et al.* demonstrated, mature donor-derived DCs are homing to T cell areas in the draining lymph nodes in the first days after transplantation, where they trigger naïve T cells by presenting graft-derived epitopes. Thus potentially contributes to acute rejection of cardiac allografts.⁽⁸⁶⁾ Similarly, Kofler *et al.* reported pronounced infiltration of recipient DCs into the cardiac allograft, picking up and processing the donor antigens, and activating the recipient's adaptive immune system in the first postoperative year after human heart transplantation.⁽⁸⁷⁾ Summarized, DCs appear to be main culprits in organ rejection, but have otherwise been shown to mediate transplant tolerance by preventing T cell-mediated immunity. Ochando *et al.* have demonstrated an essential role for pDCs as phagocytotic APCs in tolerance induction in vascularized cardiac grafts, in that adoptive transfer of tolerized pDCs induced regulatory T cell development and prolonged graft survival in mice.⁽⁸⁸⁾ Likewise, a single pre-operative infusion of donor-mobilized immature pDCs in combination with anti-CD154 monoclonal antibody was able to effectively suppress allograft rejection and prolonged graft survival in mice.⁽⁸⁹⁾ These findings are concordant with a recent study in human that examined total peripheral blood DC numbers in patients with clinical heart transplantation (HT), revealing significantly diminished DC frequency one week post HT, which probably reflects immunological quiescence through adequate immunosuppression.⁽⁹⁰⁾

In summary, adoptive transfer of pre-primed (tolerogenic) DCs seems to hold great promise for preventing graft rejection after cardiac transplantation. For all that, a better understanding of how graft-infiltrating DCs function, may also help to appreciate their contributory roles in chronic heart failure and post-myocardial infarct healing. At first glance these processes seem unrelated, but

similar immune pathways activated in the different disease settings may help to refine DC's contribution to certain CVD.

DCs as potential therapeutic tools in the treatment of atherosclerosis

As alluded to, DC-based vaccination and immunization strategies, based on application of *ex vivo* antigen loaded or genetically engineered autologous DCs to tune T cell responses have meanwhile evolved into a viable therapeutic option for cancer.⁽⁹¹⁾ This success has inspired several groups to explore the potential of DC-based vaccination for atherosclerosis. Studies in animal models so far are at least encouraging and support the notion that DC-based vaccination and immunization hold promise for therapeutic immunomodulation of atherosclerosis.⁽⁹²⁾ Kuiper and coworkers have shown that transferred oxLDL-pulsed mature DCs into *Ldlr*^{-/-} mice reduce atherosclerotic lesion size. DC vaccination led to quenched Th1 responses and elevated oxLDL-specific IgG titers indicating that oxLDL-pulsed DCs may confer protection against atherosclerosis, by favoring humoral immune responses to oxLDL.⁽⁹³⁾ Other groups have shown that repeated injection of antigen-loaded immunogenic DCs (oxLDL or malondialdehyde modified LDL (MDA-LDL)) aggravated atherosclerosis.⁽⁹⁴⁾ A recent study proposed immunotherapy with DCs, pulsed with apolipoprotein B100 in the presence of IL-10 to render them immunosuppressive, as an effective strategy to attenuate atherosclerosis. DC immunotherapy resulted in reduced proliferation of effector T cells, dampened Th1 and Th2 immunity and diminished atherosclerotic lesion formation in mice.⁽⁹⁵⁾ Taken together, the outcome of these studies indicates that DC vaccination emerges as a new, potentially powerful approach in the treatment of atherosclerosis, although translation of these largely animal experimental findings to human disease needs further investigation. A second thwart will be the establishment of exclusively atherosclerosis-specific antigens to avoid systemic immunity.

Furthermore, therapy timing and the immunological status of the patient are additional important issues that need to be considered carefully, given that atherosclerosis mainly affects the elderly. Indeed, age-dependent alterations in expression and function of innate immune receptors and signal transduction pathways may translate in defective DC activation, thus diminishing the DC-based vaccination efficacy.⁽⁹⁶⁾ Nevertheless, promising developments in the

cancer field, where *ex vivo* pulsed DC have been successfully applied in phase I/II clinical trials, stem hopeful and prompt to further investigations in DC therapy for CVD treatment.

9. Concluding remarks

This review summarizes the current state of knowledge on the role of different DC subsets in the pathogenesis of CVDs, pinpoints shortcomings/gaps and delineates the future perspectives for DCs as therapeutic target in CVDs. By now several DC subsets have been reported to accumulate not only in atherosclerotic or hypertensive vessels, but also in failing, cardiomyopathic or ischemic heart tissue and in ischemic brain, suggestive of a role in the underlying pathophysiology. DC (precursor) numbers and functionality in blood of patients with CVD have been embraced as measure of DC association with disease onset and progression, although cause and implications of these disease-associated changes in DC homeostasis still are subject of discussion. Moreover, the actual value of circulating DCs as biomarkers in CVDs needs to be established, but it is fairly improbable that DC subset numbers will offer the precision, specificity and discriminative power to be useful as biomarkers. Of all cardiovascular disorders, atherosclerosis is by far most extensively studied for impact of DC subsets in its disease ontogenesis, albeit that the majority of these studies involve murine animal models. Much less is known about the contribution of DCs to cardiovascular pathologies such as stroke, heart failure and myocardial diseases. Conceivably, also here injury associated DAMP release will lead to the recruitment and subsequent activation of DCs with T cell priming ability at the inflamed locus, skewing the adaptive immune system towards Th1/17-like immune responses or towards a state of tolerance, dependent on disease stage and environmental context.

Recent findings in mice plead for a beneficial role for pre-primed DCs as therapeutic agents in the treatment of atherosclerosis. Nevertheless, the efficacy of DC immunotherapy for preventing plaque progression and destabilisation in humans remains to be seen. In this regard, it will be vital to target the relevant disease-associated antigens for DC pulsing. Donor-mobilized DCs could be otherwise cultured *in vitro* without further adjuvants, as the use of non-pulsed immature DCs has been proven to be an attractive approach to induce tolerance. Alternatively strategies could be employed to

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instruct endogenous DCs *in situ*, f.e. by receptor-specific manipulation, albeit that the options for targeting DC subset specific surface receptors are rather limited.

Summarizing, despite the limitations in our current understanding in DC functions in various CVDs, the recently shown efficacy of DC-based tolerance and immunization strategies in ameliorating murine atherosclerosis and diminishing allograft rejection, in combination with the current dynamics in this rapidly progressing research field inspire confidence that (some of) these approaches will evolve into viable modalities for the treatment and may be even prevention of human cardiovascular disorders.

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The main goal of the following two studies presented here (**Chapter 3 and 4**) was to explore the impact of plasmacytoid dendritic cells (pDCs) in atherosclerosis.

pDCs are a rare innate immune cell population that circulates in the blood and is present in peripheral lymphoid organs. Upon stimulation through Toll-like receptor 7 (TLR7) and TLR9, pDCs release large amounts of type I interferons (IFN), which are critically involved in anti-viral defense mechanisms. Chronic pDC activation and type I IFN release in the absence of viral infections results in autoimmune diseases such as systemic lupus erythematosus (SLE), psoriasis vulgaris and multiple sclerosis. Several host factors have been shown to activate pDCs, thus breaking down innate tolerance. Of note, both SLE and psoriasis are associated with an increased risk of atherosclerosis. Otherwise, several reports have indicated that immature or alternatively activated pDCs can induce T cell tolerance, primarily through the induction of regulatory T cells. Thus, pDCs are capable to function dually. Priming of immunogenic or tolerogenic immune responses depends largely on the microenvironment in which pDCs get activated.

A potential role for pDCs in atherosclerosis has not been identified yet. pDCs were described to be present in inflammatory regions in the human atherosclerotic plaque, supposed to trigger CD4⁺ T cells and to boost conventional dendritic cell responses within the lesion. In the following two chapters we made use of different pDC depletion models to characterize a potential contributory role for pDCs to lesion formation; (1) pDC ablation by use of a specific depletion antibody resulted in significantly deteriorated lesion formation, suggestive of a protective role for pDCs in mouse atherosclerosis (**Chapter 3**). Furthermore, we examined the impact of pDCs in murine atherosclerosis by (2) use of a pDC depletion model, in which pDC ablation was based on the transgenic diphtheria toxin receptor (DTR) expression under the pDC-specific human BDCA-2 promoter (**Chapter 4**).

Contribution to the manuscript (Chapter 3):

Anette Christ was next to Dr. Daissormont and Prof. Biessen critically involved in the study design, the performance of experiments and data discussions/ interpretations. Anette Christ was responsible for the accomplishment and analysis of the immunohistochemical and RT-PCR data on human plaque tissue samples, and the blood samples received from healthy controls and patients with carotid entarterectomy. Besides, Dr. Daissormont and Anette Christ mainly performed the *in vivo* pDC depletion studies, including optimization of the used depletion antibody (120G8), *in vivo* antibody administration, sacrifice of the animals, flow cytometry experiments (analysis of leukocyte populations, sorting of plasmacytoid dendritic cells and T cell subsets for RT-PCR analysis), and histology of mouse atherosclerotic lesions (immunohistochemistry, quantification and data analysis). Additionally, Anette Christ contributed crucially to the revision of the written manuscript by Dr. Daissormont.

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3

Plasmacytoid dendritic cells protect against atherosclerosis by tuning T cell proliferation and activity.

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Abstract

Unlike conventional dendritic cells (cDCs), plasmacytoid DCs (pDCs) are poor in antigen presentation and critical for type I interferon response. While proposed to be present in human atherosclerotic lesions, their role in atherosclerosis remains elusive. We show that pDCs are scarcely present in human atherosclerotic lesions, and almost absent in mouse plaques. Surprisingly, pDC depletion by 120G8 mAb administration was seen to promote plaque T cell accumulation and exacerbate lesion development and progression in low-density lipoprotein receptor deficient (*Ldlr*^{-/-}) mice. pDC depletion was accompanied by increased CD4⁺ T cell proliferation, IFN- γ expression by splenic T cells and plasma IFN- γ levels. Lymphoid tissue pDCs from atherosclerotic mice showed increased indoleamine 2,3-dioxygenase (IDO) expression and IDO blockage abrogated the pDC suppressive effect on T cell proliferation. In conclusion, our data reveal a protective role for pDCs in atherosclerosis, possibly by dampening T cell proliferation and activity in peripheral lymphoid tissue, rendering pDCs an interesting target for future therapeutic interventions.

Introduction

Plasmacytoid dendritic cells (pDCs) are a subset of dendritic cells derived from both myeloid and lymphoid precursors in bone marrow, and constitute only 0.1-0.5% of the total leukocyte pool in blood and peripheral lymphoid tissue. As the main type I interferon (IFN) producing cells, pDCs have a critical role in detection of and host defense against bacterial and viral infection, but also in sensing RNA/DNA and immune complexes. Upon stimulation, pDCs produce large amounts of type I interferons (IFN- α , IFN- β , IFN- ω and IFN- λ) in a toll-like receptor (TLR) 7 and 9 dependent manner, thereby inducing effector T and natural killer (NK) cell activation and linking innate and adaptive immunity.⁽¹⁾ pDCs differ from conventional dendritic cells (cDCs) in that they are poor T cell activators due to low expression of major histocompatibility complex class-II (MHC-II) and co-stimulatory molecules.^(2,3)

The presence of pDCs in human atherosclerotic lesions has been documented in 1995⁽⁴⁾ and was subsequently confirmed by C. Weyand and co-workers.⁽⁵⁾ pDCs (CD4⁺CD45RA⁺IL-3 α (CD123)⁺ILT3⁺ILT1⁻CD11⁻) were reported to be expressed in the shoulder region of human plaques where they are believed to regulate T cell function, even in the absence of antigen recognition. CpG-induced IFN- α release by pDCs effected a 10-fold up regulation of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) expression on CD4⁺ T cell surface, thereby promoting vascular smooth muscle cell (vSMC) and endothelial cell (EC) apoptosis, processes that are generally deemed deleterious for plaque stability. However, these *in vitro* data leave unaddressed whether pDCs are instrumental in plaque destabilization *in vivo*.

While viral infection associated acute pDC activation results in massive type I IFN release, chronic activation of pDCs in the absence of infection was reported to cause severe autoimmune diseases. For instance, in experimental autoimmune encephalomyelitis pDCs were shown to exert overt pathogenic activity, mainly by enhancing T helper 17 (Th17) dependent immune responses.⁽⁶⁾ In patients with systemic lupus erythematosus, pDC activity and IFN- α / β release correlated with disease activity and severity.^(7,8) Conversely, evidence is culminating that immature and alternatively activated pDCs have the capacity to dampen chronic low grade inflammation and autoimmune diseases, including diabetes type I, asthma and transplant rejection, possibly by suppressing CD8⁺ effector T cell and inducing CD4⁺CD25⁺ regulatory T cell

(Treg) function by the release of tolerogenic molecules such as indoleamine 2,3-dioxygenase (IDO) or programmed death-ligand 1 (PD-L1).⁽⁹⁻¹³⁾ Altogether, these observations show that pDCs are very plastic cells with the capacity to produce high levels of type I IFN and activate the adaptive immune system in infection on the one hand and to regulate inflammation by inhibiting effector T cell and inducing regulatory T cell responses on the other hand.

In this study, we addressed the actual role of pDCs in atherosclerosis by a loss-of-function approach, providing evidence for an IDO-dependent T cell suppressive activity of pDCs in human and mouse atherosclerosis.

Methods

Atherosclerosis induction and pDC depletion in mice

Female *Ldlr*^{-/-} mice, obtained from The Jackson Laboratory and backcrossed at least 11 times to C57Bl/6, were placed on high fat diet containing 0.25% cholesterol (Special Diets Services, Witham, Essex, UK). Two weeks later, atherosclerotic lesions were induced in the carotid artery by bilateral placement of semi-constrictive collars.⁽¹⁴⁾ To study effects of pDC depletion on atherosclerosis development, a pDC depleting antibody, 120G8 (250µg/mouse/injection, Bioceros, Utrecht, the Netherlands), was administered 4 times per week i.p. for 3,5 weeks starting at the time of collar placement (n=19) after which mice were sacrificed. From these mice, also the aortic root was analyzed to study effects of pDC depletion on natural atherosclerosis development. To study effects of pDC depletion on progression of atherosclerosis, 120G8 (250µg/mouse/injection, Bioceros, Utrecht, the Netherlands) was administered 4 times per week i.p for 3 weeks starting at 4 weeks after collar placement, once initial lesions had formed (n=17). As control antibody, an isotype control (GL113) was used. All animal work was approved by the regulatory authority of Maastricht University and performed in compliance with the Dutch government guidelines.

Histology of mouse atherosclerotic lesions

Mice were euthanized by an overdose pentobarbital (115mg/kg) and perfused through the left cardiac ventricle with phosphate buffered saline (PBS) (NaCl/Na₂HPO₄/KH₂PO₄, pH 7.4) containing sodium nitroprusside

(0.1mg/ml, Sigma) and 1% paraformaldehyde (PFA). The right common carotid artery and the heart were removed, fixed overnight in 1% PFA and paraffin-embedded sections (4 μ m) were cut. To determine plaque volume in the carotid artery, plaque area was measured for consecutive cross sections at 100 μ m intervals over a carotid artery segment that covered the entire plaque. To measure plaque volume in the aortic root, plaque area was measured for each valve for consecutive sections at 20 μ m intervals that covered the entire lesion. Collagen content was detected by sirius red staining and expressed as a percentage of plaque area.⁽¹⁵⁾ Slides were analyzed in a blinded manner using a Leica DM3000 light microscope (Leica Microsystems) coupled to a computerized morphometric system (Leica Qwin 3.5.1).

Immunohistochemistry

Immunohistochemical stainings were performed on paraffin carotid artery sections for CD3 (DAKO), Siglec-H/440c (Hycult Biotechnology) and α -smooth muscle actin (ASMA) (DAKO). Slides were analyzed blindly using Leica Qwin program. The number of positive cells was measured and expressed as a percentage of total number of cells in the plaque. For ASMA, the number of positive cells was expressed as a percentage of plaque area.

Flow cytometry

Blood, spleen and peripheral lymph nodes (LN) were removed before perfusion and used for FACS analysis of pDCs (CCR9^{high} PDCA-1^{high}, eBioscience), cDCs (CD11c^{high}, eBioscience), T cells (CD3e⁺, Miltenyi), T helper cells (CD4⁺, BD), cytotoxic T cells (CD8a⁺, BD), effector T cells (CD44^{high}, eBioscience), regulatory T cells (CD4⁺CD25⁺Foxp3⁺, eBioscience), B cells (B220⁺, BD), monocytes (CD11b^{high} Ly6G⁻, BD), granulocytes (Ly6G^{high} CD11b^{high}) and NK cells (NK1.1⁺ CD3⁻). For pDC and cDC analysis, spleen and peripheral LN were enzyme treated (liberase (0.032mg/ml, Roche) and DNase (0.8 μ g/ml, Roche)) for 30 minutes in RPMI medium, before antibody incubation to release DCs from the tissue. FACS analysis was performed with FACS Canto II (BD).

Plasma cytokine and lipid analysis

Plasma cholesterol levels were measured in duplicate using a colorimetric assay (CHOD-PAP, Roche). Mouse and human plasma levels of IFN- α were measured by sandwich ELISA (PBL Interferon Source). Cytokine

analysis of mouse IFN- γ was performed using the Bio-Plex Pro assay (Bio-Rad). To verify functional ablation of pDCs after 120G8 treatment, control and 120G8 treated mice ($n=5$ per group) received subcutaneous injection with CpG (CpG-ODN 1585, type A, 40 $\mu\text{g}/\text{mouse}$, Invivogen) and were sacrificed 6 hours later to measure IFN- α plasma levels. ELISA plates were used from Greiner Bio-One (Alphen a/d Rijn, the Netherlands).

Mouse pDC and T cell isolation by Fluorescence Activated Cell Sorting (FACS)

Splenic pDCs (PDCA-1^{high} CCR9^{high}) and T cells (CD3⁺) were isolated from *Ldlr*^{-/-} mice, either on high fat diet (0,25% cholesterol) (group of atherosclerotic mice, $n=8$) or on normal diet (group of non-atherosclerotic mice, $n=8$), by FACSaria. The purity of the sorted pDCs (PDCA-1^{high} CCR9^{high}) was 98.3%, for the CD3⁺ T cells 95%.

RNA isolation and Quantitative real-time PCR

RNA from sorted cells was isolated using the [®]Trizol (Invitrogen) method. Isolated pDCs were analyzed for gene expression of mouse PD-L1 (5'-ccatcctgtgttctcctcattg-3' and 3'-gttcactcttacgatctacacct-5'), mouse IDO (5'-actgtgtcctggcaaactggaag-3' and 3'-gagataaccaccttttagcgctcgaa-5') and mouse IFN- α (5'-cattctgcaatgacctccac-3' and 3'-cacgtccttaaggggact-5'). Isolated CD3⁺ T cells were analyzed for expression of mouse IFN- γ (5'-tggtgtgtttctggctgttactg-3' and 3'-actgtacttttaggacgtctcg-5'). Isolated human pDCs from blood were analyzed for gene expression of human IFN- α (5'-aatggccttgacctttgctt-3' and 3'-cgttcagttcgacgagacac-5').

BrdU labeling and cell analysis

Ldlr^{-/-} mice on high fat diet treated either with 120G8 ($n=4$) or GL113 ($n=4$) received BrdU intraperitoneal (i.p.) injections (0.8mg/day) for 5 consecutive days. BrdU incorporation in CD4⁺ and CD8⁺ T cells was assessed using a FITC BrdU flow kit (BD) according to manufacturer's instructions. Cells were analyzed using FACS CANTO II (BD).

OT-1 and OT-2 antigen specific T cell proliferation experiment

Ldlr^{-/-} recipient mice ($n=4$ 120G8 versus $n=4$ GL113 treated mice) on high fat diet received intravenous (i.v.) 1×10^6 CFSE-labeled purified OVA-specific OT-1/CD45.1 or OT-2/CD90.1 cells together with 2×10^6 non-transgenic

purified CD8⁺/CD90.1 or CD4⁺/CD45.1 T cells that served as an internal control. The next day, mice received i.v. 10⁶ irradiated (1500 rad) Kb^{-/-}-actmOVA or C3H-actmOVA cells. Three days later, OT-1/CD90.1 and OT-2/CD45.1 proliferation and expansion were determined based on CFSE dilution and the ratio of OT-1/CD45.1 to CD90.1 control CD8⁺ T cells and OT-2/CD90.1 to CD45.1 control CD4⁺ T cells. In parallel, 5x10⁶ splenocytes were restimulated with OVA 257-264 or OVA 323-339 peptide (A&A labs, San Diego, CA) in the presence of Brefeldin A for 5 hours. Surface staining for CD8/CD4 (BD) and CD45.1/CD90.1 (eBioscience) was performed.

pDC-T cell co-culture

pDCs were isolated from spleen from a Bl6 Flt3L (3x10⁶) treated *Ldlr*^{-/-} mouse on high fat diet using PDCA-1 microbeads (Miltenyi). CD3⁺ T cells were isolated from spleen of a pDC depleted *Ldlr*^{-/-} mouse on high fat diet by staining T cells with a biotinylated CD4 (Biolegend) and CD8 (Biolegend) antibody and using a biotin isolation kit (Miltenyi). pDC and CD3⁺ T cells were co-cultured in a 96 well plate coated with anti-CD3/ CD28 (0.1μg/ml) in a 1:6 ratio (1x10⁵ pDC and 6x10⁵ T cells, respectively) for 3 days in iDMEM medium supplemented with either 1-MT (Sigma Aldrich, 100μM) or anti-PD-L1 (20μg/ml, Bioceros). Seventy-two hours later, CD3⁺ T cell proliferation was assessed by 3[H]-thymidine incorporation and plotted as relative proliferation index, being the ratio of T cell proliferation in the presence and absence of pDCs. In parallel, purified CD4⁺ T cells were CFSE labeled prior to stimulation and proliferation was determined by flow cytometry 72 hours later upon.

Human plaque analysis

For microarray analysis, total RNA was extracted from carotid artery sections obtained from autopsy (Department of Pathology, University Hospital Maastricht, Maastricht, the Netherlands) (early (n=13) and advanced stable (n=16) lesions) and surgery (Department of Surgery, Maasland Hospital Sittard, Sittard, the Netherlands) (advanced stable (n=21) and advanced unstable (n=23) lesions). For quantitative real-time PCR analysis, RNA was isolated from carotid artery sections obtained from surgery (Department of Surgery, Maasland Hospital Sittard, Sittard, the Netherlands) (advanced stable (n=12) and advanced unstable (n=12) lesions). Plaques were staged by histological analysis of adjacent slides according to Virmani *et al.* at which intimal

thickening (IT) were characterized as early, thick fibrous cap atheroma (TkfcA) as advanced stable, and intraplaque hemorrhaged lesions (IPH) as advanced unstable lesions, respectively.⁽¹⁶⁾ Immunohistochemical stainings were performed on paraffin carotid artery sections from advanced stable (n=22) and ruptured lesions (n=22) from surgery for CD123 (Sigma Prestige), BDCA-4 (R&D Systems), ASMA (Dako) and CD68 (Dako). The purity of the BDCA-4 antibody was checked by flow cytometry by comparing mean fluorescence intensity (MFI) of BDCA-4 expression on pDCs versus B cells, T cells and monocytes. Double-stained slides were analyzed with the Nuance spectral imaging system (Caliper Life Science / Cambridge Research Instrumentation, Woburn, MA, USA). Spectral imaging data sets were taken from 420-720 nm at 20 nm intervals using a DM-5000 Leica microscope system at 20x (plan apo). Spectral libraries of single-red (Vector Red), single-blue (Vector Blue), and natural occurring iron pigments were obtained from the control slides. The resulting library was applied to the double stained slides to spectrally dissect/unmix them into individual component images using the Nuance™ 3.0 software.⁽¹⁷⁾ Pseudo-color images showing co-localization, as well as an exclusive image of co-localization, were generated with the Nuance 3.0 software. Quantitative assessment of co-localization and pixel-based measurement of the individual markers per microscopic field was done with the same software.

Microarray analysis of human atherosclerotic lesions

RNA was isolated using the Guanidine Thiocyanate (GTC)/CsCl gradient method and a NucleoSpin RNA II kit (Macherey-Nagel GmbH & Co. KG).⁽¹⁸⁾ RNA concentration was determined using the Nanodrop ND-1000 and the RNA quality obtained using the RNA 6000 Nano/Pico LabChip (Agilent 2100 Bioanalyzer) where the RIN (RNA integration number) was determined. Samples, where the RIN number was lower than 5.6, were excluded from the study. The mean RIN was 7.2 ± 0.49 . Samples from autopsy were individually hybridized to HGU133 2.0 Plus arrays (Affymetrix, Santa Clara, California, USA) and samples from surgery to Illumina Human Sentrix-8 V2.0 BeadChip® (Illumina Inc., San Diego, California, USA). Analysis has been performed in R.⁽¹⁹⁾ For autopsy samples, pre-processing was performed with affy using the Robust MultiArray (RMA) normalization.⁽²⁰⁾ For surgery samples, pre-processing was performed by lumi using the Variance Stabilizing Transform (VST) normalization.⁽²¹⁾ Differential expression has been assessed separately for the

two sets of data by fitting each gene against a linear model across the given arrays with limma.⁽²²⁾ Empirical Bayesian shrinkage of the standard errors have been used to moderate t-statistics, F-statistics, and log-odds of differential expression. The resulting ordered dataset has been queried for the set of genes of interest and their results are hereby presented. The adjusted p-values presented in the results are corrected according to the Benjamini and Hochberg False Detection Rate method.⁽²³⁾

pDC isolation from human blood

pDCs were collected from healthy controls (n=15) and patients with carotid endarterectomy (n=14). pDCs were isolated from blood (Lymphoprep™ Isolation). From this, the B220 positive fraction was depleted with magnetic beads (Miltenyi Biotec) after which pDCs were isolated from the negative fraction using a BDCA-4 isolation kit (Miltenyi Biotec). The purity for BDCA-4⁺ pDCs, as assessed by flow cytometry (CD123^{high} BDCA-2^{high}) was 96%. RNA was isolated using the Trizol method. Gene expression profile was analyzed by quantitative real-time PCR. Patients for this study were recruited at the Department of Cardiology, University Hospital Oslo, Oslo, Norway. The study included 14 patients (7 male, 7 female, average age of 67) with high-grade internal carotid stenosis (≥70%), diagnosed and classified by precerebral color duplex and CT angiography according to consensus criteria. For comparison, blood was collected from 15 healthy controls, comparable to the patient group for age, sex and smoking habits. The controls were health care workers, consecutively recruited from the same area of Norway as the patients. All human work was approved by the Ethical Committee of the University Hospital Oslo. Signed informed consent for participation in the study was obtained from all individuals.

Statistical analysis

Data are expressed as mean ± SEM and Mann-Whitney U test was used to compare individual groups of animals. Data are considered statistically significant at p<0.05.

Results

pDCs are scarce in human and mouse atherosclerotic lesions

Concordant with previous observations,⁽⁵⁾ CD123⁺ pDCs were seen to be present in human atherosclerotic lesions and their presence increases with plaque progression ($P < 0.05$) (**Figure 1A**). Of note, CD123 stained cells were found to show considerable co-localization with macrophages (**Figure 1B**) and vSMC (**Figure 1C**), supporting earlier findings from van Vré *et al.* showing that CD123 is not a specific marker for human pDCs.⁽²⁴⁾ Next, we considered BDCA-4 as a more selective human pDC marker.⁽²⁵⁾ Flow cytometry on human whole blood samples confirmed the high specificity of this marker for human pDCs, as it was virtually absent on circulating monocyte, B cell, T cell and granulocyte subsets (data not shown). Surprisingly, BDCA-4⁺ staining revealed the scanty presence of pDCs in human plaques. Moreover, BDCA-4⁺ cell expression did not differ between stable and unstable advanced atherosclerotic lesions (**Figure 1D**). In agreement, microarray (**Figure 1E**) and quantitative real-time PCR analysis (data not shown) failed to demonstrate differential expression of established pDC markers during plaque progression. Likewise, pDCs were almost absent in mouse carotid and aortic artery lesions of *Ldlr*^{-/-} and *ApoE*^{-/-} mice as well (data not shown), although we did observe few scattered pDCs in the adventitia (**Figure 1F**).

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Figure 1. pDCs are scarce in human and mouse atherosclerotic lesions. Microscopic analysis of human atherosclerotic lesions. **(A)** Immunohistochemistry for CD123 and quantitative measurement of CD123⁺ pDCs in early (intimal thickening, n=12), stable advanced (thick fibrous cap atheroma, n=13) and unstable advanced (intraplaque hemorrhage, n=10) human carotid artery atherosclerotic lesions. **(B)** Co-localization for CD123 (blue) and CD68⁺ macrophages (red). Slides were analyzed with MultiSpectral Imaging and each set of two pictures on the right side represents a composite fluorescent-like image in pseudo-colors of the left picture and was obtained after unmixing the individual colors red and blue with spectral imaging. Co-localization of fluorescent red and green is represented in yellow. Spectral imaging analysis of this double-stained series clearly shows co-localization of CD123⁺ cells and macrophages. **(C)** Co-localization for CD123 (red) and αSMA⁺ smooth muscle cells (blue). **(D)** Immunohistochemistry for BDCA-4 and quantitative measurement of BDCA-4⁺ cells in advanced stable (n=16) and unstable (n=19) human carotid artery lesions. **(E)** Microarray analysis on human carotid artery lesions (early (n=13) vs advanced stable (n=16) and advanced stable (n=21) vs vulnerable (n=23)) **(F)** Immunohistochemistry for Siglec-H (440c) in mouse carotid lesions. pDCs were detected in the adventitia where they were present in small clusters near to vasa vasorum, showing a typical plasmacytoid morphology. *p<0.05; **p,0.01; ***p,0.001.

Effective and selective depletion of pDCs in mice by 120G8 antibody treatment

Next, we addressed the specificity of pDC depletion by 120G8 mAb, which recognizes PDCA-1 (also referred to as bone marrow stromal cell antigen 2 (BST2)), a marker specifically expressed on mouse pDCs. 120G8 significantly depleted (>90%) pDC numbers in blood and spleen ($P<0.05$) (**Figure 2A**). pDC repopulation started already 24 hours after a single 120G8 administration and full recovery was obtained after 72 hours (**Figure 3A**), necessitating an every two daily 120G8 dose regimen for effective and persistent pDC depletion. It has been reported that *in vitro* PDCA-1 expression is up-regulated at mRNA level in other cell types in response to viral infection or exposure to inflammatory stimuli, which theoretically could thwart the depletion specificity.⁽²⁶⁾ In our study, 120G8 treatment did neither affect cDC (**Figure 2B**), nor B cell numbers (**Figure 2C**). Also monocyte and granulocyte levels were unchanged (**Figure 3B-C**). Moreover, assessment of PDCA-1 expression by flow cytometry showed that in high fat diet fed *Ldlr*^{-/-} mice PDCA-1 expression is completely restricted to pDCs (**Figure 2D**). These data demonstrate that in our mouse model of atherosclerosis pDC depletion by 120G8 mAb was effective and specific. To investigate whether prolonged antibody administration by itself could modulate immune responses, we compared the T cell activation status between GL113 and PBS-treated mice and did not find a difference in the number of CD44^{high} T cells (**Figure 3D**). At a functional level 120G8 treatment almost abrogated CpG-induced pDC activation *in vivo*, as judged by the 6-fold attenuated induction in plasma IFN- α release upon CpG injection in 120G8 treated versus control mice ($P<0.05$; **Figure 2E**). While pDC activity and TLR7/9 function appear to be intact, under conditions of hyperlipidemia, baseline plasma IFN- α levels remained unchanged after pDC depletion, both in the plaque initiation and progression study. This suggests that an atherogenic stimulus *per se* does not increase IFN- α release by peripheral pDCs and/or that pDCs are under these conditions not the major source of circulating IFN- α .

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Figure 2. Effective and selective depletion of pDCs in *Ldlr*^{-/-} mice by 120G8 administration. Depicted are the results of flow cytometry from *Ldlr*^{-/-} mice on high fat diet treated with GL113 (n=6) or 120G8 (n=6) for 3 weeks. **(A)** pDC (PDCA-1⁺CCR9⁺) numbers in blood and spleen. **(B)** cDC (CD11c^{high}) and **(C)** B cell (B220⁺) numbers in spleen and peripheral lymph nodes. **(D)** PDCA-1 expression on pDCs, T cells (CD3⁺), B cells (B220⁺), monocytes (CD11b^{high}Ly6G⁻), granulocytes (CD11b^{high}Ly6G^{high}), cDCs (CD11c^{high}) and NK cells (NK1.1^{high}CD3⁻) from spleen. **(E)** CpG stimulation in GL113 (n=5) versus 120G8 (n=5) treated mice. *p<0.05; **p,0.01; ***p,0.001.

Figure 3. Efficiency and specificity of pDC depletion by 120G8 mAb. (A) Time-span of pDC depletion by 120G8 mAb. *Ldlr*^{-/-} mice on high fat diet received one single dose of 120G8 (250µg/mouse) via i.p. injection and pDC recovery was analyzed 24, 48 and 72 hours later in blood (n=3 per group). (B) Flow cytometry analysis of circulating monocytes (CD11b^{high}Ly6G⁺), (C) granulocytes (CD11b^{high}Ly6G^{high}) and (D) activated (CD44^{high}) CD4⁺ and CD8⁺ T cells from *Ldlr*^{-/-} mice on high fat diet treated with GL113 (n=6) versus 120G8 (n=6) for 3 weeks. *p<0.05; **p,0.01; ***p,0.001.

Depletion of pDCs accelerates atherosclerosis in *Ldlr*^{-/-} mice

To address the role of pDCs in atherosclerosis, we examined lesion development and progression in *Ldlr*^{-/-} mice fed a high fat diet. For plaque initiation, 120G8 treatment was started at time of collar placement, whereas for plaque progression, it was started at week 4 after collar placement, once initial lesions had formed. 120G8 treatment did not affect body weight, nor did it lead to overt pathogenic responses. 120G8 treatment tended to decrease plasma cholesterol levels initially (1384 ± 78.53 vs 1126 ± 46.28 mg/dl in control and 120G8 treated mice, respectively), but this effect was blunted at later stages of plaque development (1097 ± 44.83 vs 1231 ± 72.31 mg/dl in control and 120G8 treated mice, respectively) (Table 1).

Table 1. Body weight and plasma cholesterol levels

	Control Ab	120G8 mAb
Body weight initiation study (g)	21.10 ± 0.23	20.11 ± 0.42
Body weight progression study (g)	21.90 ± 0.43	22.75 ± 0.31
Plasma cholesterol levels initiation study (mg/dl)	1384 ± 78.53	1126 ± 46.2*
Plasma cholesterol levels progression study (mg/dl)	1097 ± 44.83	1231 ± 72.31

(* $P < 0.05$)

To our surprise, atherosclerosis considerably deteriorated after pDC depletion. Plaque volume was 2-fold increased in the plaque initiation study ($1.4 \times 10^7 \pm 2.6 \times 10^6$ vs $2.7 \times 10^7 \pm 4.7 \times 10^6 \mu\text{m}^3$ in control and 120G8 treated mice, respectively) ($P < 0.05$) (**Figure 4A**), while we observed a 3-fold increase in plaque progression compared to plaques at baseline ($1.4 \times 10^7 \pm 2.5 \times 10^6$ vs $5.4 \times 10^7 \pm 7.2 \times 10^6 \mu\text{m}^3$ in control baseline and 120G8 treated mice, respectively) ($P < 0.0005$) versus only 2-fold for the GL113 Ab-treated mice ($1.4 \times 10^7 \pm 2.5 \times 10^6$ vs $3.3 \times 10^7 \pm 7.5 \times 10^6 \mu\text{m}^3$ in control baseline and GL113 treated mice, respectively) ($p < 0.05$) (**Figure 4B**). Likewise, pDC depletion induced a more unstable plaque phenotype in the progression study, characterized by necrotic core expansion (**Figure 4C**) and diminished cap vSMC content (**Figure 4D**). There was no difference in plaque collagen content (data not shown). In addition to the carotid artery, we also examined atherosclerosis development in the aortic root, showing an essentially similar aggravation of lesion formation after pDC depletion ($P < 0.05$; **Figure 4E**).

Thus our data point to an unexpected protective role of pDCs in atherosclerosis, which is in contrast to the prevailing notion that pDCs might promote atherosclerosis by activating T cells in a type-I IFN dependent manner.^(5,27) Moreover, given the fact that atherosclerotic lesions are virtually devoid of pDCs, they most likely exert their athero-protective effect by modulating immune responses in the periphery and/or adventitia.

Figure 4. pDC depletion significantly deteriorates atherosclerotic plaque development and progression in *Ldlr*^{-/-} mice. (A) Effects of pDC depletion on atherosclerosis development (collar model) (GL113 (n=9) vs 120G8 (n=10) treated mice). Right carotid artery was stained with hematoxylin and eosin (HE) to analyze the extent of atherosclerosis (plaque volume, μm^3). (B) Effects of pDC depletion on atherosclerosis progression (collar model) (GL113 baseline (n=9), GL113 (n=8) and 120G8 (n=9) treated mice). GL113 baseline group represents plaque formation at start of antibody treatment. Right carotid artery was stained with HE and atherosclerotic lesion burden was analyzed (plaque volume, μm^3). (C) Necrotic core area is represented as the percentage of lipid core area over plaque area. Data are represented for the plaque progression study. (D) ASMA content is presented as the percentage of ASMA-positive cells per plaque area. Data are represented for the plaque progression study. (E) Effects of pDC depletion on atherosclerosis development in the aortic root (GL113 (n=9) vs 120G8 (n=10) treated mice). The aortic root was stained with HE to analyze the extent of atherosclerosis (plaque area, μm^2). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

pDCs exert their athero-protective effect by regulating CD4⁺ T cell proliferation and function in lymphoid tissue

As a next step, we examined effects of pDC depletion on plaque composition to address the potential mechanisms responsible for the protective effects of pDCs in atherosclerosis. We found that pDC depletion led to an increase in lesional T cell accumulation ($P < 0.05$; **Figure 5A** and **Figure**

6A). The scarce presence of pDCs in mouse atherosclerotic lesions suggests that the increased T cell infiltration into the lesions most likely reflects peripheral modulation of T cell function. Indeed, blood and spleen T cell content was increased in pDC-depleted mice ($P<0.05$; **Figure 5B**), suggesting that pDCs interfere with T cell homeostasis.

Figure 5. pDC depletion results in increased CD4⁺ T cell proliferation. (A) CD3⁺ T cell content in mouse atherosclerotic lesions (collar model) (GL113 (n=9) vs 120G8 (n=10) treated mice). Data are represented for the plaque initiation study. (B) Flow cytometry analysis for CD3⁺ T cells in blood and spleen. (C) BrdU staining of CD4⁺ T cells in spleen from GL113 (n=4) vs 120G8 (n=4) treated *Ldlr*^{-/-} mice on high fat diet. (D) OT-2 CD4⁺ T cell proliferation in spleen in response to ovalbumin challenge (OVA₃₂₃₋₃₃₉) in GL113 (n=4) versus 120G8 (n=4) treated *Ldlr*^{-/-} mice on high fat diet. (E) IFN- γ plasma levels (n=8 GL113 vs n=8 120G8). (F) IFN- γ gene expression by splenic CD3⁺ T cells isolated from GL113 (n=8) versus 120G8 (n=8) treated mice. * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

As pDCs have been reported to induce regulatory T cell (Treg) expansion,^(11,28) we investigated whether the increase in plaque CD3⁺ T cell content is owing to decreased Treg numbers or function and an associated failure to control T cell responses. However, we did not observe a difference in blood and spleen Treg numbers between 120G8 and GL113 treated *Ldlr*^{-/-} mice (**Figure 6B**). In keeping, IL-10 plasma levels did not differ as well (**Figure 6C**). To address whether the increase in T cells resulted from increased proliferation or survival, we examined T cell proliferation *in vivo*. Spleens of pDC-depleted mice were enriched in BrdU⁺ CD4⁺ ($P<0.05$; **Figure 5C**) but not CD8⁺ T cells (**Figure 6D**). Moreover, T cell proliferation appeared to be antigen-dependent in that ovalbumin (OVA) challenge led to augmented proliferation of OT-2 CD4⁺ T cells ($P<0.05$; **Figure 5D**) but not OT-1 CD8⁺ T cells (**Figure 6E**) in pDC-depleted versus non-depleted mice. In addition, these findings also show that the increased proliferation is not due to intrinsic T cell effects, as both innate and administered T cell proliferation was increased, but to changes in the environment in pDC-depleted mice. In parallel to increased T cell proliferation, IFN- γ ($P<0.0005$; **Figure 5E**) as well as IL-6 and MCP-1 plasma levels (**Figure 6F**) were seen to be increased after pDC depletion. FACS sorted CD3⁺ T cells (purity 95%) from spleens of pDC-depleted *Ldlr*^{-/-} mice tended to have increased IFN- γ expression ($P=0.07$; **Figure 5F**). In addition, CD3⁺ T cells isolated from atherosclerotic (high fat diet fed) *Ldlr*^{-/-} mice expressed higher levels of GATA-3 (Th2 marker) as well as of T-bet (Th1 marker) compared to T cells isolated from non-atherosclerotic mice (chow diet fed) (**Figure 6G**). pDC depletion abrogated this effect for GATA-3 but not T-bet, suggesting that pDCs might help to dampen the high fat diet induced Th1 shift in *Ldlr*^{-/-} mice. Overall, these data further substantiate a tolerogenic activity of pDCs under atherogenic conditions, probably by suppressing T cell proliferation and function.

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Figure 6. T cell analysis of atherosclerotic mice treated with GL113 versus 120G8. (A) CD3⁺ T cell content in mouse atherosclerotic lesions (aortic root) (GL113 (n=9) vs 120G8 (n=10) treated mice, $P=0.08$). Data are represented for the plaque initiation study. (B) Depicted are the results of flow cytometry analysis for regulatory T cell numbers in blood and spleen from *Ldlr*^{-/-} mice on high fat diet treated with GL113 (n=9) and 120G8 (n=10). (C) IL-10 plasma levels. (D) BrdU staining of CD8⁺ T cells in spleen from *Ldlr*^{-/-} mice on high fat diet treated with GL113 (n=4) versus 120G8 (n=4). (E) OT-1 CD8⁺ T cell proliferation in spleen in response to ovalbumin challenge (n=4/group). (F) IL-6 and MCP-1 plasma levels (n=8 GL113 vs n=8 120G8). (G) Quantitative real-time PCR analysis of CD3⁺ T cells sorted from spleen from *Ldlr*^{-/-} mice on chow diet (n=5) and high fat diet treated with GL113 (n=5) and 120G8 (n=5).

pDCs suppress CD4⁺ T cell proliferation in an IDO-dependent manner

To address the underlying mechanism for the T cell suppressive capacity of pDCs in atherosclerosis, we compared the expression of known key regulators of pDC tolerogenicity, such as indoleamine 2,3-dioxygenase (IDO), IL-10, programmed death ligand-1 (PD-L1) and inducible co-stimulator-ligand (ICOS-L), by pDCs isolated from chow (non-atherosclerotic mice) versus high fat diet (atherosclerotic mice) fed *Ldlr*^{-/-} mice (purity 98.3%). Expression of PD-L1 and IDO ($P<0.05$; **Figure 7A**), but not IL-10 and ICOS-L (data not shown), was significantly elevated in pDCs from high fat diet versus chow fed mice.

Importantly, CD3⁺ T cells isolated from spleen of pDC-depleted atherosclerotic mice, co-cultured with pDCs in the presence of 1-methyl-tryptophan (1-MT), an IDO blocker, but not anti-PD-L1, displayed markedly induced T cell proliferation ($P < 0.05$; **Figure 7B**), suggesting that pDCs suppress T cell proliferation in an IDO-dependent manner. The 1-MT induced CD4⁺ T cell mitogenic response was confirmed by flow cytometry (**Figure 7C**), showing a similar increment in CD4⁺ T cell proliferation after co-culture with pDCs in the presence of 1-MT. Baseline levels of IFN- α in plasma of high fat diet versus chow fed *Ldlr*^{-/-} mice were unaltered (**Figure 7D**), as well as IFN- α expression by pDCs (**Figure 7E**), firmly establishing that in mice pro-atherogenic conditions *per se* do not promote IFN- α release and immunogenic activity. Moreover, we extend these findings to the human context, as like in *Ldlr*^{-/-} mice, IFN- α plasma levels were also seen to be unchanged (**Figure 7F**) and IFN- α expression by pDCs even significantly lowered in atherosclerotic patients versus healthy controls ($P < 0.05$; **Figure 7G**). Altogether, our data indicate that both in mice and man, conditions of chronic atherosclerosis do not trigger pDC immunogenic activity, IFN- α release and ensuing T cell activation and proliferation. Rather, this milieu may even consolidate pDC's innate tolerogenic capacity to suppress T cell proliferation.⁽²⁹⁾

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Figure 7. pDCs suppress CD4⁺ T cell proliferation in an IDO-dependent manner. (A) PD-L1 and IDO gene expression by pDCs isolated from spleen from atherosclerotic (high fat diet, n=8) and non-atherosclerotic mice (chow diet, n=8). **(B)** *In vitro* co-culture of pDCs and CD3⁺ T cells (in quatro) isolated from spleen from *Ldlr*^{-/-} mice on high fat diet in the presence or absence of 1-MT (100μM) or anti-PD-L1 (20μg/ml). T cell proliferation was assessed by [3H]-thymidine incorporation and plotted as relative proliferation index, being the ratio of T cell proliferation in the presence and absence of pDCs. This experiment was reproduced twice and gave similar results. **(C)** *In vitro* co-culture of pDCs and CFSE labeled CD4⁺ T cells (in duplo) isolated from spleen from *Ldlr*^{-/-} mice on high fat diet in the presence or absence of 1-MT (100μM). **(D)** IFN-α plasma levels and **(E)** IFN-α pDC gene expression in atherosclerotic (high fat diet, n=8) versus non-atherosclerotic (chow diet, n=8) *Ldlr*^{-/-} mice. **(F)** IFN-α plasma levels and **(G)** IFN-α pDC gene expression in atherosclerotic patients (carotid endarterectomy (CEA), n=14) versus healthy controls (n=15).

Discussion

In this study, we are the first to demonstrate a contributory role for pDCs in atherosclerosis. Despite the scarce presence of pDCs in mouse

atherosclerotic lesions, depletion of pDCs in *Ldlr*^{-/-} mice by 120G8 mAb aggravated atherosclerosis development and progression. Lesions of pDC-depleted mice were characterized by increased T cell accumulation and a more unstable plaque phenotype, which, as we show, is likely linked to a deficiency in pDC-associated epitope specific dampening of T cell response.

We demonstrate selective and almost complete pDC depletion by the use of 120G8 mAb in *Ldlr*^{-/-} mice. PDCA-1 expression was exclusively restricted to the pDC population and no other leukocyte subsets other than pDCs were depleted. These findings confirm previous reports which highlight the specificity of the 120G8 mAb, all showing selective depletion of pDCs in blood, bone marrow, LN, thymus and non-lymphoid organs of C57Bl/6 mice but not of CD4/CD8 T cells, DX5⁺ NK en CD19⁺ B cells.⁽³⁰⁻³³⁾ Alternative pDC ablation or depletion models currently available such as IKAROS and IRF8 (mutant) all suffer from major effects on non-pDC subsets, while the CD11c.CRE_{E22}^{f/-} and the BDCA-2.hDTR mice are interesting new models for future ablation studies.

Our data point to an unexpected athero-protective activity of pDCs, which is in contrast to previous findings pointing towards a pro-atherogenic function.⁽⁵⁾ This notion was largely based on guilt by association, in that (1) plaques were seen to express CD123⁺ and IFN- α ⁺ cells, in particular when progressed to an unstable phenotype, (2) CpG-induced pDC activation *in vitro* led to type I IFN release, and (3) type I IFN were recently reported to contribute to atherosclerosis in *ApoE*^{-/-} and *Ldlr*^{-/-} mice by stimulating macrophage recruitment.⁽²⁷⁾ The data presented in this study justify a minor adjustment of this assumption. First, unlike BDCA-4, CD123 staining may not be entirely reflective of the plaque's pDC content as macrophages and vSMCs appear to express this marker as well and as CD123⁺ cells often lack characteristic plasmacytoid morphology. This observation concurs with recent findings by van Vré *et al.*, showing that CD123 is not a specific pDC marker staining also for endothelial cells in human atherosclerotic lesions.⁽²⁴⁾ As a result, the actual plaque pDC content may not only be lower than originally envisioned, but also does not markedly increase with progression of disease. This also implies that pDC effects may be precipitated primarily in the periphery rather than within the plaque itself. Second, we show here that pDCs are not the prime source of plasma IFN- α at baseline, and that IFN- α release by pDCs into the circulation is boosted by CpG treatment, however not by atherogenic conditions. Apparently, atherogenic stimuli *per se* do not induce

pDC activation. Moreover, in atherosclerotic mice, circulating IFN- α originates from other cell types than pDCs but may be derived from macrophages. Third, we failed to demonstrate progressively increased expression of IFN- α by microarray or real-time PCR analysis by circulating pDCs from atherosclerotic mice and by human pDCs from patients with stable versus unstable disease and by unstable versus stable endarterectomy lesions, confirming that in chronic inflammatory processes such as atherosclerosis TLR7/9 activation of pDCs is not very prominent. Collectively, our data indicate that pDCs exert their athero-protective effect primarily by modulating extravascular immune responses.

Our studies also provide a plausible mechanism by which pDCs suppress CD4⁺ T cell proliferation under conditions of atherosclerosis. pDCs isolated from spleens from atherosclerotic mice had a 2-fold increase in expression of tolerogenic molecules IDO and PD-L1 compared to pDCs isolated from non-atherosclerotic mice. IDO is an intracellular tryptophan catabolizing enzyme which has been attributed suppressive activity on cDCs and stimulatory activity on Tregs.⁽³⁴⁾ PD-L1 is an inhibitory co-stimulatory molecule which interacts with programmed death-1 (PD-1) on CD8⁺ T cells to suppress their viability and activity.⁽³⁵⁾

Moreover, co-culture of pDCs with T cells in the presence of 1-MT, an IDO blocker, but not anti-PD-L1, showed increased T cell proliferation, suggesting that pDCs suppress T cell proliferation in an IDO dependent manner. These observations correspond with previous reports in which pDCs were shown to induce tolerance in other low grade chronic inflammatory and autoimmune diseases.^(9,10,34-36) The tolerogenic function of pDCs was seen to depend on cytokine/ligand activation. For instance, B7-1 (CD80) engagement by cytotoxic T-lymphocyte Antigen-4 (CTLA-4Ig), that of CD200R1 by CD200Ig and B7-1/B7-2 (CD80/CD86) by CD28Ig all have been shown to be able to induce the release of IDO by pDCs, leading to the suppression of T cells.⁽³⁷⁾ It remains to be established which activation pathway is involved in atherosclerosis. Thus, in analogy, during atherosclerosis pDCs not only maintain their immature tolerogenic state, but even invigorate their inborn dampening activity so that they can control T cell activity. If the same also holds for brief episodes of fulminant plaque inflammation (acute myocardial infarction), remains to be established.

In conclusion, this manuscript is the first to unveil a protective role for pDCs in an established mouse model of atherosclerosis, throughout disease progression. Given the virtual absence of pDCs in the plaque itself, pDCs most likely exert their activity extravascularly, by dampening T cell proliferation and function in an IDO dependent manner. While these findings identify pDCs as an interesting new target for therapeutic intervention studies, they warrant further study to elucidate the actual pathways underlying the augmented tolerogenic activity of pDCs under conditions of atherosclerosis.

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Contribution to the manuscript (Chapter 4):

Anette Christ designed this study together with Dr. Daissormont and Prof. Biessen. Experiments were mainly accomplished and analyzed by Anette Christ. Dr. Daissormont assisted basically with the performance of the bone marrow transplantation experiment and the animal sacrifice. Animal treatments prior to sacrifice (injection of diphtheria toxin/ diphtheria toxin mutant; injection of the anti-CD4 depletion antibody/ control antibody) were conducted by Anette Christ. In addition, Anette Christ was responsible for the acquirement and the analysis of mouse flow cytometry data (influence of diphtheria toxin treatment on leukocyte subsets isolated from different lymphoid organs) and mouse histology data (aortic roots, lung, liver, heart, kidney), as well as the measurement of plasma cytokine and cholesterol levels. Data were discussed and interpreted by Anette Christ, Dr. Daissormont and Prof. Biessen. Anette Christ wrote the manuscript.

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Plasmacytoid dendritic cell ablation by diphtheria toxin administration in *Ldlr*^{-/-} mice expressing the BDCA2-DTR induces granulocytosis and over signs of pneumonia.

Abstract

Plasmacytoid dendritic cells (pDCs) play a pivotal role in orchestrating immunity and tolerance, and as such, they are key targets for immunotherapy. It has been shown that pDCs populate the inflamed human atherosclerotic plaque, proposing a pro-inflammatory role through release of type I interferons (IFNs). Studying a functional role of pDCs in atherosclerosis by use of pDC-specific depletion antibodies showed controversial results, with pDCs seen to inhibit or promote lesion development. Therefore, alternative animal models are necessary to elucidate the involvement of pDCs in atherosclerosis. The use of the inducible pDC ablation model based on the transgenic diphtheria toxin receptor (DTR) expression under the pDC specific human blood dendritic cells antigen 2 (BDCA-2) promoter may be more revealing. Here, we depleted pDCs in high fat diet fed low-density lipoprotein receptor deficient (*Ldlr*^{-/-}) mice that were reconstituted with BDCA-2 DTR derived bone marrow by administration of diphtheria toxin (DT) for 3 weeks. A subset of mice additionally received a CD4⁺ T cell depleting antibody to address the contribution of CD4⁺ T cells to pDC immunity in atherosclerosis. Unexpectedly, next to pDC depletion, repeated DT administration induced immune cell modulations, shown by peripheral granulocytosis and significant reductions in B cell and cDC numbers. Beside, DT caused systemic toxicity, featured by gradual body weight loss, generalized weakness, sudden death and over signs of pneumonia. We can conclude from our study that the BDCA-2 DTR model is not applicable to study the impact of pDCs in atherosclerosis due to undesirable side effects, pointing to a major limitation of this otherwise technically interesting approach.

Introduction

Plasmacytoid dendritic cells (pDCs) represent a rare leukocyte population present in blood and peripheral lymphoid organs. In response to viruses, and subsequent activation of the Toll-like receptor 7 (TLR7) and TLR9 pathways, pDCs secrete large amounts of type I interferons (IFNs), making pDCs refractory to viral infections.⁽¹⁻⁴⁾ Additionally, immature or alternatively activated pDCs are also equipped to trigger regulatory T cell (T_{reg}) expansion, thereby inhibiting acute graft-versus host disease⁽⁵⁾ or mediating oral tolerance to vascularized grafts.⁽⁶⁾ pDC-mediated control of inflammation was in some studies reported to depend on the type I IFN induced release of indoleamine-2,3-dioxygenase (IDO),^(7, 8) while other studies implicated IL-10, ICOS-L and PD-L1 dependent mechanisms in pDC tolerogenic capacity.^(9, 10) Thus, pDCs are essentially involved in both the control of anti-viral immune responses, characterized by a type I IFN-signature, and in tolerance induction at sites of infection in order to prevent immunopathology.

In recent years the presence of pDCs has been described in human atherosclerotic lesions, predominantly in the plaque shoulder region in close vicinity with IFN- α^+ cells, conventional DCs (cDCs) and activated T cells.⁽¹¹⁻¹⁴⁾ It is believed that plaque pDCs regulate the functional activity of cDCs and augment adaptive immune responses by CD4⁺ T cell activation through release of type I IFNs.^(11, 14) However, we failed to demonstrate co-localization of BDCA-4⁺ pDCs and IFN- α^+ cells in advanced human plaques, encouraging that pDC activation is not very prominent in atherosclerosis.⁽¹⁵⁾ Results from animal models, studying a functional role of pDCs in atherosclerosis, are controversial.⁽¹⁵⁻¹⁷⁾ We have recently shown, that selective depletion of pDCs by 120G8 monoclonal antibody (mAb) administration⁽¹⁸⁾ in low-density lipoprotein receptor deficient (*Ldlr*^{-/-}) mice fed a high fat diet protected against atherosclerosis by tuning T cell proliferation and activation.⁽¹⁵⁾ In contrast, two other groups have exhibited that pDC presence contributes significantly to diet-induced lesion formation in the aortic root and the aorta by their ability to prime and stimulate CD4⁺ T cells. Selective pDC depletion resulted in a more stable plaque phenotype and decreased systemic immune cell activation accompanied by reduced pro-inflammatory cytokine levels.^(16, 17) Recapitulatory, a functional contribution of pDCs to the pathogenesis of atherosclerosis needs to be further investigated in more advanced animal

models such as knock-out models in which the genetic deletion of transcription factors, crucial for pDC development, would specifically block pDC differentiation. An alternative strategy to address pDC function *in vivo* is based on genetic pDC ablation models, such as mice expressing the diphtheria toxin receptor (DTR) under the control of the highly specific human pDC gene promoter BDCA-2.^(19, 20) Indeed, administration of diphtheria toxin (DT) to BDCA-2 DTR transgenic mice has been shown to lead to an almost complete and selective depletion of pDCs.⁽²¹⁾

To address the impact of pDCs on atherosclerotic plaque progression and inflammation, we used bone marrow transplantation to generate *Ldlr*^{-/-} chimeras with hematopoietic BDCA-2 driven DTR expression for conditional pDC ablation. To dissect potential pDC-CD4⁺ T cell crosstalk in atherosclerosis, we additionally depleted CD4⁺ T cells in a subset of mice. Unexpectedly, long-term DT administration induced generalized body infirmness, and caused a dramatic increase in granulocyte numbers in the periphery, accompanied by tremendous immune cell infiltration in lung. We thus conclude that the BDCA-2 DTR model is not suitable to study pDC functions in atherosclerosis, due to the ectopic side-effects associated with long-term DT administration.

Methods

Animals

All animal work was approved by the regulatory authority of Maastricht University and performed in compliance with the Dutch government guidelines. BDCA-2 DTR (= diphtheria toxin receptor) transgenic mice⁽²¹⁾ on a C57Bl/6J background, maintained as heterozygotes, were provided by M. Colonna (Department of Pathology and Immunology, Washington University School of Medicine). *Ldlr*^{-/-} mice were bred at the local animal breeding facility (Maastricht University, The Netherlands). All mice were fed a standard fat diet (Cat# V1535, sniff Spezialdiäten GmbH, Soest, Germany), had *ad libitum* access to food and water, and were housed under a 12 hour light-dark cycle.

Bone marrow transplantation

Female *Ldlr*^{-/-} mice (n=37), 12 weeks of age, were housed under filter top cages and given antibiotics supplemented water (Neomycin (100mg/L; Gibco, Carlsbad, CA, USA) and Polymyxin B sulfate (60.000 U/L; Gibco), starting 2 weeks before until 6 weeks after bone marrow transplantation. To induce bone marrow aplasia, *Ldlr*^{-/-} mice were exposed to a single dose of 9Gy (0.5Gy/min, Philips MU15F/225kV, Hamburg, Germany) total body irradiation one day before bone marrow transplantation. Irradiated recipients were injected via the tail vein with bone marrow cell suspensions (10⁷ cells/mouse), prepared from BDCA-2 DTR donor mice by tibia/ femur lavage. Six weeks after bone marrow transplantation, mice were placed on a western type diet (0.25% cholesterol and 15% cacao butter; Special Diets Services, Witham, Essex, UK) for another 6 weeks to induce atherosclerosis development. To study effects of pDC depletion on atherosclerosis development, diphtheria toxin (DT, Sigma-Aldrich, Seelze, Germany; 120 ng/mouse/injection) was injected intraperitoneally (i.p.) three times per week over a period of three weeks before animals were sacrificed. A nontoxic diphtheria toxin mutant (DTM CRM197, Sigma-Aldrich, Seelze, Germany) served as control. To study effects of CD4⁺ T cell depletion in control mice compared to pDC-depleted mice, a CD4⁺ T cell depleting antibody, α CD4 (150 μ g/mouse/injection, Biocerus, Utrecht, The Netherlands), was administered 3 times per week i.p. over a period of three weeks before sacrifice. As control antibody, an isotype control (GL113, Biocerus, Utrecht, The Netherlands) was used.

Atherosclerosis experiments

Mice were euthanized by an overdose of pentobarbital (115 mg/kg) and perfused through the left cardiac ventricle with PBS (NaCl/Na₂HPO₄/KH₂PO₄; pH 7.4) containing sodium nitroprusside (0.1mg/ml; Sigma-Aldrich, Seelze, Germany) and 1% paraformaldehyde (PFA). The aortic arch and the heart were removed, fixed overnight in 1% PFA and paraffin-embedded sections (4 μ m) were cut. To measure plaque volume and necrotic core size in the aortic root, plaque area was measured for each valve for consecutive sections at 20 μ m intervals that covered the entire lesion. Plaque collagen content was quantified based on Sirius Red staining under polarized light. Slides were analyzed in a blinded manner using a Leica DM3000 light

microscope (Leica Microsystems, Wetzlar, Germany) coupled to a computerized morphometric system (Leica Qwin 3.5.1, Wetzlar, Germany).

Immunohistochemistry

Immunohistochemical stainings were performed on paraffin sections of murine heart, lung, liver and kidney: CD45 polyclonal antibody (1:5000 dilution, BD Bioscience, Bedford, MA, USA) to detect total leukocytes, CD3 polyclonal antibody (1:200 dilution, DAKO, Glostrup, Denmark) to detect T lymphocytes, Mac3 monoclonal antibody (1:30 dilution, BD Bioscience, Bedford, MA, USA) to detect macrophages, Ly6G monoclonal antibody (1:200 dilution, BD Bioscience, Bedford, MA, USA) to detect Ly6G positive granulocytes and Caspase 3 polyclonal antibody (1:20 dilution, Cell Signaling, Danvers, MA, USA) to detect apoptotic cells. Slides were analyzed using Leica Qwin program.

Plasma cytokine and cholesterol analysis

Plasma cholesterol levels were measured in duplicate using a colorimetric assay (CHOD-PAP, Roche, Mannheim, Germany). Plasma cytokine levels of IFN- γ , IL-10, IL-12p70, IL-1 β , IL-6 and TNF- α were determined with Multi-Spot assay system (Meso Scale Discovery, Rockville, MD, USA).

Flow cytometry

Blood, spleen, thymus, mesenteric lymph nodes (MLN), peripheral lymph nodes (a mixture of mandibular and axillary lymph nodes) (LN), liver and small intestines were isolated before perfusion, processed and stained for total T cells (CD3e⁺, Miltenyi Biotec, Bergisch-Gladbach, Germany), T helper cells (CD4⁺, BD Bioscience, Bedford, MA, USA), cytotoxic T cells (CD8a⁺, BD Bioscience), regulatory T cells (CD4⁺ CD25⁺ Foxp3⁺, eBioscience, San Diego, CA, USA), effector T cells (CD44^{high}, eBioscience), B cells (B220⁺, BD Bioscience), monocytes (CD11b^{high} Ly6G^{low}, BD Bioscience), granulocytes (CD11b^{high} Ly6G^{high}, BD Bioscience), NK cells (NK1.1⁺ CD3⁻), conventional DCs (CD11c^{high}, eBioscience) and plasmacytoid DCs (PDCA-1^{high} CCR9^{high}, eBioscience). For cDC and pDC analysis, spleen, peripheral LN, mesenteric LN, liver and intestines were pretreated for 30 minutes with a cocktail of liberase (0.032mg/ml, Roche, Carlsbad, CA, USA) and DNase (0.8 μ g/ml, Roche, Basel, Switzerland) in RPMI medium. Cell counts were estimated by absolute counting beads (BD

Bioscience, Bedford, MA, USA). All flow cytometry analysis was performed on a BDCanto II (BD Bioscience, Bedford, MA, USA).

Statistical analysis

Data were analyzed with the use of Graph Pad Prism 5 (Graph Pad Software Inc San Diego, CA, USA). All data are expressed as mean $\pm$ SEM. To compare individual groups, a 2-tailed Student's *t* test was used; nonparametric data were analyzed using a Mann-Whitney *U*-test. Values of $p < 0.05$ were considered statistically significant.

Results

DT administration induces effective depletion of pDCs but also influences cDC numbers in *Ldlr*^{-/-} mice on high fat diet

We generated *Ldlr*^{-/-} hematopoietic chimeras reconstituted with bone marrow from transgenic mice for the human diphtheria toxin receptor (DTR) under control of the highly pDC specific human blood dendritic cell antigen 2 (BDCA-2) (**Figure 1A**). To verify the efficacy of DT-induced pDC depletion in the chimeras, we examined pDC numbers in secondary lymphoid organs. DT administration resulted in an almost complete ablation of pDCs in spleen, peripheral and mesenteric lymph nodes ($p < 0.01$) (**Figure 1B**). Treatment with DT mutant (DTM CRM197), used as control, did not affect PDCA1⁺ CCR9⁺ pDC numbers (relative to a PBS control), establishing DTR involvement in pDC ablation (**Figure 1C**). The group of Colonna has previously shown that DT-induced pDC depletion persists for at least 2-3 days,⁽²¹⁾ corresponding with the pDC half life. However, pDCs could be depleted for several days by repeated DT administration. DT administration to BDCA-2 DTR transgenic C57Bl/6J mice resulted in selective depletion of pDCs, whereas classical cDCs as well as other leukocyte subsets were left intact.⁽²¹⁾ To investigate whether DT administration for a period of 3 weeks in *Ldlr*^{-/-} on high fat diet could modulate cDC numbers we compared percentages of cDCs between DTM and DT-injected mice. DT administration significantly diminished (~ 40 -50%) the relative cDC content in peripheral and mesenteric lymph nodes ($p < 0.01$), while splenic cDC numbers were left intact (**Figure 1D**). Thus, long term DT administration in *Ldlr*^{-/-} mice

expressing the BDCA2-DTR leads to effective depletion of pDCs, but influences also peripheral cDC numbers.

Figure 1. DT administration induces effective depletion of pDCs, but has also significant impact on the cDC subset in high fat diet fed *Ldlr*^{-/-} mice. (A) The construct for BDCA-2 DTR includes a 5kb fragment upstream of the ATG of the BDCA-2 gene, a cDNA fragment encoding DTR, and the human growth hormone (hGH) gene polyadenylation signal.⁽²¹⁾ **(B)** Flow cytometry analysis of spleen, peripheral lymph nodes (LN) and mesenteric lymph nodes (MLN) from high fat diet fed *Ldlr*^{-/-} mice treated for 3 weeks with diphtheria toxin mutant (DTM; n=10) or diphtheria toxin (DT; n=11) revealed a significant decrease of pDC (PDCA-1⁺ CCR9⁺) numbers in all three lymphoid organs. **(C)** Treatment with DTM, used as control, does not affect PDCA1⁺ CCR9⁺ pDC numbers, relative to a PBS control. **(D)** Percentage of cDCs (CD11c^{high}) in spleen, LN and MLN indicates a significant decrease in cDC numbers in LN and MLN in DT-treated compared to control mice.

* p< 0.05, ** p< 0.01, *** p< 0.001.

Effective and selective depletion of CD4⁺ T cells in *Ldlr*^{-/-} mice by administration of an anti-CD4 depletion antibody (α CD4)

Next, we addressed the specificity of CD4⁺ T cell depletion by α CD4 mAb, which recognizes the CD4 co-receptor expressed on T cells. Intraperitoneal administration of α CD4 mAb (but not GL113 isotype control) three times per week over three weeks gave almost quantitative (>95%) depletion of CD4⁺ T cell numbers in blood, spleen, peripheral and mesenteric lymph nodes (p<0.001) (**Figure 2A-C**). Depletion of CD4⁺ T cells resulted in approximately 60% reduction in absolute blood CD3⁺ T cell counts (p<0.001) compared to GL113 treated controls (**Figure 2D**). In line with the quantitative CD4⁺ T cell depletion, CD3⁺ T cells were highly enriched in CD8⁺ T cells (**Figure 2E**). Anti-CD4⁺ T cell treatment did not affect numbers of NKT cells, NK cells, B cells, monocytes, granulocytes, cDCs and pDCs (**Figure 2F**). Thus, the α CD4 mAb gave complete and specific CD4⁺ T cell depletion.

Figure 2. Effective and selective depletion of CD4⁺ T cells in *Ldlr*^{-/-} mice by administration of an anti-CD4 depletion antibody (αCD4). Depicted are the results of flow cytometry from high fat diet fed *Ldlr*^{-/-} mice treated with GL113 (n=11) or αCD4 (n=8). **(A), (B)** Absolute numbers of CD4⁺ T cells in blood are significantly decreased in high fat diet fed *Ldlr*^{-/-} mice treated for 3 weeks with αCD4 depletion antibody. **(C)** Percentage of CD4⁺ T cells in spleen, LN and MLN are significantly declined in αCD4 treated mice. **(D)** Absolute numbers of CD3⁺ T cells in blood. **(E)** Absolute numbers of CD8⁺ T cells in blood. **(F)** Percentage of NKT cells (NK1.1^{low} CD3^{low}), NK cells (NK1.1⁺), B cells (B220⁺), monocytes (CD11b⁺ Ly6G⁻), granulocytes (CD11b⁺ Ly6G⁺), cDCs (CD11c^{high}) and pDCs (PDCA1⁺ CCR9⁺) in spleen. * p< 0.05, ** p< 0.01, *** p< 0.001.

Long-term DT injection alters B cell and granulocyte numbers in *Ldlr*^{-/-} mice on high fat diet

To investigate whether prolonged DT administration by itself could modulate immune responses, we compared leukocyte subset distribution between DTM and DT treated *Ldlr*^{-/-} mice. Absolute CD45⁺ leukocyte counts in blood were unaffected (**Figure 3A**), as well as NK cell, NKT cell and monocyte levels in blood and spleen (**Figure 3A-B; Supplemental Figure 1A-B**). Comparing the T cell activation status between DTM and DT treated *Ldlr*^{-/-} mice we did not find a difference in the number of CD44^{high} effector-memory T cells (**Figure 3C**). Surprisingly, DT administration led to a significant decrease in absolute blood and, albeit to a lesser extent, relative spleen B cell numbers (p<0.01; **Figure 3D-E**), which was not in line with results previously described.⁽²¹⁾ Likewise, absolute numbers of blood B1 and B2 B cell subsets were significantly decreased in DT-treated *Ldlr*^{-/-} mice (**Supplemental Figure S1C-D**). In addition, granulocyte numbers were significantly increased (p<0.001) in blood (**Figure 3F, H**) and spleen (**Figure 3G**) after 3 weeks of DT administration. This suggests that prolonged DT treatment in high fat diet fed *Ldlr*^{-/-} mice is associated with B cell lymphocytopenia and granulocytosis. In parallel to granulocytosis, IL-6 plasma levels were seen to be significantly increased (p<0.05) in DT-treated *Ldlr*^{-/-} mice. Additionally, IFN-γ (**Figure 4A**) and CXCL1 (mKC) (**Figure 4B**) plasma levels tended to be elevated in DT treated compared to control mice (**Figure 4C**) pointing towards a systemic inflammatory status in *Ldlr*^{-/-} mice that received DT administration over a period of 3 weeks. Interestingly, plasma IL-10 levels were also significantly augmented (p<0.05) in DT-treated *Ldlr*^{-/-} mice (**Figure 4D**).

Figure 3. B cell and granulocyte numbers are altered in *Ldlr*^{-/-} mice treated with DT. Flow cytometry analysis on blood and spleen leukocyte patterns in *Ldlr*^{-/-} mice on high fat diet treated with DTM (n=10) or DT (n=11). **(A)** Absolute numbers of CD45⁺ leukocytes, NK cells (NK1.1⁺), NKT cells (NK1.1^{low} CD3^{low}) and monocytes (CD11b⁺ Ly6G⁻) in blood. **(B)** Percentage of NK cells, NKT cells and monocytes in spleen. **(C)** Numbers of effector memory CD4⁺ T cells (CD44^{high} CD62L^{low}) in blood and spleen. **(D)** Absolute numbers of B220⁺ B cells in blood and **(E)** percentage of B cells (B220⁺) in spleen decreased significantly in high fat diet fed *Ldlr*^{-/-} mice treated with DT for 3 weeks compared to controls. **(F)** Absolute numbers of circulating Ly6G^{high} granulocytes and **(G)** percentage of Ly6G^{high} granulocytes in spleen. DT treatment for 3 weeks induced granulocytosis shown by the significantly increased granulocyte numbers. **(H)** FACS plot showing monocytes (CD11b⁺, Ly6G⁻) and granulocytes (CD11b⁺, Ly6G⁺) populations in blood of DT- and DTM-treated *LDLr*^{-/-} mice. * p<0.05, ** p<0.01, *** p<0.001.

Figure 4. Long-term DT treatment induces a systemic inflammatory status in *Ldlr*^{-/-} mice. Measurement of plasma cytokine levels in high fat diet fed *Ldlr*^{-/-} mice treated for 3 weeks with DTM (n=10) or DT (n=11). (A) IFN-γ, (B) CXCL1 (mKC), (C) IL-6, and (D) IL-10 plasma levels. * p < 0.05.

Administration of DT, αCD4 antibody or DT/αCD4 antibody does not reduce atherosclerotic plaque development in *Ldlr*^{-/-} mice

To address the role of pDCs and the possible crosstalk of pDCs and CD4⁺ T cells in atherosclerosis, we examined lesion development in DT-treated, αCD4 mAb-treated, DT- and αCD4 mAb-treated, and control *Ldlr*^{-/-} mice. DT administration and/or αCD4 mAb injection were launched three weeks after start of high fat diet, once initial lesions had formed. DT treatment *per se* did not influence plasma cholesterol levels (1360 ± 83.82 mg/dl vs 1266 ± 114 mg/dl in DTM and DT treated mice, respectively), whereas αCD4 mAb treatment was seen to increase plasma cholesterol levels (1360 ± 83.82 mg/dl vs 1641 ± 67.2 in DTM/ GL113 and DTM/ αCD4 treated mice; 1266 ± 114 mg/dl vs 1647 ± 163 in DT/ GL113 and DT/ αCD4 treated mice, respectively; p < 0.05) (Table 1). To our surprise, atherosclerosis development was unchanged in the aortic root after DT-aided pDC as well as CD4⁺ T cell depletion (Figure 5A).

Unexpectedly, treatment with α CD4 mAb induced a more unstable plaque phenotype, characterized by necrotic core expansion, regardless of the pDC status of the mice (**Figure 5B**). However, collagen content was not affected in α CD4 mAb treated *Ldlr*^{-/-} mice (**Figure 5C**). These data did not corroborate our previous observations in mice, showing that pDCs function athero-protective in that they dampen peripheral CD4⁺ T cells activation and proliferation.⁽¹⁵⁾ However, given the fact that prolonged DT administration is accompanied by pronounced granulocytosis, and diminished cDC and B cell numbers in the periphery, it is conceivable that these ectopic effects have overruled the pDC intrinsic impact on atherosclerosis.

Figure 5. DT treatment in *Ldlr*^{-/-} mice conceivably overrules the intrinsic impact of pDCs and CD4⁺ T cells on atherosclerosis. (A) Effects of DT aided pDC ablation in the presence (GL113 isotype control) or absence of CD4 T cells (α CD4 mAb on atherosclerosis development in the aortic root; control mice were treated with inactive DTM instead of DT (DTM/GL113, n=10; DT/GL113, n=11; DTM/ α CD4, n=8; DT/ α CD4, n=6). Aortic roots were stained with hematoxylin and eosin (HE) to analyze plaque burden (plaque area, μm^2). **(B)** Plaque necrotic core size of the different treatment groups. Necrotic core is defined as percentage of lipid core size over total plaque area. **(C)** Plaque collagen content is expressed as the percentage of collagen over plaque area. Scale bars represent 100 μm . * $p < 0.05$.

DT administration causes significant body weight loss, body infirmity, and induces lung inflammation in *Ldlr*^{-/-} mice

As further indication of peripheral side effects of DT treatment, *Ldlr*^{-/-} mice exhibited severe progressive weight loss of up to 25% of the total body weight after 3 weeks of DT injection, both in GL113 and α CD4 mAb treated mice (**Figure 6, Table 1**) In addition, these animals exhibited generalized infirmity, reduced mobility/agility, sudden death after 18-21 days after repeated DT injection, and over signs of pneumonia.

Figure 6. DT administration causes significant body weight loss and body infirmity in *Ldlr*^{-/-} mice. Effects of DT administration on total body weight. Intraperitoneal DT application for 3 weeks caused a gradual loss of 25% body weight in *Ldlr*^{-/-} mice fed a high fat diet.

* $p < 0.05$, ** $p < 0.01$.

Table 1. Body weight and plasma cholesterol levels

	+ isotype control	+ α CD4 depletion Ab
Body weight DTM-treated mice (g)	23.3 $\pm$ 0.73	23.63 $\pm$ 0.57
Body weight DT-treated mice (g)	19.73 $\pm$ 0.57 *	18.17 $\pm$ 1.14 *
Plasma cholesterol levels DTM-treated mice (mg/ dl)	1360 $\pm$ 83.82	1641 $\pm$ 67.20 *
Plasma cholesterol DT-treated mice (mg/ dl)	1266 $\pm$ 114.0	1647 $\pm$ 163.1

DTM = diphtheria toxin mutant

DT = diphtheria toxin

(* $P < 0.05$)

Therefore, we analyzed the immune cell content in different organs in DT- and DTM-treated animals. Organ histopathology did not reveal any abnormalities in heart and kidney (data not shown) in DT-treated compared to control animals. Livers of DT-treated animals showed less fat accumulation which was in line with their weight loss (**Figure 7A**). However, lung histology demonstrated increased influx of inflammatory cells, mainly Ly6G⁺ granulocytes, Mac3⁺ macrophages and CD3⁺ T cells in the alveoli of DT-treated animals (**Figure 7A, C**). In keeping with the granulomatous lung inflammation, apoptosis was increased in lung alveoli and bronchii of DT-treated compared to control animals, as shown by Caspase 3 staining (**Figure 7D**). In conclusion, our results demonstrate that prolonged DT administration is associated with alveolar immune cell infiltration and apoptosis. It still needs to be analyzed, whether immune cells in lung of DT-treated animals display a more activated phenotype.

Figure 7. DT administration is accompanied by immune cell infiltration in lung. Microscopic analysis of mouse liver and lung. **(A)** Hematoxylin and eosin (HE) staining reveals regular histology for mouse liver of DT- versus DTM-treated animals. In contrast, lung tissue of DT- but not DTM-treated *Ldlr*^{-/-} mice shows overt features of granulomatous inflammation. **(B)** Immunohistochemistry for Ly6G (granulocytes) on mouse liver. **(C)** Immunohistochemistry for Ly6G⁺ granulocytes, Mac3⁺ macrophages and CD3⁺ T cells in lung of DTM- versus DT-treated mice to analyze leukocyte infiltration. DT-treated mice show increased influx of immune cells. **(D)** Immunohistochemistry for Caspase 3 to show content of apoptotic cells in lung in DTM- versus DT-treated mice.

Discussion

pDCs play an important role in orchestrating immunity and tolerance in a variety of diseases.⁽²²⁻²⁶⁾ In recent years pDCs gained more attention in the context of atherosclerosis. Hitherto, antibody depletion models have been used *in vivo* to uncover a functional role for pDCs in the pathophysiology of atherosclerosis. However, results are controversial, with pDCs seen to act tolerogenic or immunogenic, thus attenuating or promoting lesion formation.⁽¹⁵⁻¹⁷⁾

In the present study we used the BDCA-2 DTR model to unravel the impact of pDCs in atherosclerosis. A subset of mice additionally received a CD4⁺ T cell depleting antibody to address the contribution of CD4⁺ T cells to pDC immunity in atherosclerosis. Here, we could confirm, using the BDCA-2 DTR model that PDCA-1⁺ CCR9⁺ pDCs were indeed efficiently depleted after repeated DT administration in spleen, lymph nodes and liver of *Ldlr*^{-/-} mice fed a high fat diet. Surprisingly, under hyperlipidemic, potentially pro-inflammatory conditions in *Ldlr*^{-/-} mice, prolonged application of DT-induced systemic toxicity, manifested through elevated levels of pro-inflammatory cytokines, progressive weight loss, generalized body infirmity and sudden death between days 18 and 21 of DT administration. Moreover, DT-treated mice showed lung infiltration by inflammatory cells - mainly granulocytes, macrophages and T cells, which is not induced under conditions of hyperlipidemia.⁽²⁷⁾ Collectively, these features point to DT treatment associated acute pneumonia. This notion was supported by the elevated numbers of granulocytes and granulocyte progenitors in blood and spleen in DT-treated animals, thus reflecting inflammation elicited release of granulocyte (progenitors) from the bone marrow into the periphery. In line, plasma CXCL1 levels were markedly increased in DT-treated mice compared to control mice, promoting augmented neutrophil mobilization from the bone marrow into the circulation, most likely to lung.^(27, 28)

Our results are in seeming contrast with findings of Swiecki *et al.* showing that specific pDC depletion is not accompanied by overt systemic abnormalities.⁽²¹⁾ It has to be remarked that in the study of Swiecki *et al.*, mice received DT twice before viral challenge and splenic immune cell composition was analyzed on day 7 post-infection. Of note, they did not examine effects of prolonged DT administration. Otherwise, the potentially pro-inflammatory milieu in *Ldlr*^{-/-} mice may have favored ectopic side-effects of DT treatment and/or pDC ablation on other leukocyte subsets in our study.

Interestingly, another study has shown that the use of DT *per se* was seen to confer (sublethal) toxicity, even in toxin-resistant wild-type mice when administered during active immunization, irrespective of the immunogenic peptide applied. Respectable, DT-treated mice in this study also showed mononuclear lung infiltration.⁽²⁹⁾ In a different study, LysM-DTR mice were generated to specifically deplete myelomonocytic cells.⁽³⁰⁾ Although peritoneal macrophages were efficiently depleted after DT injection, expression of the

DTR by alveolar epithelial cells resulted in apoptosis of these cells and lethal respiratory failure. To overcome systemic toxicity and lethality in mice receiving prolonged DT administration due to DTR expression on some alternative cell subsets, bone marrow chimeras in which the DTR expression is restricted to the hematopoietic compartment, should be generated.

Another important limitation of the BDCA2-DTR model for atherosclerosis studies was the observation that during prolonged DT administration in *Ldlr*^{-/-} mice, numbers of CD11c^{high} DCs in lymph nodes as well as B cell numbers in blood and spleen were markedly decreased. These data do not correspond with previous findings of Swiecki *et al.* in which DT administration specifically depleted pDCs, leaving other immune cell subsets intact.⁽²¹⁾ We did not examine whether the BDCA2 promoter contains target sequences for transcription factors others than E2-2, that are important for B cell or cDC development, such as the zinc finger transcriptional regulator Ikaros⁽³¹⁻³³⁾ or the interferon regulatory factor 8 (IRF-8),^(34, 35) but it should be investigated to confirm specificity of the BDCA-2 DTR model. Interestingly, a recent study has described the direct involvement of E2-2 together with the HeLa E-box binding protein E2A in the early B cell development as well as in the selection of peripheral B cell subsets. Transfer of E2-2-deficient cells into irradiated mice could reconstitute all B cell subpopulations, albeit with a reduction in cellularity, requiring E2-2 for the optimal expansion of pro-B cells.⁽³⁶⁾ This could explain a direct effect on peripheral B cell (subset) numbers in our study.

By analyzing the atherosclerotic plaque size in DT-treated *Ldlr*^{-/-} mice, we could not confirm the results received from our previous study, in which we have shown an athero-protective role for pDCs in atherosclerosis.⁽¹⁵⁾ However, given the fact that prolonged DT administration is accompanied by pronounced granulocytosis, and diminished cDC and B cell numbers in the periphery, it is possible that these ectopic effects have overruled the pDC intrinsic impact on atherosclerosis.

In conclusion, our study has shown that the BDCA-2 DTR model is not appropriate to study pDC functions in atherosclerosis, due to systemic toxicity and undesirable modulations of leukocyte subsets others than pDCs after prolonged DT administration. Given the limitations of the BDCA2-hDTR model for prolonged pDC depletion, alternative models, such as the E2-2^{+/-} or E2-2^{-/-}

⁽³⁷⁾ should be considered for conditional pDC depletion studies in these chronic inflammatory disorder.

Supplemental Figures

Supplemental Figure 1. Number of B cell subsets are altered in *Ldlr*^{-/-} mice treated with DT. Flow cytometry analysis on blood and spleen leukocyte patterns in *Ldlr*^{-/-} mice on high fat diet treated with DTM (n=10) or DT (n=11). **(A)** Absolute numbers of monocyte subsets (Ly6C⁻, Ly6C⁺) in blood, and **(B)** percentage of monocyte subsets (Ly6C⁻, Ly6C⁺) in spleen reveal that DT treatment did not affect peripheral monocyte subset numbers. **(C, D)** Long-term DT administration induced a significant decrease in circulating B cell subsets. * p < 0.05.

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Dendritic cells (DCs) are specialized antigen-presenting cells that are thought to be involved in atherosclerosis adaptive immune responses. Maturation and activation of DCs induced by danger signals derived from pathogenic agents or damaged tissue via pattern-recognition receptors such as Toll-like receptors, is a crucial step in DC innate and adaptive immune actions. Hypercholesterolemia, a major risk factor in atherosclerosis development, may influence signaling pathways relevant to DC immune functions, thereby screwing host immune responses against invading pathogens. Indeed, hyperlipidemia has been shown to abrogate the activation of certain murine DC subsets, due to uncoupling of Toll-like receptor-mediated signaling, thus affecting host resistance to microbial infections.

In the following two studies (**Chapter 5** and **6**) we investigated the influence of a hyperlipidemic environment on DC precursor development, peripheral DC subset phenotype (when lipid-laden), their activation status, and functionality. In **Chapter 5** we focused on the effect(s) of hyperlipidemia on human plasmacytoid dendritic cells (pDCs), isolated from peripheral blood mononuclear cells. We indicated that excessive lipoprotein exposure led to impaired functionality of human pDCs *in vitro*. Such association might have profound implications for the host's pathogen defense capacity in hyperlipidemic patients. In **Chapter 6** we investigated the effects of early and later time points of dietary-induced hypercholesterolemia on murine DC (precursor) homeostasis, as well as the phenotype of lipid-laden peripheral DC subsets, and their T cell priming capability. In accordance with our results purchased in Chapter 5, we demonstrated in Chapter 6 that DCs were affected in their T cell priming capability upon lipoprotein uptake. These data strengthen the idea that a cholesterol-rich environment potentially interferes with DC responses to host immunity.

Contribution to the manuscript (Chapter 5):

Anette Christ designed this study together with Prof. Daemen and Prof. Biessen. Experiments were predominantly performed and analyzed by Anette Christ: Anette Christ was responsible for the accomplishment of all pDC *in vitro* culture experiments (isolation of human pDCs from PBMCs/ fresh blood, purification of pDCs by magnetic beads, stimulation of pDCs/ exposure to lipoproteins, analysis of maturation status by flow cytometry, measurement of cyto- and chemokines). LDL and oxLDL were isolated and prepared by Anette Christ. Mixed leukocyte reaction experiments were conducted in cooperation with the Department of Internal Medicine at Maastricht University (Tammy Oth, Dr. Vanderlocht). Anette Christ and Tammy Oth were responsible for the acquirement and the analysis of these data. Final data were discussed and interpreted by Anette Christ, Prof. Daemen and Prof. Biessen. Anette Christ wrote the manuscript. Prof. Daemen and Prof. Biessen revised the written manuscript.

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5

Pro-atherogenic lipid stimulation inhibits TLR9 agonist induced plasmacytoid dendritic cell activation.

- in preparation -

Abstract

Maturation and activation of plasmacytoid dendritic cells (pDCs) induced by danger signals derived from invading pathogens or damaged tissue via pattern-recognition receptors, is a pivotal step in pDC innate and adaptive immune functions. Exposure to factors, such as lipoproteins, may well influence signaling pathways relevant to pDC immune functions, thereby tuning host immune responses against endogenous, bacterial and viral pathogens. In this study, we have investigated the effect of a hyperlipidemic environment on human pDC activation, cytokine response and capacity to prime CD4⁺ T cells. We show that exposure to pro-atherogenic lipoproteins (LDL and oxidized LDL (oxLDL)), induced pDC lipid accumulation, ablated Toll-like receptor 9 (TLR9)-induced production of pro-inflammatory cytokines, including interferon (IFN)- α , interleukin (IL)-6, tumor necrosis factor (TNF)- α , CCL3 and CCL4, as well as up-regulation of the pDC maturation markers CD40, CD83, CD86 and HLA-DR. Additionally, oxLDL induced blunting of pDC activation and profoundly impaired their CD4⁺ T helper (Th) cell stimulatory capacity. These results show that a hyperlipidemic environment does interfere with pDC responses to pathogen-derived signals through abrogating pDC activation.

Introduction

Dendritic cells (DCs) are specialized antigen-presenting cells (APCs), and therefore pivotal in mediating adaptive immune responses, either inducing immunity or a state of tolerance.^(1, 2) Under healthy steady-state conditions DCs reside as immature sentinels in peripheral tissue and lymphoid organs to scan and respond to changes in their environment.^(3, 4) Upon stimulation by danger signals derived from invading pathogens and/or injured tissue through pattern-recognition receptors (PRRs), such as Toll-like receptors (TLRs), DCs get activated.^(5, 6) This process is characterized by major phenotypic changes, including the up-regulation of maturation markers, such as CD40, CD80, CD86 and major histocompatibility complex (MHC) molecules, and the release of a range of cytokines, critically implicated in the activation of naïve T cells.^(7, 8)

Plasmacytoid DCs (pDCs), an unconventional DC subset, are highly specialized in sensing viral and certain microbial infections, which is based on their expression of TLR7 and TLR9.⁽⁹⁾ TLR receptor activation leads to the unique ability of pDCs to release large amounts of type I interferons (IFNs; IFN- α , - β , - λ , - ω), which notably influences several immune responses, for example. Th1/2 differentiation, conventional DC (cDC) maturation, and stimulation of B cells to differentiate into antibody secreting plasma cells^(9, 10) Thus, pDCs are important players in initiating innate as well as adaptive immune responses. Otherwise, pDCs have also been shown to be involved in the pathophysiology of certain autoimmune diseases, such as systemic lupus erythematosus (SLE) or psoriasis, through the indelible production of type I IFNs, which stimulate autoreactive T and B cells.⁽¹¹⁾ However, immature pDCs are also contributing to peripheral T cell tolerance, as has been shown in cardiac transplantation and in oral tolerance.^(12, 13)

Interstitial fluids, lymph, and plasma compose the environment in which certain DC subsets are exposed to danger signals and they might thus steer immune responses. Lipoproteins, laden with bioactive lipids, may be particularly potent in this regard, and a perturbation of lipoprotein homeostasis by environmental or genetic factors may therefore impact (p)DC function.^(14, 15) Whether or not DC subsets are fully functional under dyslipidemic conditions is still subject to controversy. Hypercholesterolemia was seen to lead to impaired DC migration,⁽¹⁶⁾ but also to the maintenance of antigen-presentation and T cell priming ability by several DC subsets.⁽¹⁷⁻¹⁹⁾ At a

functional level, dyslipidemia was recently reported to directly affect murine CD8⁺ cDCs, impairing their response to TLR stimulation, and compromising host resistance to *Leishmania major*.⁽²⁰⁾ In keeping, overt TLR3 and 4 dependent immuno-inhibitory effects were observed for oxidized phospholipids (oxPLs) on LPS-induced DC maturation and cytokine release.⁽²¹⁾ However, no studies thus far have addressed whether a hyperlipidemic environment interferes with pDC responses to (pathogen-derived) danger signals and anti-viral host immunity.

In this study we have investigated the effect of hyperlipidemia on human pDC activation and CD4⁺ Th cell polarization capability *in vitro*. We show that modified lipoproteins impair pDC maturation and functionality. This subsequently leads to abated cell-mediated immune responses by pDCs.

Methods

Isolation and culture of pDCs

Peripheral blood mononuclear cells (PBMCs) were prepared from heparinised whole blood of healthy donors by standard Ficoll-Paque (Sigma-Aldrich, Seelze, Germany) density gradient centrifugation. pDCs were isolated from PBMCs using the BDCA-4 Cell Isolation Kit (Miltenyi Biotec, Bergisch-Gladbach, Germany) and phosphate buffered saline (PBS)/ EDTA Buffer (Miltenyi Biotec) supplemented with 0.5% bovine serum albumine (BSA; Sigma-Aldrich). Labeled cells were enriched using permanent magnets yielding a high-gradient magnetic field (midiMACS separators, Miltenyi Biotec). The positively sorted pDC fraction was analyzed by flow cytometry on a BD Canto II (BD Bioscience, Bedford, MA, USA) according to their BDCA-2⁺, CD123⁺, CD11c⁻, CD3⁻, CD14⁻, CD19⁻, CD20⁻ and CD56⁻ phenotype and reached > 85% purity. Isolated pDCs were seeded in round-bottomed 96-well cell culture plates at a density of 5x10⁴ cells/ well in AIM-V medium (Invitrogen, Darmstadt, Germany), and cultured in the presence of 10μM CpG (CpG-ODN 2216, type A; Invivogen, San Diego, CA, USA), CpG (10μM) plus LDL (50μg/ml), CpG (10μM) plus oxLDL (10μg/ml or 50μg/ml), LDL (50μg/ml) or oxLDL (10μg/ml or 50μg/ml) for 24 hours at 37°C and 5% CO₂.

OxLDL preparation

Low-density lipoprotein (LDL) ($1.006 \leq d \leq 1.063$ g/ml) was isolated from human plasma of normolipidemic healthy individuals by ultracentrifugation. The plasma density was raised to 1.055 g/ml using KBr. After ultracentrifugation at 32,000 rpm at 4°C for 16 hours using a SW41Ti rotor Beckman Instrument, the light fraction, containing LDL, was collected and stored under nitrogen. LDL concentration was determined using the bicinchoninic acid (BCA) protein kit (Thermo Scientific, Rockford, IL, USA). LDL concentration was adjusted to 0.5 mg/ml of protein by dilution in PBS. Cu^{2+} -mediated oxidation was conducted at 37°C for 24h against 0.32mM CuSO_4/PBS . The reaction was stopped by addition of 50 μM ethylene-diamid-tetraacetate (EDTA) and extensive dialysis at 4°C against PBS containing 0.5M EDTA for 24 hours. LDL or modified LDL were tested for endotoxin using a Limulus Amebocyte Lysate (LAL) assay (Hycult Biotechnology, Uden, The Netherlands), according to the manufacturer's instructions. Endotoxin concentration of all samples was below 5pg/ml.

Labeling of LDL and oxLDL with Dil

LDL and oxLDL were labeled with the fluorescent lipid 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (Dil; Invitrogen, Darmstadt, Germany), by incubating 3mg/ml Dil per ml of LDL or oxLDL, overnight and in the dark, while continuously stirring in a 37°C water bath. Free Dil was removed by size exclusion chromatography using PD-10 columns (GE Healthcare, Uppsala, Sweden). Dil-labeled LDL and oxLDL uptake was determined by flow cytometry on a BD Canto II (BD Bioscience, Bedford, MA, USA).

Oil-Red-O staining

5×10^4 pDCs were cytopspun at 700rpm for 5 minutes on glass slides. After drying slides for 1 hour, sections were incubated in PBS for 5 minutes, dipped 10x in 60% 2-propanol and stained with Oil-Red-O solution for 20 minutes. After dipping 10x in 60% 2-propanol, sections were cleared under tap water. Cell nuclei were stained with haematoxylin. Sections were mounted with IMSOL and covered.

Flow cytometry

Isolated pDCs were stained with fluorescein isothiocyanate (FITC)-conjugated CD3 (1:100, DAKO, Glostrup, Denmark), CD14 (1:100, eBioscience, San Diego, CA, USA), CD16 (1:100, DAKO), CD19 (1:100, DAKO), CD56 (1:100, eBioscience), CCR7 (1:20, BD Bioscience, Bedford, MA, USA) and CD40 (1:100, BD Bioscience), R-phycoerythrin (PE)-conjugated CD86 (1:500, BD Bioscience) and CD83 (1:20, BD Bioscience), PerCP-Cy5.5-conjugated CD123 (1:50, BD Bioscience), allophycocyanin (APC)-conjugated BDCA-2 (1:50, Miltenyi Biotec, Bergisch-Gladbach, Germany), phycoerythrin-Cyanine7 (PE-Cy7)-conjugated CD11c (1:20, eBioscience), allophycocyanin-H7 (APC-H7)-conjugated HLA-DR (BD) and Pacific Blue-conjugated CD4 (1:20, eBioscience) monoclonal antibody (mAb). Life-dead cell staining was accomplished using 7-AAD, a marker that is intercalating in non-vital cell DNA. All flow cytometry analyses were performed on a BD Canto II (BD Bioscience).

Mixed leukocyte reaction

Autologous naïve CD4⁺ T cells were isolated from pDC-depleted PBMCs by use of the human naïve CD4⁺ T Cell Isolation Kit (Miltenyi-Biotec, Bergisch-Gladbach, Germany). As determined by staining with V500-conjugated CD3 mAb, APC-H7-conjugated CD4 mAb, FITC-conjugated CD8 mAb, PE-conjugated CD45RO mAb, V450-conjugated CD45RA mAb and APC-conjugated CD25 mAb (BD Bioscience, Bedford, MA, USA), populations containing 99,9% CD3⁺ CD4⁺ CD45RA⁺ CD45RO⁻ CD8⁻ T cells were routinely obtained. For the mixed leukocyte reaction (MLR), autologous purified naïve CD4⁺ T cells (5x10⁵ cells/well) were incubated in 96-well cell culture plates with 1x10⁵ pDCs, pre-cultured for 24 hours as described above and pre-incubated 1 hour before adding the T cells with Staphylococcus Enterotoxin B (SEB; 10ng/ml). MLRs were cultured for 7 days. The assay was performed in duplicate.

Cytokine measurement

pDCs were treated as indicated, and cytokine concentrations of IFN- α , IL-6, IL-12p70, IL-10, TNF- α , RANTES (CCL5), MIP-1 α (CCL3), MIP-1 β (CCL4) and CXCL10 (IP10) in cell-free supernatants were measured after 24 hours using the Cytometric Bead Array (CBA) human soluble protein kit (BD Bioscience, Bedford, MA, USA), according to the manufacturers instructions. For the T cell polarization assay, MLR supernatants were harvested at day one to seven of

co-culture and analyzed for IFN- γ , TNF- α (T helper 1 cytokines), IL-4, IL-5, IL-13 (T helper 2 cytokines), IL-17A (T helper 17 cytokine), and IL-10 (Treg cytokine) by CBA kit. ELISA plates were used from Greiner Bio-One (Alphen a/d Rijn, The Netherlands).

Statistical analysis

Data were analyzed with the use of Graph Pad Prism 5 (Graph Pad Software Inc., San Diego, CA, USA). All data are expressed as mean $\pm$ SEM. To compare several groups, a one-way ANOVA with multiple testing correction was used. Values of $p < 0.05$ were considered statistically significant. Values of $p < 0.05$ were considered statistically significant.

Results

Human pDCs are able to engulf lipoproteins

First we examined whether pDCs, freshly isolated from human PBMCs by BDCA-4 magnetic beads, are able to take up oxLDL. Enrichment of isolated immature pDCs was confirmed by flow cytometry according to the surface marker expression of BDCA-2 and CD123 and reached $>85\%$, represented by flow cytometry (**Figure 1A**). Immature, as well as CpG-matured pDCs were incubated for 24 hours in the absence or presence of Dil-labeled oxLDL (10 and 50 $\mu\text{g}/\text{ml}$). Dil-oxLDL exposed pDCs exhibited modest lipoprotein uptake, as assessed by flow cytometry ($p < 0.05$; **Figure 1B, C**). CpG-stimulated pDCs showed markedly increased Dil-oxLDL uptake ($p < 0.001$; **Figure 1B, C**). OxLDL accumulation by pDCs could be confirmed by Oil-Red-O staining, showing the characteristic presence of Oil-Red-O stained vacuoles in oxLDL exposed pDCs (**Supplemental Figure 1**). Overnight incubation of immature pDCs with oxLDL did not lead to increased cell death (**Supplemental Figure 2A, B**), and average cell viability amounted 25-30%. pDCs stimulated for 24 hours with CpG and oxLDL (10 and 50 $\mu\text{g}/\text{ml}$) showed much less cell death (10-15%) (**Supplemental Figure 2A, B**).

Figure 1. Human pDCs take up lipoproteins. pDCs, isolated from human PBMCs, were cultured for 24 hours with CpG-ODN 2216, CpG-ODN 2216 plus Dil-oxLDL (10µg/ml or 50µg/ml), or Dil-oxLDL alone (10µg/ml or 50µg/ml). Non-supplemented medium was used as control, and Dil fluorescence was determined by flow cytometry as a measure of oxLDL uptake. Results are representative of five independent experiments. **(A)** Enrichment of CD123⁺ BDCA-2⁺ pDCs from isolated PBMCs by BDCA-4 positive magnetic beads. Purity was determined by flow cytometry. **(B)** FACS plots demonstrating oxLDL uptake by pDCs. **(C)** Mean fluorescence intensity (MFI) of Dil⁺ pDCs. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Lipoproteins inhibit CpG-induced up-regulation of maturation markers and cytokine production by pDCs

To test the impact of oxLDL accumulation on pDC maturation, we incubated human pDCs for 24 hours with oxLDL (0, 10 and 50µg/ml) in the absence or presence of CpG. CpG-activated pDCs acquired a characteristic mature phenotype compared to non-stimulated controls, as demonstrated by the significantly increased expression of maturation markers, including CD83, CD86, HLA-DR and the lymphoid-homing receptor CCR7 (**Figure 2A**). BDCA-2, a pDC specific type II C-type lectin,⁽²²⁾ is expressed on immature pDCs, but abated

on mature pDCs, rendering it a useful marker of immature pDCs. As expected, CpG-stimulated pDCs showed significantly decreased BDCA-2 expression ($p < 0.001$; **Figure 2A**). OxLDL treated pDCs exhibited a low surface expression of CD83, CD86, HLA-DR and CCR7, comparable to that of immature pDCs (**Figure 2A**). Interestingly, co-incubation of pDCs with oxLDL dose-dependently inhibited CpG-induced pDC maturation (**Figure 2A**). BDCA-2 surface expression remained unexpectedly low in CpG/oxLDL co-stimulated pDCs (**Figure 2A**). Bioactive oxidized lipids, such as oxidized phospholipids, are present in oxLDL, and can profoundly modify the course of an immune response,⁽²³⁾ as has also been observed for pDC activation. To test the impact of LDL on pDC maturation, human pDCs were incubated for 24 hours with CpG, in the absence or presence of LDL (50 $\mu\text{g/ml}$), with LDL alone (50 $\mu\text{g/ml}$), or in non-supplemented medium (control). While treatment with LDL did not influence pDC maturation status, it abrogated CpG-induced pDC maturation as was indicated by the significant down-regulation of CD83, CD86, HLA-DR and CCR7, as well as the concomitant up-regulation of BDCA-2 (**Supplemental Figure 3A**).

The oxLDL and LDL associated inhibition of CpG-induced pDC maturation led us to test their impact on pDC cytokine production. In the context of systemic viral or bacterial infections pDCs rapidly release large amounts of type I IFNs, as well as other pro-inflammatory cyto- and chemokines, which promote lymphocyte recruitment to sites of infection.^(24, 25, 27, 28) Here, simultaneous treatment of immature pDCs with LDL or oxLDL (50 $\mu\text{g/ml}$) and CpG for 24 hours almost completely prevented the CpG-induced production of the pro-inflammatory cytokines IFN- α , TNF- α and IL-6. Additionally, the release of the chemokines CCL3, CCL4 and CXCL10 was nearly entirely abrogated, when stimulated with the lipoproteins and CpG ($p < 0.01$; **Figure 3A**). Concentrations of IL-10, IL-12p70, and CCL5 were not measurable under all conditions tested (data not shown).

Altogether, these data reveal that the presence of lipoproteins do not induce *in vitro* maturation of human pDCs. Moreover, CpG-induced pDC activation is abrogated when co-cultured with LDL or oxLDL.

Figure 2. OxLDL inhibits CpG-induced expression of maturation markers in pDCs. Freshly isolated human pDCs were stimulated with 10µg/ml CpG-ODN 2216 either with or without oxLDL (10 and 50µg/ml) (oxLDL and CpG-ODN 2216 were added simultaneously). Non-stimulated pDCs were used as control. After 24 hours, cells were harvested and the surface expression levels of the indicated markers were measured by flow cytometry. **(A)** Induction of maturation markers (CD83, CD86, HLA-DR, CCR7, BDCA-2) on pDCs shown as MFI. The presence of oxLDL dose-dependently inhibits the up-regulation of pDC maturation markers. **(B)** Maturation marker expression on non-stimulated pDCs (grey peak), CpG-ODN 2216 stimulated pDCs (red peak), or CpG-ODN 2216 plus oxLDL stimulated pDCs (10µg/ml: green peak; 50µg/ml: blue peak). Data are shown as mean ± SEM of five independent experiments using different donor pDCs. *p<0.05, **p<0.01, ***p<0.001.

Figure 3. LDL and oxLDL inhibit CpG-induced pro-inflammatory cytokine production by pDCs.

(A) Freshly isolated human pDCs were stimulated with 10µg/ml CpG-ODN 2216 either with or without LDL (50µg/ml) or oxLDL (50µg/ml). Non-stimulated pDCs were used as control. After 24 hours, cell supernatants were analyzed for the indicated cytokines by use of the CBA kit. Data are shown as mean ± SEM of three independent experiments using different donor pDCs.

*p< 0.05, **p< 0.01, ***p<0.001.

Prior lipoprotein treatment inhibits CpG-induced pDC maturation

Next, we addressed the question whether lipoproteins inhibit CpG effects by preventing CpG-uptake, or by shielding CpG to interact with TLRs. pDCs express TLR7 and TLR9, which recognize single-stranded RNA and double-stranded DNA, respectively.⁽²⁹⁾ Upon TLR stimulation, pDCs undergo major phenotypic changes, including the up-regulation of maturation markers. Pre-treatment of pDCs with the lipoproteins prevented CpG-induced up-regulation of maturation markers, including CD40, CD86, HLA-DR, and CCR7 (**Figure 4A**). BDCA-2 surface expression remained up-regulated in CpG-stimulated pDCs, pretreated with lipoproteins. In contrast, CpG-induced pDC activation was not affected by post-treatment with LDL or oxLDL. Cells displayed elevated expression levels of the aforementioned pDC maturation markers (**Figure 4A**), and increased IFN-α production (data not shown). To conclude, pre-incubation of pDCs with LDL or oxLDL ablates TLR9-induced activation by CpG.

Figure 4. Lipoprotein treatment inhibits TLR9-induced pDC maturation. Freshly isolated human pDCs were stimulated for 4 hours with (ox)LDL (50µg/ml), and subsequently for 16 hours with 10µg/ml CpG-ODN 2216 (red bars), or conversely (blue bars). As controls, pDCs were stimulated with 10µg/ml CpG-ODN 2216 (positive control) or incubated in non-supplemented medium. Subsequently, cells were harvested and the surface expression levels of the indicated markers were measured by flow cytometry. **(A)** Induction of maturation markers (CD40, CD86, HLA-DR, CCR7, BDCA-2) on pDCs shown as MFI. Data are shown as mean ± SEM of three independent experiments using different donor pDCs. *p < 0.05, **p<0.01, ***p<0.001.

OxLDL treatment suppresses TLR9-induced pDC activation and T cell stimulation capacity

To analyze the effect of LDL and oxLDL on T cell stimulatory capacity of pDCs, activated with CpG, we performed autologous mixed leukocyte reactions. Non-activated pDCs were used as control. As expected CpG-activated pDCs augmented Th1 polarization of naïve CD4⁺ T cells after 7 days of co-culture, as judged by the markedly increased IFN- γ and TNF- α cytokine release (**Figure 5A**). Similarly, CpG-matured pDCs also enhanced cytokine induced Th2 polarization, illustrated by significantly elevated IL-13 production on day 7 of co-culture (**Figure 5B**). Of note, Th2 cytokine levels of IL-5 and IL-4 remained unaffected in CpG-matured pDC co-cultures (**Figure 5B**). Importantly, T cell stimulatory capacity of CpG-stimulated pDCs was almost or completely blunted by co-treatment with oxLDL (**Figure 5A, B**), consistent with the observed impaired maturation and activation capacity of (ox)LDL treated pDCs. Surprisingly, pDCs, pre-treated with LDL, were able to induce a Th1 polarization of naïve CD4⁺ T cells (**Figure 5 A**), comparable to the Th1 response elicited by CpG-activated pDCs. IL-17A and IL-10 cytokine levels in the cell supernatants were nearly or completely undetectable in all conditions (**Figure 5C, D**), except for IL-10 cytokine concentrations that were significantly up-regulated on day 7 of co-culture with immature pDCs (control). This might reflect a preferential T cell differentiation into regulatory T cells (Treg's) (**Figure 5D**).

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Figure 5. OxLDL inhibits T cell stimulatory capacity of CpG-activated pDCs. Human autologous naïve CD4⁺ T cells were stimulated with immature, CpG-ODN 2216 pre-incubated (with or without 25 μ g/ml LDL or oxLDL), or lipoprotein pre-incubated (25 μ g/ml LDL or oxLDL) pDCs, and T cell polarization capacity (Th1, Th2, Th17 or Treg) was analyzed on day 1, 3, 5 and 7 of co-culture by measuring T cell cytokines in the cell supernatant (CBA kit). **(A)** Measurement of Th1 cytokines IFN- γ and TNF- α ; **(B)** measurement of Th2 cytokines IL-5, IL-4 and IL-13; **(C)** measurement of Th17 cytokine IL-17A; **(D)** measurement of Treg cytokine IL-10. Data are shown as mean $\pm$ SEM of two independent experiments using different donor pDCs and T cells.

Discussion

DC activation, induced by receptor-mediated recognition of pathogen-derived and endogenous danger signals is a crucial step during infections to trigger innate and adaptive immunity. Hyperlipidemia has been shown to impair host immune responses against yeast, bacterial, and viral infections.⁽³⁰⁻³³⁾ However, the underlying mechanisms are not well understood. pDCs have a critical role in anti-viral defense through production of large amounts of type I IFNs. Additionally, pDCs are fully capable to present endogenous antigens to naïve T cells, thereby inducing effective T cell responses.^(34, 35) Here, we address the question whether a hyperlipidemic environment affects pDC activation and their T cell stimulatory capacity. We demonstrate that human pDCs are hypo-responsive after (ox)LDL exposure, and fail to induce Th cell polarization *in vitro*. These effects might have striking implications for pDC-mediated pathogen responses, as well as general immunity *in vivo* under pathological conditions such as atherosclerosis or obesity, which are strongly associated with dyslipidemia.⁽¹⁴⁾

As we show, human pDCs are able to take up and accumulate lipoproteins. Lipoprotein (LDL or oxLDL) accumulation dose-dependently dampened the CpG-induced IFN and cytokine response. Additionally, the hyperlipidemic environment abrogated the up-regulation of co-stimulatory molecules, such as CD40, CD83 and CD86, as well as HLA-DR, markers which are pivotal for T cell priming.⁽⁴⁾ As a consequence, CD4⁺ T cell polarization was completely quenched. This is in keeping with the recently reported ability of murine pDCs to incorporate oxLDL.⁽¹⁹⁾ Interestingly, *in vitro* oxLDL exposure enhanced the phagocytic efficiency of murine pDCs, but did neither alter surface expression of the maturation markers CD86 and MHC II, nor secretion of IFN- α protein.⁽¹⁹⁾ Surprisingly, *ex vivo* ovalbumin-pulsed pDCs, additionally pre-treated with oxLDL, were able to induce strong antigen-specific OT-II CD4⁺ T cell proliferation *in vivo*.⁽¹⁹⁾ One explanation might be that additional *in vivo* stimuli have boosted pDCs to trigger T cell proliferation. Otherwise, it needs to be determined how long pDCs were pre-treated *ex vivo* with oxLDL in the referred study. Long-term lipoprotein exposure possibly quenches pDC activation, whereas short incubation times preserve their antigen processing and presenting capability.

Moreover, we demonstrated that lipoprotein treatment (LDL and oxLDL) markedly decreased surface expression of the lymphoid-homing receptor CCR7 in CpG-stimulated pDCs. Down-regulation of this receptor may limit pDC migration from injured tissue to secondary lymphoid organs, thereby impairing the induction of effective T cell responses.^(36, 37) Then again, other chemokine receptors may regulate pDC trafficking.⁽³⁸⁾ pDC migration in the steady-state is mediated through the expression of L-Selectin, CXCR4 and ChemR23.⁽³⁸⁾ Under inflammatory conditions this chemokine receptor repertoire is enlarged by the expression of CCR5, CXCR3 and CCR7.⁽²²⁾ Next to the latter, the ChemR23/chemerin axis seems to play a key role in pDC migration towards secondary lymphoid organs, especially under inflammatory conditions.⁽³⁹⁾ It remains to be demonstrated, whether a hyperlipidemic environment will impact the surface expression of other chemokine receptors on pDCs, thereby interfering their trafficking routes.

Additionally, lipoprotein treatment potently quenched CpG-induced IFN- α production by pDCs. Moreover, the release of IL-6 and TNF- α , as well as of the T cell attracting chemokines CCL3 and CCL4 was impaired by lipoprotein exposure, identifying LDL and oxLDL as strong negative regulators of inflammatory cytokine and chemokine production in human pDCs.^(27, 28) In line, we have previously shown that CpG-induced IFN- α release by pDCs into the circulation was dampened in high fat diet fed low-density lipoprotein deficient (*Ldlr*^{-/-}) mice, suggesting that hyperlipidemia dampens murine pDC activation.⁽⁴⁰⁾ At least two studies in humans also observed an impaired functionality of circulating pDCs, isolated from patients with carotid endarterectomy (CEA),⁽⁴⁰⁾ or from coronary artery disease (CAD) patients, revealed by the derogated expression and secretion of IFN- α .⁽⁴¹⁾ Nevertheless, data interpretation has to be considered cautious, since hyperlipidemia was present only in a minor fraction of included patients. It will be of interest in future studies to analyze phenotype and functionality of pDCs, isolated from patients with familial hypercholesterolemia.

The actual mechanism underlying (ox)LDL mediated interference with TLR9 activation remains elusive. (Ox)LDL may act by interfering with CpG-binding to TLR9, thereby inhibiting TLR9-induced NF- κ B, and IRF7 activation.⁽⁴²⁻⁴⁴⁾ Alternatively, oxLDL could bind to other surface receptors, such as BDCA-2, dendritic cell immuno-receptor (DCIR), or immunoglobulin-like receptor 7

(ILT7), which potentially suppress IFN- α / β production upon ligand binding.^(22, 45, 46)

Previous studies corroborate the idea that oxidized lipoproteins directly affect TLR-ligand binding. For instance, Kopf and coworkers demonstrated that dyslipidemia inhibits murine CD8 α^+ cDC activation. OxLDL appeared to be responsible for this effect, as it uncoupled TLR2, 3, 4, 7-mediated signaling in cDCs, and inhibited NF- κ B binding to DNA.⁽²⁰⁾ Similarly, Bluemel *et al.* have shown that oxidized phospholipids (oxPLs) negatively regulate LPS-induced maturation of human monocyte-derived DCs, and their lymphocyte stimulatory capacity.⁽²¹⁾ The authors suggest a direct interaction of oxPLs with TLR4, thereby blocking LPS binding to its receptor.⁽⁴⁷⁾ A similar mechanism may apply for TLR9 as well.

Our data suggest that hyperlipidemia could induce immuno-tolerance in pDCs. This may require the production of anti-inflammatory cytokines, such as IL-10 or TGF- β , crucial for such immuno-suppression by dampening immunogenic T cell responses, and favoring Treg differentiation.^(48, 49) We were however unable to detect elevated levels of IL-10 or TGF- β after lipoprotein exposure, nor did lipoprotein treatment induce the generation of Treg's. As we have previously shown, hyperlipidemia can also induce tolerogenic pDCs in mice by dampening T cell proliferation in an IDO-dependent manner, an option that requires further study.⁽⁴⁰⁾

Taken together, our data show that a hyperlipidemic environment inhibits TLR9-dependent maturation and cytokine production by human pDCs. Moreover, the presence of oxLDL abrogates the ensuing Th cell responses *in vitro*. It will be of importance to dissect the lipoprotein uptake associated culprit pathways and the downstream lipid metabolites responsible for the inhibitory effect of (ox)LDL. Additionally, it remains elusive, whether hyperlipidemia renders pDCs functionally impaired and non-responsive to pathogen-derived signals *in vivo*.

Supplemental Figures

Supplemental Figure 1. Human pDCs are able to take up oxLDL. pDCs, isolated from human peripheral blood mononuclear cells, were cultured for 24 hours with CpG-ODN 2216, CpG-ODN 2216 plus oxLDL (10µg/ml), with oxLDL (50µg/ml)) alone (10 and 50µg/ml) or in non-supplemented medium (control). Oil-Red-O staining confirmed oxLDL accumulation by pDCs (red droplets). Cell nuclei were counterstained with haematoxylin.

Supplemental Figure 2. OxLDL does not affect pDC viability. Depicted are the results of flow cytometry analysis of isolated human pDCs, cultured with CpG-ODN 2216, CpG-ODN 2216 and oxLDL (10µg/ml or 50µg/ml), oxLDL alone (10µg/ml or 50µg/ml) or in non-supplemented medium (control), and stained with 7-AAD for the presence of dead/ apoptotic cells. **(A)** Percentage of viable (white bars) versus dead (black bars) pDCs. **(B)** Histograms representing viability (red histograms) versus cell death (green histograms) in pDCs. Results are representative of five independent experiments.

Supplemental Figure 3. LDL inhibits CpG-induced expression of pDC maturation markers. Freshly isolated human pDCs were stimulated with 10 µg/ml CpG-ODN 2216 in the absence or presence of LDL (50 µg/ml). Non-stimulated pDCs were used as control. After 24 hours, the cells were harvested and the surface expression levels of the indicated markers were measured by flow cytometry. **(A)** Induction of maturation markers on pDCs shown as MFI. The presence of LDL inhibits up-regulation of maturation markers on pDCs. Data are shown as mean ± SEM of three independent experiments using different donor pDCs. *p < 0.05, **p < 0.01, ***p < 0.001.

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Contribution to the manuscript (Chapter 6):

Anette Christ designed this study together with Erwin Wijnands, Dr. Temmerman and Prof. Biessen. Experiments were mainly accomplished and analyzed by Anette Christ and Erwin Wijnands. All animal work prior to sacrifice was executed by Anette Christ (placing animals on high fat diet, BrdU injections). Anette Christ and Erwin Wijnands were mainly responsible for the acquirement and the analysis of mouse flow cytometry data (influence of high fat diet feeding on numbers and phenotype of different leukocyte populations; lipid uptake by different dendritic cell populations). In addition, Anette Christ and Erwin Wijnands performed the *in vitro* dendritic cell-T cell co-culture experiments. Anette Christ analyzed these data. Plasma cholesterol levels were measured by Anette Christ. Final data were discussed and interpreted by Anette Christ, Erwin Wijnands, Dr. Temmerman and Prof. Biessen. Anette Christ wrote the manuscript. Dr. Temmerman, Prof. Daemen and Prof. Biessen revised the written manuscript.

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6

Murine lipid-laden peripheral dendritic cell subsets display an immature phenotype and are impaired in their capability to prime naïve T cells.

- in preparation -

Abstract

Immune cell activation, proliferation and recruitment are key mechanisms in the onset and progression of hypercholesterolemia-associated atherosclerosis. Dendritic cells (DCs), highly professional antigen-presenting cells (APCs), are thought to contribute to atherosclerosis related adaptive immune responses. However, their functionality under hypercholesterolemic conditions, a major risk factor in disease development, remains to be established. Here, we addressed the immediate impact of high fat diet-induced hypercholesterolemia in low-density lipoprotein receptor deficient (*Ldlr*^{-/-}) mice on DC subset patterns, their compartmentalization and functionality. Hypercholesterolemia induced a significant rise in bone marrow myeloid and dendritic cell (MDP) frequency and proliferation rate after 5 weeks of high fat diet feeding. DC subset numbers in peripheral compartments, such as blood and lymphoid organs, remained largely unchanged. Hypercholesterolemia led to augmented granularity of pDCs, CD8⁺ and CD8⁻ DCs, which for the cDC subsets correlated with intracellular lipoprotein accumulation. Lipid loading did not affect DC maturation levels, as witnessed by an unaltered co-stimulatory molecule expression. Remarkably, lipid-laden CD8⁻ cDCs displayed a profoundly reduced capacity to stimulate naïve T cells. Conclusively, our data provide evidence that under conditions of hypercholesterolemia peripheral CD8⁻ and CD8⁺ cDC subsets engulf lipoproteins *in situ*. In addition, lipid-laden cDCs, while maintaining their trafficking capacity, show sharply impaired T cell priming ability, a finding that may have repercussions for host defense against pathogens and/or endogenous (neo) antigens.

Introduction

An established risk factor in atherosclerosis related cardiovascular diseases (CVD), hypercholesterolemia, was seen to trigger a range of effects that contribute to its pro-atherogenic activity. Amongst others it was seen to activate not only endothelial cells, but also myeloid lineage subsets relevant to disease, and to induce an expansion of circulating inflammatory monocytes in atherosclerosis mouse models.^(1, 2) The causal contribution of this subset to plaque formation, due to their permanent recruitment to the growing lesion, where they differentiate into macrophages, has been studied extensively.⁽³⁻⁶⁾ Recent evidence from mouse atherosclerosis models has proven that dietary-induced hypercholesterolemia can also modify neutrophil homeostasis and phenotype, rendering them more prone to infiltrate the lesion during early stages of atherosclerosis.⁽⁷⁻⁹⁾ Hypercholesterolemia-associated myelocytosis may be linked to an ablated high-density lipoprotein (HDL) or apolipoprotein E (ApoE)-mediated suppression on stem and multipotential progenitor cell proliferation in the bone marrow.^(6, 10) Additionally, hypercholesterolemia was seen to promote extramedullary myelopoiesis resulting in continuous ectopic production of inflammatory cells that potentially contribute to plaque burden.⁽¹¹⁾

Several bodies of evidence from patient studies as well as from murine atherosclerosis models suggest a significant contribution of another myeloid subset, dendritic cells (DCs), to atherosclerotic lesion formation.⁽¹²⁻¹⁴⁾ DCs are present in non-diseased arteries,^(15, 16) and accumulate within atherosclerotic plaques.⁽¹⁷⁻¹⁹⁾ In mice, hypercholesterolemia has been shown to elicit rapid lipoprotein incorporation by vascular resident DCs, which initiates their foam cell formation,⁽²⁰⁾ while conventional DCs (cDCs) were reported to favor cholesterol clearance, possibly from the circulation.⁽¹⁴⁾ Whether lipid-laden DCs are able to present antigens and prime T cells remains controversial. Hypercholesterolemia was seen to inhibit the activation of particular DC subsets,⁽²¹⁻²³⁾ but did not alter DCs capacity to prime naive T cells.^(13, 14) Altogether, systematic investigation of how hypercholesterolemia impacts bone marrow and peripheral DC (progenitor) counts, phenotype, migratory behaviour, as well as functionality is largely lacking.

In the present study, we addressed temporal effects of diet-induced hypercholesterolemia in *Ldlr*^{-/-} mice on DC (precursor) homeostasis, on DC lipid

accumulation and its consequences for phenotype and T cell priming capability, analyzing cell composition, cell proliferation, and cell activation status in bone marrow and peripheral lymphoid organs at different time points after diet change.

Methods

Animals

All animal work was approved by the regulatory authority of Maastricht University and performed in compliance with the Dutch government guidelines. Female age-matched *C56BL/6J Ldlr^{-/-}* mice (originally obtained from The Jackson Laboratory) were bred at the local animal breeding facility (Maastricht University, The Netherlands). Mice were fed a standard fat diet (SFD; Cat# V1535, sniff Spezialdiäten GmbH, Soest, Germany) unless indicated otherwise, had *ad libitum* access to food and water, and were housed under a 12 hour light-dark cycle. To study early adaptation responses to diet induced hypercholesterolemia on DC development, homeostasis, and phenotype in peripheral lymphoid organs, *Ldlr^{-/-}* mice were placed for 0,5 weeks (n=6), 1 week (n=6), 3 weeks (n=6) or 5 weeks (n=6) on high fat diet (HFD; 0.25% cholesterol and 15% cacao butter; Special Diets Services, Witham, Essex, UK). Standard fat diet fed mice served as controls (n=6). At sacrifice mice were euthanized by an overdose of pentobarbital. Blood was subsequently obtained from the left cardiac ventricle and plasma was used for cholesterol measurements and cytokine profiles. Bones, blood, spleen, a pool of peripheral (a mixture of mandibular and axillary lymph nodes) and mesenteric lymph nodes, and small intestines were used for flow cytometric analysis.

Plasma cytokine and cholesterol analysis

Plasma cholesterol levels were measured in duplicate using a colorimetric assay (CHOD-PAP, Roche, Mannheim, Germany). Plasma cytokine levels of IL-10, IL-12p70, IL-1 β , IL-6 and mKC (CXCL10) were determined with a Multi-Spot assay system (Meso Scale Discovery, Rockville, MD, USA).

Flow cytometry

Bone marrow, blood, spleen, mesenteric lymph nodes, peripheral lymph nodes (a mixture of mandibular and axillary lymph nodes), and small intestines were isolated, processed and stained for total leukocytes (CD45⁺, BioLegend, Uithoorn, The Netherlands), total T cells (CD3⁺, eBioscience, San Diego, CA, USA), T helper cells (CD4⁺, BD Bioscience, Bedford, MA, USA), cytotoxic T cells (CD8a⁺, BD Bioscience), B cells (B220⁺, BD Bioscience), NK cells (CD3⁻ NK1.1⁺, BD Bioscience) monocytes (CD11b^{high} Ly6G^{low}, BD Bioscience; or CD115⁺, BioLegend), granulocytes (CD11b^{high} Ly6G^{high}, BD Bioscience), conventional dendritic cells (cDCs; CD11c^{high}, CD8⁻ or CD8⁺, eBioscience) and plasmacytoid dendritic cells (pDCs; PDCA-1^{high} B220⁺, eBioscience). DC maturation was analyzed performing cell surface staining on CD40 (eBioscience), CD86 (eBioscience), and major histocompatibility complex (MHC-) II (eBioscience). For cDC and pDC analysis, spleen, peripheral lymph nodes, mesenteric lymph nodes, and small intestines were pretreated for 30 minutes with a cocktail of liberase (32µg/ml; Roche, Basel, Switzerland) and DNase (0.8µg/ml; Roche, Basel, Switzerland) in RPMI medium. Absolute cell numbers in blood were calculated by use of Trucount tubes (BD Bioscience, Bedford, MA, USA). All flow cytometry analysis was performed on a BDCanto II (BD Bioscience, Bedford, MA, USA) using FACS Diva Analysis Software.

BrdU labeling and cell analysis

Ldlr^{-/-} mice on standard fat diet (n=6) or high fat diet (0,5 weeks, 1 week, 3 weeks, or 5 weeks; n=6 per group) received two 5-bromo-2'-deoxyuridine (BrdU; Sigma-Aldrich, Seelze, Germany) i.p. injections (1mg/twice per day every 12 hours) for 1 day before sacrifice (last injection 12 hours before sacrifice). BrdU incorporation in various leukocyte (precursor) subsets, isolated from bone marrow, blood and spleen, was assessed using an allophycocyanin (APC) BrdU flow kit (BD Bioscience, Bedford, MA, USA) according to the manufacturer's instructions. Cells were analyzed using the BDCanto II.

Reactive oxygen species (ROS) activity measurement

Splenic DC subsets, isolated from standard or high fat diet (3weeks) fed *Ldlr*^{-/-} mice (n=11 per group), were stained for 20 minutes at 37°C with 20µm 2',7'-dichlorofluorescein diacetate (DCFDA), which is first deacetylated and

after oxidized by ROS to 2', 7' -dichlorofluorescein (DCF), visible as a fluorescent signal at 525nm. Fluorescently labeled cells were analyzed using the BDCanto II.

Mouse DC isolation by Fluorescence Activated Cell Sorting (FACS)

Splenic DC subsets, including cDCs ($\text{lin}^- \text{CD11c}^{\text{high}} \text{CD8}^-$ or CD8^+) and pDCs ($\text{lin}^- \text{CD11c}^{\text{low}} \text{PDCA-1}^{\text{high}} \text{B220}^+$) were sorted by FACS Aria from *Ldlr*^{-/-} mice, either on high fat diet (n=3) or on normal standard fat diet (control, n=3). The purity of the isolated DC subsets was > 98%, assessed by flow cytometry.

Oil-Red-O staining

DC subsets, sorted as described above from different lymphoid organs (spleen, peripheral lymph nodes, mesenteric lymph nodes) and small intestines from standard fat diet fed (n=3) and high fat diet fed (n=3; 3 weeks of high fat diet) *Ldlr*^{-/-} mice were cytospun at 700 rpm for 5 minutes on glass slides. After drying slides, sections were incubated in phosphate buffered saline (PBS) for 5 minutes, dipped 10x in 60% 2-propanol and stained with Oil-Red-O solution for 20 minutes. Subsequently, sections were cleared under tap water. Cell nuclei were stained with haematoxylin. Images were analyzed by ImageJ.

Nile Red lipid staining

DC subsets (cDCs and pDCs), isolated from spleen and peripheral lymph nodes from standard fat diet fed (n=3) and high fat diet fed (n=3; 3 weeks of high fat diet) *Ldlr*^{-/-} mice were stained with Nile Red (AdipoRed™; Lonza, Walkersville, MD, USA) to quantify intracellular lipid droplet accumulation. Cells were incubated with AdipoRed (1:1000 dilution in PBS) for 10 minutes at room temperature, washed twice in PBS, and were immediately analyzed using the BDCanto II (BD Bioscience, Bedford, MA, USA).

cDC-T cell co-culture

Low and high granular cDC subsets (CD8^- and CD8^+) were sorted from spleen from *Ldlr*^{-/-} mice, either fed a standard (n=3) or a high fat diet for 3 weeks (n=3). DC subsets were gated as described above, but additionally low/high granular DC subsets were separated, by gating for side scatter (SSC). Naïve CD4^+ and CD8^+ T cells were sorted from spleen from *Ldlr*^{-/-} mice on standard fat diet (n=3). The purity of the isolated cDC and T cell subsets was >

98%, assessed by flow cytometry. Subsequently, purified cDC and T cell subsets were co-cultured in a 96 well plate in a 1:5 ratio (1×10^5 cDCs and 5×10^5 T cells, respectively) for 3 days in complete RPMI medium (Hepes, glutamax, Pen/Strep) (Gibco LifeTechnologies, Carlsbad, CA, USA) supplemented with 10% heat-inactivated fetal calf serum (Gibco), 2ng/ml β -mercaptoethanol, and 0,5 μ g/ml anti-CD3 beads (Bioceros, Utrecht, The Netherlands). Purified T cell subsets were CFSE labeled prior to co-culture, according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA), and proliferation was determined by flow cytometry 72 hours later using the BDCanto II (BD Bioscience, Bedford, MA, USA).

Statistical analysis

Data were analyzed with the use of Graph Pad Prism 5 (Graph Pad Software Inc., San Diego, CA, USA). All data are expressed as mean $\pm$ SEM. To compare individual groups, a 2-tailed Student's *t* test was used (if normally distributed); nonparametric data were analyzed using a Mann-Whitney *U*-test. To compare several groups, a one-way ANOVA with multiple testing correction was used. Values of $p < 0.05$ were considered statistically significant.

Results

Hypercholesterolemia alters myeloid and dendritic cell precursor homeostasis in the bone marrow

Hypercholesterolemia has been shown to affect counts and phenotype of circulating cells of the myeloid lineage, however systemic investigation on how a cholesterol-rich environment affects DC (precursor) behaviour is lacking.^(4, 7, 24, 25) In this study we examined whether very early and more advanced stages of hypercholesterolemia alter DC (precursor) homeostasis, DC maturation status and DC functionality.

We primarily made a temporal analysis of abundance of DC subsets in bone marrow and peripheral lymphoid organs from *Ldlr*^{-/-} mice in response to shift from standard fat diet (SFD) to high fat diet (HFD). As expected, plasma cholesterol levels rapidly increased upon the diet change to reach plateau values after 1 week (**Figure 1A**). This was accompanied by a consistent increase in body weight (**Table 1**). Plasma levels of relevant inflammatory cytokines, such as IL-6, IL-12p70, IL-1 β , and IL-10 remained largely unchanged during HFD feeding (**Supplemental Figure 1A-D**). Congruent with previous studies,⁽⁷⁾ plasma levels of CXCL1 (mKC), a neutrophil chemoattractant,^(26, 27) significantly increased with HFD feeding (**Supplemental Figure 1E**). Next, we assessed whether diet-induced hypercholesterolemia influences bone marrow content of myeloid and dendritic cell precursors (MDP), the most differentiated clonogenic precursors of conventional DCs (cDCs), plasmacytoid DCs (pDCs), and monocytes (CD115⁺ CD135⁺ CX₃CR₁⁺ within the lin⁻ IL7Ra⁻ and CD117^{low} population).^(28, 29) Bone marrow MDP numbers were increased two fold after 5 weeks of HFD feeding (**Figure 1B, D**). In contrast to previous findings,⁽¹¹⁾ we were unable to detect the presence of splenic MDPs in hypercholesterolemic *Ldlr*^{-/-} mice (data not shown). Next, we examined whether MDP expansion was associated with increased cell proliferation. Cell cycle analysis as assessed by 5-bromo-2'-deoxyuridine (BrdU) incorporation, revealed a slight, but significant increase in bone marrow MDP proliferation upon diet change (**Figure 1C**). To summarize, HFD feeding leads to an MDP expansion in bone marrow, possibly due to increased cell proliferation.

Figure 1. Hypercholesterolemia induces an augmentation in bone marrow MDP numbers. Effects of standard (SFD; n=6) versus high fat diet feeding (HFD; 0.5 weeks, 1 week, 3 weeks, or 5 weeks; n=6 per group) on **(A)** plasma cholesterol levels. Depicted are the results of flow cytometry analysis on bone marrow from *Ldlr*^{-/-} mice on SFD or HFD. **(B)** Relative MDP abundance (lin⁻, c-kit⁺, CD115⁺, CD135⁺) in bone marrow is significantly increased after 5 weeks of HFD. **(C)** BrdU staining on bone marrow isolated MDPs shows a slight increase in proliferating cells in HFD fed animals. *p<0.05, **p<0.01, ***p<0.001.

Table 1. Body weight

	SFD	3 days HFD	1 wk HFD	3 wks HFD	5 wks HFD
Body weight (g)	26.0 ± 0.43	26.2 ± 0.59	27.1 ± 0.55	27.8 ± 0.57*	28.2 ± 0.61*

* $p < 0.05$, ** $p < 0.01$

Early adaptive response to hypercholesterolemia does not alter peripheral DC subset patterns

As HFD feeding resulted in increased MDP numbers in bone marrow, we sought to investigate whether this was associated with raised DC frequency in peripheral lymphoid organs and/ or small intestines. Splenic pDCs as well as CD8⁻ and CD8⁺ cDCs, were unaltered under hypercholesterolemic conditions (**Figure 2A, E; Supplemental Figure 3A**). Similarly, we did not observe any effects on DC numbers in peripheral lymph nodes, except for an initial transient 2-fold decline in total cDCs (**Figure 2B, E**). Peripheral lymph node CD8⁻ cDC numbers increased slightly immediately after onset of HFD feeding. In contrast, CD8⁺ cDC frequency was significantly decreased after 0.5 and 1 week of HFD, but normalized to control values as seen in SFD fed *Ldlr*^{-/-} mice afterwards (**Supplemental Figure 3B**). In line with these results, we did not observe an increase in proliferating splenic and peripheral lymph node DC subsets (data not shown). Interestingly, HFD feeding resulted in a 2-fold significant decrease in small intestinal cDC and pDC counts, which reverted with prolonged HFD feeding to numbers seen in normolipidemic mice (**Figure 2D, E**). This effect was paralleled by a significant increase in mesenteric lymph node pDC numbers after 0,5 weeks of HFD feeding (**Figure 2C, E**)

We next investigated whether acute hypercholesterolemia affects peripheral T and B cell content. CD45⁺ leukocyte counts in blood and spleen, as well as T cell (subset), and B cell levels in bone marrow, blood and spleen were essentially unaffected throughout the high fat diet adaptation phase (0,5-5 weeks; **Supplemental Figure 2A-C**), contrary to previously reported data in *ApoE*^{-/-} mice.^(4, 24, 25) As expected,⁽⁴⁾ we observed a mild monocytosis in response to high fat diet exposure (data not shown).

Collectively our data show that early stages of HFD-induced hypercholesterolemia in *Ldlr*^{-/-} mice do not modify peripheral DC subset patterns, as well as T and B cell levels in bone marrow and periphery.

6 | Effects of hypercholesterolemia on peripheral DC phenotype and functionality

Figure 2. Hypercholesterolemia leads to a rapid decline in DC subset content in small intestine but not lymphoid organs. To study immediate adaptation response of peripheral DCs to hypercholesterolemia, *Ldlr*^{-/-} mice were placed for 0-5 weeks on HFD (n=6 per group) or left on SFD (control; n=6). Depicted are the results of flow cytometry analysis on spleen, peripheral lymph nodes (LN), mesenteric lymph nodes (mesenteric LN) and small intestines. **(A)** Absolute numbers of conventional DCs (cDCs; lin^- , $\text{CD11c}^{\text{high}}$ MHC II⁺) and plasmacytoid DCs (pDCs; PDCA-1⁺, B220⁺) in spleen. **(B-D)** Percentage of lin^- $\text{CD11c}^{\text{high}}$ MHC II⁺ cDCs and PDCA-1⁺ B220⁺ pDCs in peripheral LN, mesenteric LN and small intestines. **(E)** FACS plots representing percentages of DC subsets in lymphoid organs and small intestines at different time point of HFD feeding. *p< 0.05, **p<0.01.

Peripheral cDC subsets accumulate lipids during hypercholesterolemia

As previously shown *in vitro* and *in vivo*, DCs have the capacity to take up lipoproteins.⁽¹³⁾ The finding that cDC depletion led to increased plasma cholesterol levels⁽¹⁴⁾ led the authors to suggest that DCs are important for lipoprotein elimination from the periphery/ circulation. Here, we investigated lipid loading capacities of several peripheral DC subsets, isolated from lymphoid organs or small intestine, in response to a HFD shift. Splenic, peripheral lymph node, and mesenteric lymph node CD8⁻ and CD8⁺ cDC subsets, and total cDCs in small intestine all exhibited a pronounced increase in cellular granularity already in the first week of HFD feeding. Granularity peaked after 1-3 weeks of HFD (**Figure 3A-E**). Of note, lymphoid organ resident CD8⁺ cDCs were more granular at baseline, under normolipidemic conditions, compared to CD8⁻ cDCs, possibly reflecting their intracellular antigen-processing machinery (**Figure 3E**). Cell granularity in pDCs from mesenteric lymph nodes and small intestines, increased as well with the onset of HFD feeding, and remained high (**Figure 3C, D**). Interestingly, splenic and peripheral lymph node pDCs presented a temporary, but striking increase in cellular granularity after 3 days of HFD feeding, normalizing to values of normolipidemic mice at 1 week (**Figure 3A, B**). In accordance with these data, cell granularity in peripheral cDC subsets, as depicted in **Figure 4** for splenic and lymph node CD8⁻ (**Figure 4A, C**) and CD8⁺ cDCs (**Figure 4B, D**), but not in pDCs (data not shown), correlated positively with increasing plasma cholesterol levels, suggesting that the aforementioned effects reflected lipid loading of these cells.

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Figure 3. HFD feeding is associated with increased cellular granularity of various DC subsets. Depicted are the results of flow cytometry analysis on DC subsets isolated from diverse lymphoid organs and small intestines of *Ldlr*^{-/-} mice fed a SFD (control; n=6) or HFD (3 days, 1 week, 3 weeks or 5 weeks; n=6 per group). Percentage of granularity (measured as increase in granularity on side scatter (SSC) in **(A)** spleen, **(B)** lymph nodes (LN), **(C)** mesenteric lymph nodes (mesenteric LN), and **(D)** small intestinal DC subsets (CD8⁻ cDCs, CD8⁺ cDCs, PDCA-1⁺ B220⁺ DCs) is markedly increasing with the onset of HFD feeding. **(E)** FACS plots revealing splenic DC subset granularity after different time points of HFD feeding. *p<0.05, **p<0.01.

Figure 4. Cellular granularity of splenic cDC subsets correlates with plasma cholesterol levels.

To investigate whether DC granularity is increasing with increasing plasma cholesterol levels, a non-linear correlation analysis was performed comparing the granularity of cDC subsets with plasma cholesterol levels in *Ldlr*^{-/-} mice on SFD (control; n=6) versus HFD (0.5 weeks, 1 week, 3 weeks, 5 weeks; n=6 per group). Correlation analysis (non-linear correlation) of splenic (A) CD8⁻ cDCs ($R^2=0.51$), (B) CD8⁺ cDCs ($R^2=0.16$) and lymph node (C) CD8⁻ cDCs ($R^2=0.61$), (D) CD8⁺ cDCs ($R^2=0.68$). R^2 = non-linear correlation coefficient.

The straight correlation between plasma cholesterol levels and granularity change suggests that the latter is a measure of lipid loading of the cells. Therefore, we sorted CD8⁻ cDCs, CD8⁺ cDCs and pDCs from spleen and peripheral lymph nodes from *Ldlr*^{-/-} mice fed a standard or high fat diet, and performed Oil-Red-O staining. Indeed, splenic and lymph node CD8⁻ and CD8⁺ cDCs from hypercholesterolemic mice were lipid-laden and had foam cell-like morphology (**Figure 5A**), whereas pDCs did not reveal any lipid deposits, concordant with their low cell granularity after 3 weeks of HFD feeding (**Figure 5A**). Quantification of Oil-Red-O staining demonstrated a 13-fold increase in intracellular lipid content in splenic CD8⁻ and to a lesser extent in CD8⁺ cDCs, from hypercholesterolemic mice, whereas pDCs stained negative (**Figure 5B**). The total number of lipid-laden cDCs (CD8⁻ and CD8⁺ cDCs) was around 15%, suggesting that only a small fraction of peripheral cDCs had ingested

lipoproteins. These data were confirmed by flow cytometry analysis of Nile Red lipid staining, showing Nile Red signal in high granular splenic cDC subsets from hypercholesterolemic mice but not in low granular cDCs nor in pDCs (**Figure 5C**). Again, high granular cDCs were sharply induced in hyperlipidemia, while hypercholesterolemic mouse pDCs retained a low granular cell phenotype (**Figure 5C**). Taken together, our data demonstrate that unlike pDCs, peripheral CD8⁻ and less so CD8⁺ cDCs accumulate lipids, converting them into DC-derived foam cells already in the first week of HFD feeding.

Figure 5. Peripheral cDC subsets from hypercholesterolemic *Ldlr*^{-/-} mice engulf lipoproteins. DC subsets (PDCA-1⁺ B220⁺ pDCs, CD8⁻ cDCs, CD8⁺ cDCs), sorted from lymphoid organs of hyperlipidemic (3 weeks of HFD; n=3) but not normolipidemic *Ldlr*^{-/-} mice (SFD; n=3) show pronounced lipoprotein accumulation (**A**) Microscopic analysis of Oil-Red-O staining performed on cytospun splenic DC subsets. Red droplets represent intracellular accumulated lipoproteins. Cell nuclei are counterstained with haematoxylin. (**B**) Quantitative measurement of Oil-Red-O⁺

cells. Slides were analyzed blindly using the Image J program. **(C)** Flow cytometry analysis of Nile Red lipid staining on low- and high-granular DC subsets ($CD8^-$ cDCs, $CD8^+$ cDCs, pDCs) was performed to confirm intracellular lipoprotein storage. High-granular splenic cDC subsets show a clearly increased Nile Red signal, associating granularity with lipoprotein accumulation.

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

OxLDL has been shown to induce apoptosis in resident monocytes and macrophages, an effect that was attributable to the pro-oxidant capacity of lipoprotein-entrapped agents, generating reactive oxygen species (ROS), such as superoxide anions.^(30, 31) Therefore, we investigated whether lipoprotein uptake was accompanied by ROS accumulation in cDCs.⁽³²⁾ Flow cytometry of DCFDA-stained splenic cDC subsets, isolated from normo- and hypercholesterolemic *Ldlr*^{-/-} mice, revealed markedly elevated intracellular ROS activity in $CD8^-$ cDCs from HFD compared to standard fed *Ldlr*^{-/-} mice (**Figure 6A**). Similarly, ROS activity in splenic $CD8^+$ cDCs of HFD fed mice tended to be increased as well (**Figure 6C**). ROS signals were significantly augmented in high granular (lipid-laden) compared to low granular $CD8^-$ and $CD8^+$ cDCs from HFD mice, suggesting a clear association between lipid ingestion and ROS accumulation (**Figure 6B, D**).

Figure 6. Lipoprotein accumulation in cDCs is associated with induced intracellular ROS activity. Depicted are the results of flow cytometry analysis on splenic cDC subsets isolated from *Ldlr*^{-/-} mice fed a SFD (control; n=11) or HFD (3 weeks; n=11). **(A, D)** Percentage of ROS activity in cDC subsets from normo- and hyperlipidemic mice represents an increase in ROS activity in cDCs from HFD fed mice. **(B-F)** Percentage of ROS activity in low and high granular **(B, C)** CD8⁻ and **(E, F)** CD8⁺ cDCs from HFD fed mice. High-granular cDCs show a marked 3-fold increase in ROS activity compared to low-granular cDCs. *p< 0.05, **p< 0.01, ***p< 0.001.

Peripheral lipid-laden DC subsets display impaired maturation

To determine whether lipid loading has affected DC phenotype and maturation, we analyzed surface expression of co-stimulatory molecules (CD40, CD86) and major histocompatibility complex (MHC) class II on splenic DC subsets from normo- and hypercholesterolemic *Ldlr*^{-/-} mice. As has been shown before,⁽³¹⁾ cDCs expressed intermediate levels of MHC II under normolipidemic conditions. MHC II surface expression was unaltered in high granular lipid-laden CD8⁻ cDCs, and even tended to decrease in high granular CD8⁺ cDCs (**Figure 7A, B**). Strikingly, expression of CD40 and CD86 on high granular CD8⁻ cDCs was significantly diminished (**Figure 7A**), and the same held for CD40 expression by high granular CD8⁺ cDCs from hypercholesterolemic mice (**Figure 7B**). pDCs displayed unaltered expression of MHC II, CD40 and CD86 upon HFD feeding (**Figure 7C**). To summarize, these data strongly support the idea that a hypercholesterolemic environment, not only results in the formation of lipoprotein-laden DCs, particularly of the CD8⁻ subset, but also impairs DC maturation, a crucial step towards T cell priming.

Figure 7. Peripheral DC subsets display impaired maturation under hypercholesterolemic conditions. (A-C) Induction of maturation markers (CD40, CD86, MHC II) on high granular DC subsets (CD8⁺ and CD8⁺ cDCs, PDCA-1⁺ B220⁺ pDCs) isolated from spleen from normolipidemic (SFD; n=3) and hyperlipidemic (HFD; n=3) *Ldlr*^{-/-} mice. Expression levels are shown as mean fluorescence intensity (MFI). *p < 0.05.

Lipid-laden cDC subsets reveal a reduced ability to stimulate naïve T cells

The unaltered or even diminished co-stimulatory molecule expression by lipid-laden DCs led us to investigate the T cell priming capacity of DCs under the hypercholesterolemic conditions generally associated with atherosclerosis. Splenic low and high granular (lipid-laden) CD8⁻ and CD8⁺ cDCs from HFD and standard fed *Ldlr*^{-/-} mice were co-cultured with naïve T cells (from standard diet fed mice). Importantly, splenic lipid-laden CD8⁻ cDCs had a significantly reduced ability to induce naïve CD4⁺ (-40%) and CD8⁺ T cell proliferation (-20%), compared to low granular cDCs from HFD mice or CD8⁻ cDCs from SFD mice (**Figure 8A, B, E**). T-cell mitogenic capacity, expressed as number of cell divisions, was also quenched in the presence of lipid-laden vs lipid-free CD8⁻ cDCs (**Supplemental Figure 4A, B**). Lipid-laden CD8⁺ cDCs from hypercholesterolemic mice were also less capable of priming naïve CD4⁺ (-34%) and CD8⁺ T cell proliferation (-25%) (**Figure 8C, D, E; Supplemental Figure 4C, D**). Conclusively, our data clearly demonstrate that lipid-laden CD8⁺ and CD8⁻ cDCs are less functional, as judged by their attenuated ability to stimulate naïve T cells.

Figure 8. Lipid-laden cDC subsets reveal a reduced ability to stimulate naïve T cells.

(A, B) Splenic (low and high granular) CD8⁻ cDCs, or (C, D) splenic (low and high granular) CD8⁺ cDCs, isolated from SFD or HFD fed *Ldlr*^{-/-} mice (n=3) were co-cultured *in vitro* with CFSE labeled naïve splenic CD4⁺ or CD8⁺ T cells (in triplo) from SFD fed *Ldlr*^{-/-} mice. T cell proliferation was assessed by CFSE signal dilution, as a measure of T-cell divisions. (E) Flow cytometry plots illustrating CFSE signal dilution (T cell proliferative response; blue: 1st division; red: 2nd division; green: 3rd division; yellow: 4th division; grey: 5th division). *p<0.05, **p<0.01.

Discussion

Recent work suggests a prominent role for DCs in atherosclerosis, as they are key players in triggering adaptive immune responses.⁽³³⁾ The decreased levels of circulating DC (precursors) in patients with coronary artery disease inversely correlated with their progressive accumulation in atherosclerotic plaque during lesion progression.⁽³⁴⁾ However, little is known about peripheral DC homeostasis and behaviour during conditions of hyperlipidemia, a key determinant in atherosclerosis. Here, we studied the early adaptive responses of DC (precursors) to high fat diet feeding, as well as the functionality of lipid-laden peripheral DC subsets. We have shown that in an immediate response to hypercholesterolemia, splenic and lymph node cDC subsets accumulated lipids and, possibly related, reactive oxygen species. Interestingly lipid-laden cDCs featured an immature phenotype and an impaired capacity to prime naïve T cells.

High fat diet feeding was in our study shown to induce rapid bone marrow myeloid and dendritic cell precursor (MDP) expansion, in keeping with previous findings.⁽²⁾ Splenic MDP numbers however, previously reported to increase during atherogenesis in *ApoE*^{-/-} mice,⁽³⁵⁾ did not change in *Ldlr*^{-/-} mice, potentially related to the more modest myeloopoietic effects seen in the latter model.⁽¹⁰⁾ Interestingly, while DC lymphoid organ distribution patterns were not profoundly affected, we did observe an initial decrease in small intestinal DC content (CD8⁺ cDCs, pDCs) upon high fat diet feeding to normalize within 1 week after diet change. Conceivably, this apparent drop could reflect prompt recruitment of DCs to mesenteric lymph nodes or thymus to present dietary epitopes to T cells.⁽³⁶⁾ Unaltered peripheral DC counts might be explained by MDP differentiation mainly into monocytes/ macrophages. FMS-like tyrosine kinase 3 ligand (Flt3L) and macrophage colony-stimulating factor (M-CSF) are the two main regulators of the development of DCs and macrophages, respectively.⁽³⁷⁾ A disbalance of these two signaling mediators under hyperlipidemic conditions potentially leads to monocytosis.⁽³⁸⁻⁴⁰⁾

A second objective of this study was to address how peripheral DC subsets (lymphoid organ and small intestinal DC subsets) behave in response to chow- to high-fat diet change. Interestingly, recent findings of Lesnik and coworkers argued for a contributory role of cDCs to cholesterol homeostasis under hyperlipidemic conditions, possibly by taking up lipoproteins from

peripheral organs.⁽¹⁴⁾ Our study indeed showed that granularity of lymphoid organ and small intestinal pDCs and CD11c^{high} cDC subsets is rapidly increased in response to high fat diet feeding to peak at 1-3 weeks. Notably, for splenic and peripheral lymph node pDCs augmented granularity only manifested immediately after onset of high fat diet feeding. Increased granularity of cDCs clearly reflected lipid accumulation as witnessed by the increased Nile Red lipid (flow cytometry) and Oil-Red-O staining (cytospin). However, only a small fraction of cDCs actually accumulated lipoproteins, potentially representing a (1) more long-lived cDC subset, (2) a population with highest capacity for or lowest mobilization/ apoptosis response to lipoprotein uptake, or (3) a DC population with a more activated intracellular antigen-uptake/ processing machinery. Of relevance in this regard is that resident intimal DCs were shown to account for lipoprotein accumulation and rapid formation of foam cells during very early stages of atherogenesis (5 days of high fat diet feeding).⁽²⁰⁾

A further intriguing finding was the increased reactive oxygen species (ROS) build-up in splenic cDC subsets in the immediate response to high fat diet feeding, particularly in the high granular (lipid-laden) cDCs. Accumulation of oxidized low density lipoproteins (oxLDLs) has been shown to promote apoptosis in macrophages, amongst others due to the generation of cytotoxic ROS, f.e. superoxide anions.⁽³¹⁾ Interestingly, DCs express high levels of antioxidant enzymes, such as Manganese superoxide dismutase (Mn-SOD) in response to oxLDL accumulation, which confers resistance to oxLDL induced apoptosis.⁽³²⁾ Whether or not the respiratory burst in high granular peripheral cDCs induces cell apoptosis, remains unclear.

Lipid-laden cDC subsets, as well as pDCs, from lymphoid organs from hypercholesterolemic mice featured an immature phenotype, with low or even abated surface expression of co-stimulatory molecules (CD40, CD86) and MHC class II. Additionally, hyperlipidemia led to impaired ability of splenic CD8⁻ and CD8⁺ cDCs to stimulate naïve T cells, as indicated by the reduced T cell proliferative responses *in vitro*. As non lipid-laden cDC subsets retained their CD4⁺ and CD8⁺ priming ability, this effect obviously is resultant of lipid ingestion. Support for this notion may be derived from a study by Kopf and coworkers showing abrogated Toll-like receptor (TLR)-induced cytokine production, reduced co-stimulatory molecule expression by CD8⁻ cDCs, and abated Th cell responses under hyperlipidemic conditions.⁽²³⁾ Herber *et al.* similarly evidenced that lipid-laden DCs in cancer were markedly impaired in

their capacity to process and present antigens to T cells, thus disturbing immune responses.⁽⁴¹⁾ Otherwise, several studies have previously demonstrated that adaptive immune responses prevail in a cholesterol-rich environment in that DCs fully maintain their antigen-presenting and T cell priming ability, even when cholesterol-loaded.^(13, 14, 42) It remains to be elusive which DC subsets are involved in cholesterol homeostasis and how excessive lipoprotein uptake quenches DC's T cell priming capacity, potentially by impeding intracellular antigen processing and presentation machinery.⁽⁴³⁻⁴⁵⁾

Altogether, these data indicate that high fat diet feeding acutely interferes with cDC activation, impairing their T cell stimulating capacity. This might compensate for the pro-atherogenic effect by priming T cells within the atherosclerotic plaque. Besides, it is conceivable that this reduced DC functionality will affect host's immunity against pathogen-derived signals under dyslipidemic conditions, making the host more vulnerable/fragile to infectious diseases.

Supplemental Figures

Supplemental Figure 1. Inflammatory plasma cytokine levels remain mainly unaltered under early conditions of hypercholesterolemia. *Ldlr*^{-/-} mice were either placed for 3 days (n=6), 1 week (n=6), 3 weeks (n=6) or 5 weeks (n=6) on HFD (0.25% cholesterol) or left on SFD (control; n=6). **(A)** Effects of HFD on plasma cytokine levels of IL-6, IL-12p70, IL-1β, IL-10, and CXCL1 (mKC). *p<0.05, **p<0.01.

Supplemental Figure 2. Hypercholesterolemia does not alter peripheral lymphocyte numbers.

Depicted are the results of flow cytometry analysis on bone marrow, spleen and blood from *Ldlr*^{-/-} mice on SFD (control; n=6) versus HFD (3 days, 1 week, 3 weeks or 5 weeks; n=6 per group). **(A, B)** Percentage of CD45⁺ leukocytes, NKT cells (NK1.1^{low} CD3^{low}), total T cells (CD3⁺), CD4⁺ and CD8⁺ T cell subsets, and B cells (B220⁺) in bone marrow and spleen. **(C)** Absolute numbers of CD45⁺ leukocytes, total T cells (CD3⁺), CD4⁺ and CD8⁺ T cell subsets, and B cells (B220⁺) in blood.

*p<0.05.

Supplemental Figure 3. Immediate temporal response of cDC subset counts in peripheral lymphoid organs to high fat diet feeding. Depicted are the results of flow cytometry analysis on spleen, peripheral LN, and mesenteric LN from *Ldlr*^{-/-} mice on SFD (control; n=6) versus HFD (3 days, 1 week, 3 weeks or 5 weeks; n=6 per group). **(A)** Absolute numbers of splenic CD8⁻ and CD8⁺ cDCs. **(B, C)** Percentage of CD8⁻ or CD8⁺ CD11c^{high} cDCs in peripheral LN and mesenteric LN. *p< 0.05.

Supplemental Figure 4. Lipid-laden cDC subsets have reduced ability to stimulate naïve T cells. (A, B) Splenic (low and high granular) CD8⁻ cDCs, or (C, D) splenic (low and high granular) CD8⁺ cDCs, both isolated from SFD or HFD fed *Ldlr*^{-/-} mice were co-cultured *in vitro* with CFSE labeled naïve splenic CD4⁺ or CD8⁺ T cells isolated from SFD fed *Ldlr*^{-/-} mice. T cell proliferation was assessed by CFSE signal dilution. This experiment was reproduced in triplicate and gave similar results.

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In the previous chapters we investigated a potential functional role for plasmacytoid dendritic cells (pDCs) in murine atherosclerosis, although they are almost absent within the mouse plaque. Besides, we examined phenotype and activation status/ functionality of murine and human (p)DCs in a hyperlipidemic environment. In the present study (**Chapter 7**) we finally addressed the question whether pDCs have an impact on its local immune cell context within the human atherosclerotic plaque.

In the recent years pDCs have been described in inflammatory foci in the human atherosclerotic plaque, where they are thought to execute pro-inflammatory actions through a type I interferon signature. Stimuli that potentially trigger pDC maturation and activation at lesion site are unknown. It is supposable that immunocomplexes, composed of self-DNA released from necrotic/ dying cells and joined with antimicrobial peptides or oxidized (lipo)proteins, formed in the advanced plaque, have the capacity to stimulate pDCs. Otherwise, pDCs may exert tolerogenic functions during processes of low-grade inflammation. To dissolve the question whether pDCs manipulate their cellular neighbourhood we employed Laser Capture Microdissection on pDC-enriched and pDC-deficient human plaque fractions, isolated RNA and performed whole genome arraying and bioinformatics analyses. This study allowed us to acquire pDC gene expression patterns within the human atherosclerotic lesion.

Contribution to the manuscript (Chapter 7):

Anette Christ designed this study primarily with Prof. Daemen (and Prof. Biessen). Anette Christ was responsible for the collection of human plaque tissue samples. Anette Christ and Veronica Herias accomplished the laser capture microdissection experiments, including the optimization of the anti-BDCA-4 staining on frozen human plaque tissue sections, the isolation of pDC-enriched and pDC-deficient plaque areas by LCM, and subsequent RNA-isolation. RNA-amplification and microarray hybridization were performed in cooperation with the Genetics Department at Maastricht University. Bioinformatics analyses were performed by Marco Manca. Final data were discussed and interpreted by Anette Christ, Marco Manca and Prof. Biessen. Anette Christ and Marco Manca wrote the manuscript. Prof. Daemen, Prof. Zenke and Prof. Biessen revised the written manuscript.

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7

An immune response network associated with plasmacytoid dendritic cells in the human atherosclerotic plaque.

- submitted -

Abstract

Background: Plasmacytoid dendritic cells (pDCs) are scarcely present in the inflamed human atherosclerotic plaque, where they are presumed to exert pro-inflammatory functions through release of type I interferons. However, the precise role of pDCs in human atherosclerosis yet remains to be established. Here, we investigated the impact of human plaque pDCs on its local context, applying state of the art genomics and bioinformatics.

Methods and Results: pDC-enriched and pDC-deficient plaque fractions, isolated by laser capture microdissection from human carotid endarterectomy tissue samples, were used for genome-wide micro-array analysis. We determined gene expression pattern of pDC-enriched and pDC-deficient plaque fractions and employed network and module-trait correlation analyses to characterize clusters of tightly co-expressed gene modules for macrophages, T cells and microvessels, associating with the presence of pDCs. We uncovered five significantly overexpressed immune clusters in the T-cell module, highly enriched in genes involved in regulation of neutrophil chemotaxis and regulation of T cell differentiation/ proliferation. Moreover, we were able to identify two microRNAs, which target the pDC-T cell gene cluster.

Conclusions: The present study reveals a direct cross-talk between pDCs and its immune cell context in the plaque, but also led to the unravelling of potential novel central modifiers in pDC-mediated immune responses in atherosclerosis.

Introduction

Cardiovascular diseases (CVDs) are the number one killer in western countries, and projections say that by 2030 they will become the first cause of death and morbidity in Low-Middle-Income Countries (LMIC).⁽¹⁾ The pathogenesis of this complex disease is still subject of much debate, with models for plaque progression and destabilization/instability alternatively based on lipid and glucidic metabolism, tissue ageing, calcification, gut microbiota, and global and local immunity competing for our understanding and ability to influence the course of CVDs. Here, we are focusing on profiling local immunity to further our understanding of plaque physiology and to identify potential clinical targets.

Among the leukocyte subsets that contribute to atherosclerosis (the main underlying lesion of CVDs), dendritic cells (DCs) are receiving growing attention.^(2, 3) DCs are a family of antigen presenting cells displaying considerable diversity in trafficking modalities, anatomical location, phenotype, and functionality.^(4, 5) While previous studies have focused mainly on conventional DCs (cDCs),⁽⁶⁻⁸⁾ plasmacytoid DCs (pDCs) recently emerged as important players in the pathophysiology of atherosclerosis as well.⁽⁹⁻¹²⁾ pDCs develop in bone marrow and migrate to lymphoid tissues.⁽¹³⁾ They are highly specialized sensors of viral and bacterial infections, due to their selective expression of Toll-like receptor 7 (TLR7) and TLR9.^(14, 15) Upon receptor activation, pDCs are capable to produce large amounts of type I interferons (IFNs), which amongst others can trigger T cell responses.^(16, 17) Chronic pDC activation and type I IFN release in the absence of viral infection can result in the breakdown of tolerance, potentially leading to the development of autoimmune diseases.^(18, 19) Vascular pDCs have been identified in human atherosclerosis.⁽⁹⁾ Their presence in the plaque shoulder of advanced human lesions is supposed to drive inflammatory processes and plaque destabilization by the release of type I IFNs.^(9, 10) However, a clear functional role for pDCs in human atherosclerosis has not been reported. Here, we investigated the impact of plaque pDCs on their local inflammatory context in human atherosclerosis. To this end, we employed laser capture microdissection (LCM) on human carotid endarterectomy tissue samples to isolate pDC-enriched and adjacent pDC-deficient plaque tissue fractions from the same plaque, and extracted RNA for genome-wide expression profiling. Differential gene

expression analysis between pDC-enriched and pDC-deficient plaque fractions revealed gene sets implicated in pDCs' role in atherosclerosis. Furthermore, co-expression network reverse engineering and module-trait correlation analysis focused our attention on T-cell associated gene clusters, which are strongly linked to/imprinted by the presence of pDCs. To the best of our knowledge our analysis provides the first insights into the action/impact of pDCs on its local immune environment in the advanced atherosclerotic plaque, obtained in human tissue.

Material and Methods

Tissue samples

Fresh atherosclerotic tissue samples (human carotid arteries) were obtained from endarterectomy (n=6). Collection, storage in the Maastricht Pathology Tissue Collection (MPTC) and use of tissue and patient data were performed in agreement with the "Code for Proper Secondary Use of Human Tissue in the Netherlands" (<http://www.fmwv.nl>). Detailed tissue and patient characteristics are added in **Supplemental Table 1**. Immediately after resection, tissue samples were snap-frozen in Tissue-Tek® OCT compound (Sakura Finetek Zoeterwoude, the Netherlands) and stored at -80°C. The stage of atherosclerosis was determined according to Virmani *et al.*⁽²⁰⁾ based on hematoxylin-eosin (HE) stained frozen tissue flanking sections. The samples were as all advanced plaques, stable (n=5; thin or thick fibrous cap atheroma) or unstable (n=1; complicated type VI lesions, with luminal thrombus and/or intraplaque hemorrhage (IPH)). The localization of pDCs was inferred from anti-BDCA-4 immunohistochemical staining of flanking frozen tissue sections. We evaluated RNA quality of the tissues before pDC isolation. Total RNA of whole mount plaque tissue sections was isolated using RNeasy Micro kit (Qiagen, Hilden, Germany). RNA integrity was assessed using Agilent's 2100 Bioanalyzer System (Agilent Technologies, Palo Alto, CA, USA). From the same slice, inflammatory foci in the plaque shoulder area containing >5 BDCA-4⁺ pDCs/ μm^2 (pDC-enriched) or 0 BDCA-4⁺ pDCs/ μm^2 (pDC-deficient), were isolated by LCM. As quality control, the aforementioned procedure for RNA quality was repeated on RNA extracted from LCM fractions; patient samples were only included for microarray analysis if the RNA integrity value of both

pDC-enriched and pDC-deficient plaque fractions was higher than 4.5. All tools for LCM work were UV-sterilized before experiment to increase RNA quality.

Tissue preparation and LCM

For isolation of pDC-enriched and pDC-deficient plaque fractions, 4 µm thick OCT frozen sections were cut at -20°C in a cryostat (Leica CM 3050S) and placed on MembraneSlides (NF 1.0 PEN; Carl Zeiss Micro Imaging, Munich, Germany). Sections were fixed and dehydrated for 10 minutes in ice-cold sterile acetone before LCM (Palm Microlaser Technologies, Zeiss Microscope Palm Robo 4.3, Munich, Germany) to improve tissue catapulting. pDC-enriched and pDC-deficient plaque fractions from the very same plaque slices were isolated by LCM from 6-10 frozen tissue sections, respectively pooled and collected in nuclease and nucleid acid free tubes with an adhesive cap covered with tissue lysis buffer (RLT buffer) containing β-mercaptoethanol to improve RNA yield and minimize RNA degradation. pDC-enriched plaque fractions were harvested using BDCA-4 immuno-positivity on parallel sections as a navigator (pDC-enriched tissue fractions contained between 5 and 16 BDCA-4⁺ cells). pDC-deficient plaque fractions did not contain any BDCA-4⁺ cells.

RNA isolation and amplification

Total RNA was extracted form LCM drive pDC-enriched and pDC-deficient plaque fractions immediately after dissection using the RNeasy Micro kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol for extraction of RNA from microdissected cryosections. DNA was removed by using 50U DNase I (Qiagen). RNA samples were speed vacuum centrifuged to increase RNA concentration. Total RNA (500-750pg) of pDC-enriched and pDC-deficient plaque fractions was cDNA transcribed (double strand cDNA synthesis), purified (RNAClean beads) and amplified (Ribo-SPIA amplification process) using the Ovation Pico WTA System V2 (NuGEN Technologies, Bemmell, The Netherlands).

Array hybridization and analysis

For array hybridization single primer isothermal amplified (SPIA) cDNA samples were enzymatically fragmented (input: 3 µg per sample) using the Ovation Pico WTA System V2. Size and quality of the amplified fragments were examined by gel shift assay and Agilent's 2100 Bioanalyzer System.

Subsequently, cDNA samples were biotin-labeled to the 3-hydroxyl end of the fragmented cDNA using the Encore Biotin Module (NuGEN Technologies). 2.5 µg of labeled cDNA of each sample was applied to Affymetrix GeneChip Human Gene 1.0 ST arrays (Affymetrix, Santa Clara, CA, USA). Images were acquired using the Affymetrix Gene Chip Scanner 7G and feature extraction was done by Gene Chip® Command Console 3.2 (AGCC) software.

Raw data (available on Gene Expression Omnibus: GSE49670) were imported in R (R Core Team (2012), URL <http://www.R-project.org>) and pre-processed by GCRMA (Wu *et al.*, *Journal of the American Statistical Association* 2004) for background correction, normalization and gene level summarization (**Supplemental Figure 1**). Data were annotated using the last updated annotation files available on the manufacturer's website. Differential expression analysis of pDC-enriched versus pDC-deficient plaque fractions was performed by fitting each gene against a linear model with limma (Smyth *et al.*, (2005). Limma: linear models for microarray data; Gentleman *et al.*, *Springer*, New York). Empirical Bayes shrinkage of the standard errors has been used to moderate t-statistics, F-statistic, and log-odds of differential expression. To correct for repeated measurements False Discovery Rate (Benjamini, Yekutieli, *Roy Statist Soc Ser B (Methodological)* 1995) was used. An explorative gene ontology enrichment analysis of the 500 top differentially expressed genes was conducted using the Database for Annotation, Visualization and Integrated Discovery (DAVID).

Reverse engineering of a co-expression network was performed by leveraging on the tools included in the R package WGCNA (Langfelder *et al.*, *BMC Bioinformatics* 2008). The soft-threshold, used to reconstruct the network architecture, was optimized by inferential statistics to evaluate the scale-freeness of the resulting network architecture for threshold values from 12-30 (**Supplemental Figure 2**). Modules, clusters of densely interconnected genes, were identified using the default method of hierarchical clustering (Kaufman *et al.*, *John Wiley & Sons Inc*, 1990) and the function cutree Dynamic (Langfelder *et al.*, *Bioinformatics* 2008) to "prune" the branches of the tree. Module's Eigengene were calculated and those too highly correlated were merged to optimize the following statistical analyses. We then reconstructed the consensus network between the pDC-enriched and pDC-deficient plaque fractions. We focused our attention on those modules which were unique to

the pDC-enriched samples, since we hypothesized that these gene clusters would be the most reflective of the biological changes induced by pDCs on their surrounding microenvironment. The relationship between quantitative phenotypic traits and gene clusters was estimated by canonical correlation of the Eigengenes and quantitative traits, and significance was estimated by Fisher's asymptotic p-value. Within each module, genes with high module membership in modules related to traits were selected for further investigation (Langfelder, Horvath, *BMC Bioinformatics* 2008). Module network data were uploaded into Cytoscape (Smoot *et al.*, *Bioinformatics* 2011) for visualization and further analyses. The plugin ClueGene Ontology (GO) (Bindea *et al.*, *Bioinformatics* 2009) was used to enrich the interesting modules for over-represented GO terms and pathways.

Immunohistochemistry

Human carotid endarterectomy tissue sections, obtained as above reported, were analyzed for expression of (1) pDCs (BDCA-4⁺; for LCM guidance), and (2) T cells (CD3⁺), macrophages (CD68⁺) and microvessels (CD31⁺) in pDC-enriched and pDC-deficient plaque fractions (analysis of immune cell expression patterns). Immunohistochemistry was performed on acetone-fixed frozen tissue sections (4μm thick) using the following primary antibodies: sheep anti-human BDCA-4 (1/40; R&D Systems, Minneapolis, MN, USA), rabbit anti-human CD3 (1/200; Dako, Glostrup, Denmark), mouse anti-human CD68 (1/100; Dako) and mouse anti-human CD31 (1/50; Dako). Tissue sections were incubated with primary antibody for 60 minutes at room temperature. For CD31 staining, non-specific antigens were blocked for 30 minutes in TBS containing 5% BSA prior to primary antibody incubation. For CD3 staining, endogenous peroxidases were blocked in methanol/H₂O₂. Secondary antibodies were added as follows: poly-horseradish peroxidase (HRP) conjugated goat anti-rabbit IgG (1/50, Immunologic, Duiven, the Netherlands) for CD3, biotin-labeled rabbit anti-sheep IgG (1/200, Zymed, Vienna, Austria) for BDCA-4, biotin-labeled sheep anti-mouse IgG (1/250, Amersham Pharmacia, Uppsala, Sweden) for CD68, or poly-alkaline phosphatase (AP) conjugated goat anti-mouse IgG (1/50, Immunologic) for CD31. Color development was assessed by use of Strep-ABC-alkaline (1/200, VectorLaboratories, Burlingame, CA, USA) and Vectastain Red (VectorLaboratories), or 3-amino-9-ethylcarbazole (AEC; Dako) according to

the manufacturer's instructions. Cell nuclei were counterstained with hematoxylin. Slides were analyzed using the Leica DM3000 light microscope (Leica Microsystems, Wetzlar, Germany) coupled to a computerized morphometric system (Leica Qwin 3.5.1, Wetzlar, Germany).

Statistical analysis of IHC

Data were analyzed with the use of Graph Pad Prism 5 (Graph Pad Software Inc San Diego, CA, USA). All data are expressed as mean $\pm$ SEM. To compare individual groups, a Wilcoxon paired test was used. Values of $p < 0.05$ were considered statistically significant.

Results

Presence of plasmacytoid dendritic cells in the human atherosclerotic plaque

To identify pDC associated gene signatures in advanced human atherosclerotic plaques, we used the LCM technology to isolate similarly inflamed pDC-enriched and pDC-deficient plaque fractions in the same plaque of the same patient (**Figure 1A, B**). BDCA-4⁺ staining revealed the presence of pDCs in inflammatory regions in the plaque shoulder and at the plaque base close to plaque microvessels. Adjacent regions from the flanking section were subsequently captured by LCM. Quantitative measurement demonstrated that less than 2% of total cells in the pDC-enriched fractions were BDCA-4⁺ pDCs (**Figure 1C**) (number of BDCA-4⁺ pDCs in enriched tissue fractions diversified between 5 and 16 per fragment). pDC-deficient plaque fractions did not show any BDCA-4 positivity (**Figure 1C**). To exclude that expression patterns would be biased by differences in major cellular constituents in pDC-enriched vs. pDC-deficient plaque fractions, we assessed T cell and macrophage content, as well as the presence of microvessels in both groups by immunohistochemistry. Quantitative measurement did not reveal any differences in T cell ($p=0.1875$), macrophage ($p=0.625$) and microvessel ($p=1.0$) presence in pDC-enriched compared to pDC-deficient plaque fractions (**Figure 2A, B, C**).

Figure 1. Presence of pDCs in human atherosclerotic plaques, mainly in regions of inflammation and neovascularization. Microscopic analysis of human atherosclerotic plaque tissue specimens from carotid endarterectomy used for LCM. **(A)** Immunohistochemistry for BDCA-4⁺ pDCs in pDC-enriched plaque fractions. **(B)** Inflammatory region in the plaque shoulder of advanced lesions, negative for BDCA-4⁺ pDCs. **(C)** Percentage of BDCA-4⁺ pDCs per total cell number in pDC-enriched (n=6) and pDC-deficient fractions of advanced plaques (n=5).

Figure 2. Cellular composition and presence of vessels in pDC-enriched compared to pDC-deficient plaque fractions. Microscopic analysis of human atherosclerotic plaque tissue specimen from carotid endarterectomy used for LCM. Immunohistochemistry and quantitative measurement of **(A)** CD68⁺ macrophages, **(B)** CD3⁺ T cells and **(C)** CD31⁺ vessels in pDC-enriched (n=6) versus pDC-deficient (n=5) plaque fractions of advanced plaques.

Human atherosclerotic plaques, enriched in pDCs, reveal distinct patterns of immune pathways

Differential expression analysis yielded ambiguous results. As shown in the volcano plot of **Figure 3A**, several promising genes appeared in the results. However, after multiple testing adjustment, no gene had an adjusted p-value $\leq .05$. A top list of 200 over- or underexpressed genes was extracted for exploratory purposes (**Supplemental Table S2**). Importantly, this tentative signature perfectly sets apart the pDC-enriched and pDC-deficient plaque fractions, as shown in the heatmap in **Figure 3B**. Subsequent pathway analysis resulted in a top 7 of networks overrepresented in the pDC-enriched plaque fractions, including amongst others T cell receptor and mitogen-activated protein kinase (MAPK) signaling pathways (**Supplemental Table S3**), suggesting that pDCs are present in an activated immune microenvironment.

Figure 3. Unsupervised analysis of differential gene expression in pDC-enriched versus pDC-deficient plaque fractions. Gene array data were analyzed using the Affymetrix metadata software. **(A)** Volcano plot for pDC-enriched versus pDC-deficient plaque fractions, used to measure changes in the gene expression levels. The log 2-fold change is plotted on the x-axis (up or down), while the $-\log_{10}$ p-value is plotted on the y-axis. The probesets shown in blue are the top-25 probesets ordered by log-ratio. The probesets highlighted in red are the top-25 ordered by p-value. **(B)** Hierarchical clustering of the top 200 genes that are over - or underexpressed (green and red, respectively) in pDC-enriched versus pDC-deficient plaque fractions results in four clusters, representing different expression patterns. Results are shown in heat map format. pDC-enriched plaques, samples 1-5, 11; pDC-deficient plaque samples 6-10.

Inspired by these findings we sought to assess the impact of pDCs on its local immune context. Hereto, by co-expression networks analysis and subsequent module trait correlation, specific pDC-associated gene modules were identified and correlated with quantified phenotypes (T cells, macrophages and microvessels) using the methods first described by Langfelder and Horvath (*BMC Bioinformatics*, 2008). To differentiate strong and weak correlations and best approximate scale-free network topology, we conducted a scale-freeness estimation (**Supplemental Figure S2**). The module identification routine resulted in twenty-six distinct modules, whose individual Eigengenes were tested for correlation with T cells, macrophages and microvessels (**Figure 4**). The strongest association for T cells resulted in module MEbrown ($p=0.02$), consisting of 732 genes. The strongest associations for macrophages were with modules MELightcyan ($p=0.03$), consisting of 140 genes, and METurquoise ($p=0.02$; 846 genes). The strongest associations for

microvessels were with modules MEtan ($p=0.01$; 238 genes), MEblack ($p=0.003$; 431 genes), MEorange ($p=3e^{-06}$; 38 genes), and MEcyan ($p=0.01$; 182 genes). Because we selected modules which were unique to the pDC-enriched fractions (**Supplemental Figure S3**), the above mentioned correlation patterns for macrophages, T cells and microvessels are to be considered reflective of the interaction between pDCs and that particular trait.

Figure 4. Network module association with macrophage, T cell and vessel traits in pDC-enriched plaque fractions. For each trait and each module expression profile, a Spearman rank correlation was performed. Each row corresponds to a module (lettered from MEorange to MEdarkred) and each column to a particular trait (macrophages, T cells, microvessels). Each cell contains the probability that a correlation exists by chance and is colour-coded with red indicating a strong positive correlation and green a strong negative correlation. The modules most strongly associated with the macrophage trait are MElightcyan and METurquoise, while the module most strongly associated with the T cell trait is MEbrown. The modules most strongly associated with the microvessel trait are modules METan, MEblack, MEorange and MEcyan (for all strong positive correlation).

Gene significance and module membership in the ‘T cell module’ are connected

As previous studies have alluded to an interaction between pDCs and $CD4^+$ T cells in the human atherosclerotic plaque, driving Th1 polarization of $CD4^+$ T cells,⁹ we further focused on the pDC-associated gene module that correlates with the presence of T cells (MEbrown, 732 genes), hereafter

referred to as the ‘T cell module’. In the ‘T cell module’ highly connected genes (referred to as intramodular hub genes) also had a high gene significance for T cells, meaning that these genes constitute the core of the module and are the most correlated with the T cell trait ($\text{cor.} = 0.28$; $p = 1.2\text{e-}14$; **Figure 5**). Genes that are most associated with the T cell trait are encircled in red (**Supplemental Table 3**).

Figure 5. Connectivity and trait association within the T cell module. In the T cell module, there is a strong positive correlation between the intramodular connectivity and the gene significance for particular T cell genes, revealing that these genes are the most associated with the T cell trait ($\text{cor.} = 0.28$; $p = 1.2\text{e-}14$). Genes that are most associated with the T cell trait are encircled in red.

We exported the T cell module data to Cytoscape to integrate our results (module structure and gene expression profiles) (**Figure 6A**). Furthermore, by importing gene functional annotations from the Gene Ontology (GO) and KEGG and BioCarta pathways databases to the network in the T cell module, five immune clusters were significantly overrepresented: (1) regulation of leukocyte chemotaxis, (2) neutrophil chemotaxis, (3) regulation of neutrophil chemotaxis, (4) positive regulation of Th1/ Th17 cell differentiation, and (5) regulation of Th1/ Th17 cell differentiation (**Table 1**). We could also distinguish two major axes within the topology of the network that is representing the interactive genes, and the attributed pathways:

regulation of neutrophil chemotaxis and regulation of T cell activation/proliferation (**Figure 6B**). Beyond, we could infer two microRNAs specifically overrepresented in the 'T cell module': microRNA-518A-5P and miRNA-382-5P. MicroRNA-382 has been implicated in CD4 T cell activation²¹, thus again pointing to the privileged immune microenvironment in pDC-enriched plaques.

A

B

Figure 6. Network and molecular pathway analysis in the T cell module. (A) Molecular interaction networks in the T cell module are visualized by Cytoscape. Nodes in blue represent genes whose association did not surpass multiple testing adjustment. Nodes in various shades of red represent genes associated to the phenotype. The darker the node the stronger is the association to the phenotype. Sizes of the nodes illustrate a continuous mapping of the importance of the gene in the architecture of the module. **(B)** Within the topology of the network, used to make strong correlations clear, two major axes are standing out: regulation of neutrophil chemotaxis and regulation of T cell proliferation.

Table 1. Immune clusters in the ‘T cell module’

Discussion

The presence of pDCs in the human advanced plaque, mainly in the inflamed plaque shoulder area and in regions with neovascularization, has been firmly documented.^(9, 11, 12) Still, plaque pDCs’ association with plaque progression stage, as well as their precise role in disease progression, remain controversial. So far, pDCs are believed to induce Th1 polarization of naïve CD4⁺ T cells by release of type I IFNs, albeit some studies pointed to a tolerogenic role in low grade plaque inflammation in mouse models of

disease.⁽⁹⁻¹¹⁾ In this study we employed a whole genome expression analysis to investigate the impact of plaque pDCs on their local immune context within the human atherosclerotic plaque. Laser capture microscopy was used to isolate pDC-enriched and pDC-deficient plaque fractions for transcriptomics analysis, to investigate pDC-mediated context imprinting in human advanced atherosclerosis, *ex vivo*. We employed a co-expression network analysis from the same plaque to extract and characterize co-expressed gene modules from pDC-enriched plaque segments, strongly associated with T cells, macrophages, and/ or microvessels. Transcriptional profiling of pDC-enriched vs. pDC-deficient plaque fractions did not identify a pDC-specific gene signature, probably due to the low number of our cohort, the interpatient heterogeneity and the subtle effects exerted by low pDC abundance. However, further exploration of the transcriptomics data highlighted a list of genes in pDC-enriched plaque area and identified 7 overrepresented functions, amongst others T cell receptor signaling pathway and MAPK pathway. Given the similar T cell presence in both study groups this possibly reflects a more activated phenotype in the pDC-enriched advanced human plaque. p38 MAPK pathway activation in pDCs- enriched fractions might be associated with enhanced pDC recruitment to these areas. p38 MAPK pathway activation has furthermore been shown to be associated with IFN- α production, due to increased expression of the IFN regulatory factor (IRF) 7.⁽²²⁾ However, we were unable to identify the increased expression of genes encoding the IFN pathway (or IFN response genes) in pDC-enriched fractions, suggesting that other mediators rather than IFN- α are responsible for pDC-induced immunity in the human plaque. To investigate the impact of pDCs on the immune microenvironment of the atherosclerotic plaque in finer details, co-expression network reverse engineering and gene modules-traits correlation were performed. This analysis highlighted certain gene clusters for T cells, macrophages and microvessels that particularly emerged in the pDC-enriched plaque fractions and not in the pDC-deficient ones. Inspired by the above pathway analysis and the IFN/ tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-mediated interplay between pDCs and CD4⁺ T cells suggested by Weyand and coworkers,^(9, 10) we focused on the T cell gene module, ascertaining gene pathways being involved in the possible pDC-T cell crosstalk in the human plaque. Of note, adoptive transfer of human plaque-sorted CD4⁺ T cells into mice engrafted with human atherosclerotic plaque resulted in TRAIL-dependent vascular smooth muscle

cell (vSMC) apoptosis.⁽²³⁾ These studies suggest a link between vascular pDCs and possible TRAIL-dependent T cell-induced fibrous cap thinning and plaque destabilization, a hypothesis worth further investigation also in light of our results.

Here, we identified five immune functions, over-expressed in the ‘T cell module’, including positive regulation of Th1/Th17 differentiation and regulation of leukocyte chemotaxis. These data support the idea of a key role for pDCs on its immune context in atherosclerosis. Likewise, previous studies demonstrated the involvement of pDCs in the initiation of autoimmune Th17- and Th1-cell responses in experimental autoimmune encephalomyelitis.⁽²⁴⁾ Similarly, in patients with psoriasis and systemic lupus erythematosus (SLE), pDC activation and type I IFN release were associated with disease severity, due to an activation of monocyte-derived DCs, T cells and B cells.^(19, 25) Once again, in this study we were unable to link pDC presence in the advanced human plaque with a type I IFN signature. It needs to be further validated whether pDCs are bystanders or the actual driving force inducing Th1/Th17 differentiation in pDC-enriched plaque regions. In fact, Th1 cells may release CXCL10, which might amplify the CXCL12-dependent pDC recruitment.⁽²⁶⁾ Further experiments are needed to support this notion. In any case, human inflammatory DCs are probably responsible for the induced immune response,^(27, 28) and might be interesting targets for translational research. Furthermore, we identified two microRNAs, i.e. microRNA-382 and microRNA-518a, targeting the pDC-T cell genes cluster. In literature, microRNA-382 was amongst others seen to be enriched in resting CD4⁺ T cells as compared to activated CD4⁺ T cells, to confer potent inhibition of human immunodeficiency virus type 1 (HIV-1) production.⁽²¹⁾ In parallel, it is up-regulated by IFN- α and IFN- β in HIV-1 infected macrophages, inhibiting viral replication.⁽²⁹⁾ Further studies are awaited to address a functional role for these two microRNAs and their implication in pDC- T cell interplay in the context of atherosclerosis.

Conclusively, our study has uncovered a previously uncharacterized gene network associated with the presence of pDCs in the human atherosclerotic plaque, suggesting an extensive pDC/T cell cross-talk. We extracted five overrepresented functional immune pathways, including T cell polarization and leukocyte chemotaxis, in the T cell module, which are linked to the presence of pDCs. Moreover, two microRNAs were identified, targeting a subset of the pDC/T cell gene cluster. Thus, this study not only offers new

insights into pDCs imprinting on the immune microenvironment in advanced human atherosclerosis, but also exposes pDCs as potential therapeutic targets in human atherosclerosis. Further prospective validation experiments have to firmly establish the reported findings, thus consolidating how pDCs impact the immune context of human plaque.

Supplemental Figures

Supplemental Figure 1. Post-robust multi array average (GCRMA) normalization. Array data were pre-processed using the GCRMA algorithm to correct background and normalize signal intensities. **(A)** Correlation matrix of pDC-enriched and pDC-deficient plaque fractions based on all detectable genes. **(B)** Boxplots after GCRMA normalization, showing the distribution of the observed log₂-intensities on the sample dataset. **(C)** Smoothed histograms for samples in the dataset post-robust multi array average normalization.

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Supplemental Figure 2. Scale-free topology analysis within the pDC-enriched and pDC-deficient plaque fractions. (A) The panel shows the scale free topology fit index R^2 as a function of the soft-thresholding power. **(B-D)** The panels show the mean, median and max network connectivity. The networks become approximately scale-free when the soft-thresholding power becomes 12 (for pDC-deficient fractions), or >20 (for pDC-enriched fractions).

Correspondence of DC-enriched plaque set-specific and DC-enriched/ control consensus modules

Supplemental Figure 3. Overlap of modules in the pDC-enriched and pDC-deficient plaque fractions. Each row corresponds to a module related to the pDC-enriched fractions, indicated by name and number of genes in the module. Conversely, each column corresponds to a module related to the pDC-deficient fractions, indicated by name and number of genes in the module. Numbers in the table represent the number of genes in the overlap. The table is coloured according to the log10 p-value, with a colour scale indicated on the right. Most modules related to the pDC-enriched plaque fractions do not or weakly overlap with modules related to the pDC-deficient fractions.

Supplemental Tables

Supplemental Table 1. Patient and tissue characteristics

Patient code	Age (years)	Sex	stage of atherosclerosis in carotid artery sample	RIN values	
1298.3	65	male	IPH	enriched:	4,5
1299.2	53	female	IPH	non-enriched:	6,0
				enriched:	4,5
1309.5	65	male	IPH	non-enriched:	7,0
				enriched:	5,2
1310.5	83	male	IPH	non-enriched:	6,0
				enriched:	6,4
1311.7	75	male	IPH/ TnFCA	non-enriched:	4,6
				enriched:	6,7
1330.6	53	female	TkFCA	non-enriched:	6,6
				enriched:	5,7

IPH = intraplaque hemorrhage
TkFCA = thick fibrous cap atheroma
TnFCA = thin fibrous cap atheroma

Supplemental Table 2. Top of 179 genes over- or underexpressed in the pDC-enriched plaque regions with $p < 0.01$ as obtained by independent students t-test (Ensembl Annotation, individual p-value and fold-change).

Ensembl ID	Description	P value	FC
ENSG00000165659	dachshund homolog 1 (Drosophila) [Source:HGNC Symbol;Acc:2663]	8.41E-05	0.94
ENSG00000185055	EF-hand calcium binding domain 10 [Source:HGNC Symbol;Acc:34531]	1.80E-04	0.89
ENSG00000176105	v-yes-1 Yamaguchi sarcoma viral oncogene homolog 1 [Source:HGNC Symbol;Acc:12841]	2.08E-04	0.92
ENSG00000167037	small G protein signaling modulator 1 [Source:HGNC Symbol;Acc:29410]	2.16E-04	1.05
ENSG00000138101	dystrobrevin, beta [Source:HGNC Symbol;Acc:3058]	2.51E-04	1.05
ENSG00000112473	solute carrier family 39 (zinc transporter), member 7 [Source:HGNC Symbol;Acc:4927]	4.65E-04	0.94
ENSG00000153140	centrin, EF-hand protein, 3 [Source:HGNC Symbol;Acc:1868]	4.79E-04	0.90
ENSG00000162881	oxoecicosanoid (OXE) receptor 1 [Source:HGNC Symbol;Acc:24884]	6.05E-04	1.17
ENSG00000142541	ribosomal protein L13a [Source:HGNC Symbol;Acc:10304]	6.40E-04	0.92
ENSG00000088682	coenzyme Q9 homolog (S. cerevisiae) [Source:HGNC Symbol;Acc:25302]	6.58E-04	1.09
ENSG00000207001	small nucleolar RNA, C/D box 116-2 [Source:HGNC Symbol;Acc:33068]	7.48E-04	0.80
ENSG00000133606	makorin ring finger protein 1 [Source:HGNC Symbol;Acc:7112]	1.00E-03	0.90
ENSG00000207442	small nucleolar RNA, C/D box 116-6 [Source:HGNC Symbol;Acc:33072]	1.07E-03	0.78
ENSG00000167280	endo-beta-N-acetylglucosaminidase [Source:HGNC Symbol;Acc:24622]	1.18E-03	1.13
ENSG00000188403		1.19E-03	1.10
ENSG00000188343	family with sequence similarity 92, member A1 [Source:HGNC Symbol;Acc:30452]	1.23E-03	1.30
ENSG00000087299	L-2-hydroxyglutarate dehydrogenase [Source:HGNC Symbol;Acc:20499]	1.37E-03	0.94
ENSG00000196772	olfactory receptor, family 14, subfamily A, member 16 [Source:HGNC Symbol;Acc:15022]	1.40E-03	0.95
ENSG00000114784	eukaryotic translation initiation factor 1B [Source:HGNC Symbol;Acc:30792]	1.44E-03	0.89
ENSG00000182551	acireductone dioxygenase 1 [Source:HGNC Symbol;Acc:30576]	1.54E-03	0.90
ENSG00000150776	chromosome 11 open reading frame 57 [Source:HGNC Symbol;Acc:25569]	1.62E-03	0.91
ENSG00000154518	ATP synthase, H+ transporting, mitochondrial Fo complex, subunit C3 (subunit 9) [Source:HGNC Symbol;Acc:843]	1.64E-03	0.96
ENSG00000133895	multiple endocrine neoplasia I [Source:HGNC Symbol;Acc:7010]	1.79E-03	1.08
ENSG00000114573	ATPase, H+ transporting, lysosomal 70kDa, V1 subunit A [Source:HGNC Symbol;Acc:851]	1.84E-03	0.90
ENSG00000197849	olfactory receptor, family 8, subfamily G, member 7 pseudogene [Source:HGNC Symbol;Acc:19625]	1.91E-03	0.94
ENSG00000204228	hydroxysteroid (17-beta) dehydrogenase 8 [Source:HGNC Symbol;Acc:3554]	1.99E-03	1.10
ENSG00000169075	bromodomain containing 7 pseudogene 3 [Source:HGNC Symbol;Acc:24171]	2.02E-03	0.94
ENSG00000152266	parathyroid hormone [Source:HGNC Symbol;Acc:9606]	2.10E-03	0.95
ENSG00000120533	enhancer of yellow 2 homolog (Drosophila) [Source:HGNC Symbol;Acc:24449]	2.29E-03	0.89
ENSG00000200902	U6 spliceosomal RNA [Source:RFAM;Acc:RF00026]	2.31E-03	0.95
ENSG00000155249	olfactory receptor, family 4, subfamily K, member 1 [Source:HGNC Symbol;Acc:14726]	2.34E-03	0.94
ENSG00000104881	protein phosphatase 1, regulatory subunit 13 like [Source:HGNC Symbol;Acc:18838]	2.47E-03	1.11
ENSG00000073737	dehydrogenase/reductase (SDR family) member 9 [Source:HGNC Symbol;Acc:16888]	2.52E-03	0.84
ENSG00000102174	phosphate regulating endopeptidase homolog, X-linked [Source:HGNC Symbol;Acc:8918]	2.55E-03	0.94
ENSG00000138032	protein phosphatase, Mg2+/Mn2+ dependent, 1B [Source:HGNC Symbol;Acc:9276]	2.56E-03	0.91

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ENSG00000185222	WW domain binding protein 5 [Source:HGNC Symbol;Acc:30084]	2.57E-03	0.91
ENSG00000146378	trace amine associated receptor 2 [Source:HGNC Symbol;Acc:4514]	2.60E-03	0.89
ENSG00000174942	olfactory receptor, family 5, subfamily R, member 1 [Source:HGNC Symbol;Acc:14841]	2.62E-03	0.95
ENSG00000207386	Y RNA [Source:RFAM;Acc:RF00019]	2.64E-03	0.91
ENSG00000179010	Morf4 family associated protein 1 [Source:HGNC Symbol;Acc:24549]	2.66E-03	0.95
ENSG00000202089	U6 spliceosomal RNA [Source:RFAM;Acc:RF00026]	2.66E-03	0.62
ENSG00000074966	TXK tyrosine kinase [Source:HGNC Symbol;Acc:12434]	2.71E-03	0.89
ENSG00000202103	U4 spliceosomal RNA [Source:RFAM;Acc:RF00015]	2.72E-03	0.95
ENSG00000166987	methyl-CpG binding domain protein 6 [Source:HGNC Symbol;Acc:20445]	2.74E-03	1.08
ENSG00000184785	chromosome X open reading frame 69 [Source:HGNC Symbol;Acc:41913]	2.76E-03	1.21
ENSG00000140262	transcription factor 12 [Source:HGNC Symbol;Acc:11623]	2.83E-03	1.08
ENSG000000020129	neurochondrin [Source:HGNC Symbol;Acc:17597]	2.84E-03	1.09
ENSG00000188315	chromosome 3 open reading frame 62 [Source:HGNC Symbol;Acc:24771]	2.93E-03	1.13
ENSG00000175309	alanine-glyoxylate aminotransferase 2-like 2 [Source:HGNC Symbol;Acc:28249]	2.94E-03	1.07
ENSG00000175262	chromosome 1 open reading frame 127 [Source:HGNC Symbol;Acc:26730]	3.02E-03	1.03
ENSG00000200034	U4 spliceosomal RNA [Source:RFAM;Acc:RF00015]	3.08E-03	0.94
ENSG00000185728	YTH domain family, member 3 [Source:HGNC Symbol;Acc:26465]	3.23E-03	0.94
ENSG00000161180	coiled-coil domain containing 116 [Source:HGNC Symbol;Acc:26688]	3.25E-03	1.04
ENSG00000133731	inositol(myo)-1(or 4)-monophosphatase 1 [Source:HGNC Symbol;Acc:6050]	3.34E-03	0.90
ENSG00000047644	WWC family member 3 [Source:HGNC Symbol;Acc:29237]	3.37E-03	1.08
ENSG00000170509	hydroxysteroid (17-beta) dehydrogenase 13 [Source:HGNC Symbol;Acc:18685]	3.46E-03	0.93
ENSG00000200313	RNA, 5.8S ribosomal 7 [Source:HGNC Symbol;Acc:41962]	3.50E-03	0.90
ENSG00000102678	fibroblast growth factor 9 (glia-activating factor) [Source:HGNC Symbol;Acc:3687]	3.54E-03	0.92
ENSG00000132429	popeye domain containing 3 [Source:HGNC Symbol;Acc:17649]	3.68E-03	0.93
ENSG00000049618	AT rich interactive domain 1B (SWI1-like) [Source:HGNC Symbol;Acc:18040]	3.70E-03	1.14
ENSG00000135506	osteosarcoma amplified 9, endoplasmic reticulum lectin [Source:HGNC Symbol;Acc:16994]	3.96E-03	0.97
ENSG00000116478	histone deacetylase 1 [Source:HGNC Symbol;Acc:4852]	4.16E-03	0.90
ENSG00000202044	RNA, 5S ribosomal 223 [Source:HGNC Symbol;Acc:43123]	4.16E-03	0.97
ENSG00000075239	acetyl-CoA acetyltransferase 1 [Source:HGNC Symbol;Acc:93]	4.20E-03	0.94
ENSG00000113643	arginyl-tRNA synthetase [Source:HGNC Symbol;Acc:9870]	4.21E-03	0.92
ENSG00000131584	ArfGAP with coiled-coil, ankyrin repeat and PH domains 3 [Source:HGNC Symbol;Acc:16754]	4.28E-03	1.06
ENSG00000162390	acyl-CoA thioesterase 11 [Source:HGNC Symbol;Acc:18156]	4.41E-03	1.09
ENSG00000174032	solute carrier family 25, member 30 [Source:HGNC Symbol;Acc:27371]	4.46E-03	0.89
ENSG00000160563	mediator complex subunit 27 [Source:HGNC Symbol;Acc:2377]	4.50E-03	0.89
ENSG00000006530	acylglycerol kinase [Source:HGNC Symbol;Acc:21869]	4.60E-03	0.94
ENSG00000072736	nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 3 [Source:HGNC Symbol;Acc:7777]	4.64E-03	1.04
ENSG00000133111	regulatory factor X-associated protein [Source:HGNC Symbol;Acc:9988]	4.66E-03	0.96
ENSG00000185390	fibroblast growth factor 7 pseudogene 2 [Source:HGNC Symbol;Acc:17193]	4.74E-03	0.76
ENSG00000143624	integrator complex subunit 3 [Source:HGNC Symbol;Acc:26153]	4.88E-03	1.08
ENSG00000178972		4.95E-03	1.11
ENSG00000100139	MICAL-like 1 [Source:HGNC Symbol;Acc:29804]	5.00E-03	1.05
ENSG00000033011	asparagine-linked glycosylation 1, beta-1,4-mannosyltransferase homolog (S. cerevisiae) [Source:HGNC Symbol;Acc:18294]	5.04E-03	1.12

ENSG00000144711	IQ motif and Sec7 domain 1 [Source:HGNC Symbol;Acc:29112]	5.15E-03	1.06
ENSG00000223037	U6 spliceosomal RNA [Source:RFAM;Acc:RF00026]	5.25E-03	0.96
ENSG00000053918	potassium voltage-gated channel, KQT-like subfamily, member 1 [Source:HGNC Symbol;Acc:6294]	5.27E-03	1.05
ENSG00000159761	chromosome 16 open reading frame 86 [Source:HGNC Symbol;Acc:33755]	5.30E-03	1.04
ENSG00000160094	zinc finger protein 362 [Source:HGNC Symbol;Acc:18079]	5.30E-03	1.08
ENSG00000206862	Y RNA [Source:RFAM;Acc:RF00019]	5.37E-03	0.95
ENSG00000173517	Pseudopodium-enriched atypical kinase 1 [Source:UniProtKB/Swiss-Prot;Acc:Q9H792]	5.37E-03	1.11
ENSG00000164037	solute carrier family 9, subfamily B (NHA1, cation proton antiporter 1), member 1 [Source:HGNC Symbol;Acc:24244]	5.39E-03	0.93
ENSG00000171517	lysophosphatidic acid receptor 3 [Source:HGNC Symbol;Acc:14298]	5.41E-03	0.91
ENSG00000105122	RAS protein activator like 3 [Source:HGNC Symbol;Acc:26129]	5.56E-03	1.08
ENSG00000150768	dihydrolipoamide S-acetyltransferase [Source:HGNC Symbol;Acc:2896]	5.59E-03	0.94
ENSG00000222997	Y RNA [Source:RFAM;Acc:RF00019]	5.62E-03	0.94
ENSG00000146049	kidney associated antigen 1 [Source:HGNC Symbol;Acc:21031]	5.64E-03	0.93
ENSG00000150977	Rab interacting lysosomal protein-like 2 [Source:HGNC Symbol;Acc:28787]	5.67E-03	0.91
ENSG00000205755	cytokine receptor-like factor 2 [Source:HGNC Symbol;Acc:14281]	5.74E-03	0.88
ENSG00000212560	U6 spliceosomal RNA [Source:RFAM;Acc:RF00026]	5.77E-03	0.82
ENSG00000182447	otolin 1 [Source:HGNC Symbol;Acc:34071]	5.79E-03	0.94
ENSG00000067533	ribosomal RNA processing 15 homolog (S. cerevisiae) [Source:HGNC Symbol;Acc:24255]	5.86E-03	1.04
ENSG00000173486	FK506 binding protein 2, 13kDa [Source:HGNC Symbol;Acc:3718]	5.94E-03	0.96
ENSG00000103227	lipase maturation factor 1 [Source:HGNC Symbol;Acc:14154]	6.03E-03	1.09
ENSG00000187630	dehydrogenase/reductase (SDR family) member 4 like 2 [Source:HGNC Symbol;Acc:19731]	6.05E-03	1.10
ENSG00000212829	ribosomal protein S26 pseudogene 3 [Source:HGNC Symbol;Acc:23411]	6.08E-03	0.96
ENSG00000204389	heat shock 70kDa protein 1A [Source:HGNC Symbol;Acc:5232]	6.08E-03	0.91
ENSG00000095139	archain 1 [Source:HGNC Symbol;Acc:649]	6.22E-03	0.93
ENSG00000198728	LIM domain binding 1 [Source:HGNC Symbol;Acc:6532]	6.23E-03	1.11
ENSG00000197275	RAD54 homolog B (S. cerevisiae) [Source:HGNC Symbol;Acc:17228]	6.38E-03	1.06
ENSG00000196152	zinc finger protein 79 [Source:HGNC Symbol;Acc:13153]	6.43E-03	1.07
ENSG00000207093	small nucleolar RNA, C/D box 116-8 [Source:HGNC Symbol;Acc:33074]	6.46E-03	0.68
ENSG00000178654		6.47E-03	0.94
ENSG00000092531	synaptosomal-associated protein, 23kDa [Source:HGNC Symbol;Acc:11131]	6.49E-03	0.94
ENSG00000146425	dynein, light chain, Tctex-type 1 [Source:HGNC Symbol;Acc:11697]	6.50E-03	0.90
ENSG00000174891	arginine/serine-rich coiled-coil 1 [Source:HGNC Symbol;Acc:24152]	6.53E-03	0.90
ENSG00000084764	microtubule-associated protein, RP/EB family, member 3 [Source:HGNC Symbol;Acc:6892]	6.71E-03	1.10
ENSG00000197045	glia maturation factor, beta [Source:HGNC Symbol;Acc:4373]	6.81E-03	0.92
ENSG00000172375	C2CD2-like [Source:HGNC Symbol;Acc:29000]	6.83E-03	1.05
ENSG00000108100	cyclin Y [Source:HGNC Symbol;Acc:23354]	6.88E-03	0.97
ENSG00000132522	G protein pathway suppressor 2 [Source:HGNC Symbol;Acc:4550]	6.92E-03	1.04
ENSG00000116350	serine/arginine-rich splicing factor 4 [Source:HGNC Symbol;Acc:10786]	7.00E-03	0.97
ENSG00000160584	SIK family kinase 3 [Source:HGNC Symbol;Acc:29165]	7.01E-03	1.10
ENSG00000137364	thiopurine S-methyltransferase [Source:HGNC Symbol;Acc:12014]	7.01E-03	0.86
ENSG00000127527	epidermal growth factor receptor pathway substrate 15-like 1 [Source:HGNC Symbol;Acc:24634]	7.05E-03	1.06

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ENSG00000148362	chromosome 9 open reading frame 142 [Source:HGNC Symbol;Acc:27849]	7.11E-03	1.06
ENSG00000206718	U6 spliceosomal RNA [Source:RFAM;Acc:RF00026]	7.14E-03	0.95
ENSG00000165694	FERM domain containing 7 [Source:HGNC Symbol;Acc:8079]	7.19E-03	0.92
ENSG00000171703	transcription elongation factor A (SII), 2 [Source:HGNC Symbol;Acc:11614]	7.23E-03	1.04
ENSG00000171790	schlafen-like 1 [Source:HGNC Symbol;Acc:26313]	7.27E-03	1.05
ENSG00000108298	ribosomal protein L19 [Source:HGNC Symbol;Acc:10312]	7.28E-03	1.03
ENSG00000201436	U6 spliceosomal RNA [Source:RFAM;Acc:RF00026]	7.37E-03	0.85
ENSG00000105325	fizzy/cell division cycle 20 related 1 (Drosophila) [Source:HGNC Symbol;Acc:24824]	7.41E-03	1.07
ENSG00000127152	B-cell CLL/lymphoma 11B (zinc finger protein) [Source:HGNC Symbol;Acc:13222]	7.41E-03	1.04
ENSG00000100258	lipase maturation factor 2 [Source:HGNC Symbol;Acc:25096]	7.45E-03	1.12
ENSG00000206970	U6 spliceosomal RNA [Source:RFAM;Acc:RF00026]	7.50E-03	0.93
ENSG00000087884	chromosome 11 open reading frame 67 [Source:HGNC Symbol;Acc:30205]	7.51E-03	0.95
ENSG00000221954	olfactory receptor, family 4, subfamily C, member 12 [Source:HGNC Symbol;Acc:15168]	7.51E-03	0.91
ENSG00000207439	Y RNA [Source:RFAM;Acc:RF00019]	7.53E-03	0.92
ENSG00000092841	myosin, light chain 6, alkali, smooth muscle and non-muscle [Source:HGNC Symbol;Acc:7587]	7.67E-03	0.97
ENSG00000144381	heat shock 60kDa protein 1 (chaperonin) [Source:HGNC Symbol;Acc:5261]	7.77E-03	0.95
ENSG00000173451	THAP domain containing, apoptosis associated protein 2 [Source:HGNC Symbol;Acc:20854]	7.84E-03	0.90
ENSG00000154079	chromosome 6 open reading frame 57 [Source:HGNC Symbol;Acc:20957]	7.90E-03	1.09
ENSG00000173531	macrophage stimulating 1 (hepatocyte growth factor-like) [Source:HGNC Symbol;Acc:7380]	7.94E-03	1.17
ENSG00000143344	ral guanine nucleotide dissociation stimulator-like 1 [Source:HGNC Symbol;Acc:30281]	7.95E-03	0.94
ENSG00000174028	family with sequence similarity 3, member C2 (pseudogene) [Source:HGNC Symbol;Acc:34501]	8.02E-03	0.80
ENSG00000197171	olfactory receptor, family 2, subfamily B, member 4 pseudogene [Source:HGNC Symbol;Acc:8239]	8.06E-03	0.95
ENSG00000143382	ADAMTS-like 4 [Source:HGNC Symbol;Acc:19706]	8.13E-03	1.07
ENSG00000124702	kelch domain containing 3 [Source:HGNC Symbol;Acc:20704]	8.19E-03	1.11
ENSG00000113073	solute carrier family 4, sodium bicarbonate cotransporter, member 9 [Source:HGNC Symbol;Acc:11035]	8.32E-03	0.97
ENSG00000146067	family with sequence similarity 193, member B [Source:HGNC Symbol;Acc:25524]	8.36E-03	1.08
ENSG00000185250	peptidylprolyl isomerase (cyclophilin)-like 6 [Source:HGNC Symbol;Acc:21557]	8.51E-03	0.95
ENSG00000168591	transmembrane and ubiquitin-like domain containing 2 [Source:HGNC Symbol;Acc:28459]	8.51E-03	1.05
ENSG00000222644	U2 spliceosomal RNA [Source:RFAM;Acc:RF00004]	8.55E-03	0.91
ENSG00000207242	U6 spliceosomal RNA [Source:RFAM;Acc:RF00026]	8.56E-03	0.64
ENSG00000103037	SET domain containing 6 [Source:HGNC Symbol;Acc:26116]	8.56E-03	1.05
ENSG00000114491	uridine monophosphate synthetase [Source:HGNC Symbol;Acc:12563]	8.58E-03	1.11
ENSG00000141655	tumor necrosis factor receptor superfamily, member 11a, NFkB activator [Source:HGNC Symbol;Acc:11908]	8.63E-03	1.07
ENSG00000090520	DnaJ (Hsp40) homolog, subfamily B, member 11 [Source:HGNC Symbol;Acc:14889]	8.70E-03	0.93
ENSG00000203691		8.80E-03	0.94
ENSG00000086189	DIM1 dimethyladenosine transferase 1 homolog (S. cerevisiae) [Source:HGNC Symbol;Acc:30217]	8.82E-03	0.95
ENSG00000161217	phosphate cytidylyltransferase 1, choline, alpha [Source:HGNC Symbol;Acc:8754]	8.87E-03	0.92
ENSG00000109814	UDP-glucose 6-dehydrogenase [Source:HGNC Symbol;Acc:12525]	8.92E-03	0.92
ENSG00000200606	Y RNA [Source:RFAM;Acc:RF00019]	8.92E-03	0.88
ENSG00000142599	arginine-glutamic acid dipeptide (RE) repeats [Source:HGNC Symbol;Acc:9965]	8.96E-03	1.04

ENSG00000109436	TBC1 domain family, member 9 (with GRAM domain) [Source:HGNC Symbol;Acc:21710]	9.02E-03	1.05
ENSG00000139572	G protein-coupled receptor 84 [Source:HGNC Symbol;Acc:4535]	9.10E-03	1.12
ENSG00000164818	HEAT repeat containing 2 [Source:HGNC Symbol;Acc:26013]	9.20E-03	1.07
ENSG00000198886	mitochondrially encoded NADH dehydrogenase 4 [Source:HGNC Symbol;Acc:7459]	9.21E-03	0.98
ENSG00000199222	Y RNA [Source:RFAM;Acc:RF00019]	9.22E-03	0.94
ENSG00000164106	stimulator of chondrogenesis 1 [Source:HGNC Symbol;Acc:17036]	9.33E-03	0.84
ENSG00000105205	Charcot-Leyden crystal protein [Source:HGNC Symbol;Acc:2014]	9.42E-03	0.92
ENSG00000152078	transmembrane protein 56 [Source:HGNC Symbol;Acc:26477]	9.47E-03	0.85
ENSG00000081791	KIAA0141 [Source:HGNC Symbol;Acc:28969]	9.56E-03	1.10
ENSG00000198874	tRNA-yW synthesizing protein 1 homolog (S. cerevisiae) [Source:HGNC Symbol;Acc:25598]	9.63E-03	0.93
ENSG00000149547	etoposide induced 2.4 mRNA [Source:HGNC Symbol;Acc:13276]	9.68E-03	0.96
ENSG00000174989	F-box and WD repeat domain containing 8 [Source:HGNC Symbol;Acc:13597]	9.68E-03	1.07
ENSG00000169224	chromosome 1 open reading frame 150 [Source:HGNC Symbol;Acc:29583]	9.69E-03	0.91
ENSG00000158092	NCK adaptor protein 1 [Source:HGNC Symbol;Acc:7664]	9.91E-03	0.91
ENSG00000113327	gamma-aminobutyric acid (GABA) A receptor, gamma 2 [Source:HGNC Symbol;Acc:4087]	9.93E-03	0.98
ENSG00000204020	lipase, family member N [Source:HGNC Symbol;Acc:23452]	9.93E-03	0.87
ENSG00000140691	armadillo repeat containing 5 [Source:HGNC Symbol;Acc:25781]	9.94E-03	1.04
ENSG00000155876	Ras-related GTP binding A [Source:HGNC Symbol;Acc:16963]	9.94E-03	0.96
ENSG00000184471	C1q and tumor necrosis factor related protein 8 [Source:HGNC Symbol;Acc:31374]	9.96E-03	1.02
ENSG00000116251	ribosomal protein L22 [Source:HGNC Symbol;Acc:10315]	9.96E-03	0.92
ENSG00000110917	malectin [Source:HGNC Symbol;Acc:28973]	9.98E-03	0.96

Supplemental Table 3. Overrepresentation of the top 7 networks in the pDC-enriched plaque regions.

Pathways	Genes
Natural killer cell mediated cytotoxicity	NFAT5, FYN, IFNA14, IFNA16, NFATC2, NFATC3, NFATC4, MAP2K1, TNFRSF10D
B cell receptor signaling pathway	NFAT5, BLNK, NFATC2, NFATC3, NFATC4, MAP2K1
Axon guidance	NFAT5, EFNA4, EFNB1, PLXNB2, FYN, NFATC2, NFATC3, NFATC4
VEGF signaling pathway	NFATC4, MAP2K1, NFATC3, NFAT5, FYN, NFACTC2
T cell receptor signaling pathway	NFATC4, MAP2K1, NFATC3, NFAT5, FYN, NFACTC2
Wnt signaling pathway	NFACTC4, CREBBP, CSNK1E, NFATC3, NFAT5, NFATC2, WNT5A
MAPK signaling pathway	MKNK1, HSPA2, PPM1B, NFATC4, HSPA1A, HSPA1B, ATF2, MAP2K1, FGF9, NFATC2

Supplemental Table 4. Genes that are the most associated with the T cell trait in the T cell modules (encircled in red in Figure 5)

EntrezID	p-value
26777	7.25E+08
2217	0.000188462086830394
5224	0.000242516790962645
6293	0.000382305933654084
2796	0.000682946053090609
5982	0.000833397240535827
57122	0.000889817922496262
92285	0.000990655535173302
160851	0.0010064578366297
128229	0.00115587755070108
54482	0.00133679356133274
9448	0.00134562535876311
54148	0.0013933284697501
340252	0.00139550575853281
11198	0.00153569120797477
3995	0.0015686216372194
10572	0.00162908708427192
84668	0.00169828161209434
57609	0.00176971882666819
23398	0.00219639462803895
2752	0.00243357571599038
7182	0.00244673075655807
112939	0.0025555281157694
9871	0.00316443304724345
22978	0.00333071793788981
133619	0.0043009249631973
51313	0.00470120320472875
407013	0.0047618176506613
941	0.00480336618005059
2054	0.00496711134782501
441108	0.00519561491608452
22848	0.00520292668870337
145282	0.00531442710689766
10111	0.00545681563161602
2937	0.00547508622734234
729080	0.00572589205802052
1487	0.00599141238018546
84876	0.00612337784993039
5360	0.00615160076303719
51187	0.00616594382472369
63920	0.00620469988110582
59277	0.00637388771760107
51167	0.00672466089009627
153396	0.00672525162841608
55729	0.00721796042515293
9167	0.00730424694809579

50862	0.00732351996668049
3136	0.00753626031690917
2137	0.00754148255637059
29945	0.00764029098391836
149951	0.00789112636607362
3096	0.00830898419516174
84129	0.0084704368458084
3384	0.00848487357230459
1678	0.0084965463750001
11333	0.00854783022445309
153527	0.0086180635255337
5533	0.00868551686692155
84267	0.00878752305550031
10897	0.00895092494954742
79053	0.0091852222422393
23654	0.00932697170353974
9839	0.00951845960169084
7328	0.00993222476780401

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8

General Discussion

General Discussion

The main goal of the studies presented here was to explore the impact of certain DC subsets within hyperlipidemia-associated atherosclerosis in experimental animal models and in human. One DC subtype that we studied in detail, are plasmacytoid dendritic cells (pDCs), which have previously been identified in the shoulder region of human atherosclerotic lesions.⁽¹⁾ Here, we demonstrated that specific pDC depletion in low-density lipoprotein receptor deficient (*Ldlr*^{-/-}) mice significantly deteriorated lesion formation, suggestive of a protective role for pDCs in mouse atherosclerosis (**Chapter 3**). Furthermore, we examined the impact of pDCs in murine atherosclerosis by use of a second pDC depletion model, in which pDC ablation was based on the transgenic diphtheria toxin receptor (DTR) expression under the pDC-specific human BDCA-2 promoter (**Chapter 4**).

Based on previous findings^(1, 2) and our findings in Chapter 3, we hypothesised that pDCs, despite their scarce presence in the human atherosclerotic plaque, probably act by impacting their local immune context. To address this notion, we used Laser Capture Microdissection (LCM), to isolate pDC-enriched and pDC-less plaque segments from human carotid endarterectomy tissue samples, and performed genome-wide microarraying (**Chapter 7**). Co-expression cluster analyses revealed gene module traits of T cells, macrophages and microvessels that were specifically associated with the presence of pDCs in the human plaque.

In **Chapter 5** we addressed the question, whether a hyperlipidemic environment interferes with pDC functionality. Therefore, we stimulated human pDCs with lipoproteins (LDL or oxLDL) and a Toll-like receptor 9 (TLR9) agonist. Interestingly, lipoprotein pretreatment inhibited the TLR9 agonist induced activation of pDCs, and their T cell priming ability *in vitro*, reflecting an impaired activation status of pDCs under hyperlipidemic conditions. In **Chapter 6** we additionally addressed the role of hyperlipidemia on murine DC (precursor) homeostasis, and their phenotype and maturation status in peripheral (lymphoid) organs. Splenic lipid-laden cDCs (CD8⁻ and CD8⁺ cDCs) showed an impaired ability to prime naïve CD4⁺ and CD8⁺ T cells.

Plasmacytoid dendritic cells protect against atherosclerosis by tuning T cell proliferation and activity

In 2006 the group of Weyand and coworkers characterized increasing numbers of pDCs in the advanced human atherosclerotic plaque by using an anti-CD123 antibody. Additionally, they associated interferon- α (IFN- α) secretion, most likely by pDCs, with plaque destabilization by promoting apoptosis of vascular smooth muscle cells (vSMC).⁽¹⁾ IFN- α was furthermore seen to trigger the surface expression of TLR4 on conventional DCs (cDCs), thereby potentially amplifying inflammatory processes within the plaque.⁽²⁾ In keeping, Levy *et al.* confirmed IFN- α as a critical contributor to lesion formation in that repeated IFN- α injections into *Ldlr*^{-/-} mice enhanced lesion formation.⁽³⁾ Our group confirmed the presence of pDCs in the human plaque (Chapter 3), however pDCs were only marginally present, mainly in regions of inflammation, and pDC numbers did not differ between stable and vulnerable atherosclerotic lesions. Additionally, we failed to show co-localization of pDCs with IFN- α in the human plaque, thus challenging the concept that pDCs are the prime source of IFN- α . Other cells of the myeloid lineage, such as macrophages, might rather be the type I IFN producing cells within the atherosclerotic lesion.⁽⁴⁾

Of note, we revealed an athero-protective function for pDCs in mouse atherosclerosis, despite their virtual absence within the mouse plaque (Chapter 3). Specific pDC depletion resulted in amplified peripheral T cell proliferation and activity, which led to increased T cell invasion into atherosclerotic lesions. In effect, pDC depletion significantly aggravated lesion formation. We showed that pDCs were able to suppress T cell proliferation in an indoleamine 2,3-dioxygenase (IDO)-dependent manner in the context of atherosclerosis. IDO expression in DCs has been shown to be a highly versatile modulator of T cell immunity.^(5, 6) Due to the lack of pDCs in mouse atherosclerotic lesions, it is conceivable that they exert their protective functions in peripheral lymphoid organs, such as spleen or lymph nodes, by regulating T cell responses. Alternatively, pDCs might bear their effects in mucosa-associated lymphoid organs, the liver, or the thymus. Indeed, it has been demonstrated that pDCs are responsible for systemic tolerance by inducing T cell anergy in the liver, thus preventing oral sensitization to dietary antigens.⁽⁷⁾ Likewise, peripheral pDCs were able to mediate central tolerance

by transporting self-antigens to the thymus for presentation to developing T cells.⁽⁸⁾

As against our results, two other groups demonstrated recently that pDC depletion in ApolipoproteinE deficient (*ApoE*^{-/-}) mice significantly decreased plaque development, accompanied by a more stable plaque phenotype, and a reduced systemic inflammatory state, associating the presence of pDCs with aggravated lesion formation.^(9, 10) Both groups were able to detect pDCs within the mouse atherosclerotic plaque. Interestingly, Doering *et al.* demonstrated elevated IFN- α levels in murine plaques and serum in high fat diet fed *ApoE*^{-/-} mice, being reduced after pDC depletion, thus linking an immunogenic IFN- α signature to pDCs in mouse atherosclerosis.⁽⁹⁾

pDCs show rapid release of large amounts of type I IFNs in response to viral or certain bacterial antigens, attributing pDCs a crucial role in anti-viral defense.^(11, 12) Moreover, pDCs are considered to be involved in the pathomechanisms of autoimmune diseases, such as psoriasis or systemic lupus erythematosus (SLE), characterized by a type I IFN-signature and dsDNA-targeted immune responses.⁽¹³⁾ Nuclear antimicrobial peptides, f.e. LL-37 or antigens that are derived from necrotic cells, form immunocomplexes with self-DNA which have been shown to stimulate IFN- α production through activation of TLR9, expressed on pDCs.^(14, 15) Hence, it is conceivable that these immunocomplexes, composed of self-DNA released from necrotic cells and assembled with antimicrobial peptides or oxidized proteins, form in the advanced plaque, and have the capacity to stimulate pDCs in a TLR9-dependent manner. Indeed, Edfeldt *et al.* confirmed the presence of LL-37 in human atherosclerotic plaques.⁽¹⁶⁾ In line, Zerneck and coworkers visualized lesional Cramp in murine plaques, and Cramp-DNA complexes stimulated IFN- α production by pDCs *in vitro*, albeit that induction capacity was rather poor and required high concentrations of Cramp-DNA.⁽⁹⁾ In addition, *Cramp*^{-/-}/*Ldlr*^{-/-} mice displayed diminished lesion sizes, whereas *ApoE*^{-/-} mice treated with Cramp revealed an increase in plaque formation.⁽⁹⁾ The authors proposed that Cramp-induced recognition of self-DNA may trigger pDC activation and IFN- α secretion, thus driving inflammatory processes in atherosclerosis.⁽⁹⁾

The divergent findings in the different studies might be explained by the stage of lesion formation, and the use of different animal models. Indeed, our findings refer to conditions of low-grade inflammation, as we investigated the effect of pDC ablation in more progressed murine lesions, whereas Doering

et al. and MacRitchie *et al.* analyzed the impact of pDC depletion after 4 weeks of high fat diet feeding.^(9, 10) It has to be noted that the injection regime of the pDC depletion antibody differed between the three studies. We injected our depletion antibody every two days over a period of four weeks, whereas Doering *et al.* only injected the depletion antibody on day 1 and 7 during 32 days of high fat diet feeding. Mac Ritchie *et al.* injected the antibody every fifth day during high fat diet. Considering the antibody kinetics and the fast and entire repopulation of pDCs already after 24-48 hours,⁽¹⁷⁾ it has to be mentioned that pDC depletion in both studies was performed only partially and not continuously throughout the experiment, thus carefully viewing the interpretation of their data. Moreover, both groups used the *ApoE*^{-/-} mouse, a more aggressive atherosclerosis model than the *Ldlr*^{-/-} mouse, to study effects of pDC depletion on plaque development. In more fulminant inflammatory stages during early lesion development pDCs potentially act immunogenic in response to triggers in their environment,⁽¹⁸⁾ most likely immunocomplexes or even modified lipoproteins. In sharp contrast, chronic low-grade inflammatory processes have been shown to enhance the expression of tolerogenic markers, such as CTLA-4, CD200 or B7-1, by pDCs.^(19, 20) We confirmed the increased expression of tolerogenic markers such as IDO and programmed death-ligand 1 (PD-L1) by pDCs, isolated from atherosclerotic *Ldlr*^{-/-} mice. Supportively, we were unable to detect differences in IFN- α expression in lymphoid pDCs either isolated from non-atherosclerotic or atherosclerotic mice. Similarly, we failed to reveal differences in IFN- α in circulating human pDCs in carotid endarterectomy patients compared to healthy controls, providing evidence that pDC activation during chronic inflammation is not very prominent.

Regarding the complex pathophysiology of atherosclerosis and the considerable plasticity of pDCs, they could well elicit dual functions during different stages of atherosclerosis. This might be reflected by the rapid release of type I IFNs and other pro-inflammatory cytokines during inflammatory episodes, whereas in the context of chronic inflammation pDCs possibly act athero-protective by suppressing T cell mediated immune responses. The exact trigger(s) for pDC immunogenicity and/ or tolerance, and the actual underlying pathways that are activated in pDCs during different stages of atherosclerosis remain to be established. Certainly, local and peripheral environmental changes are critical in driving pDC phenotype and functionality in

atherosclerosis. Mechanisms, crucial for pDC activation in autoimmune diseases, might as well be substantial in atherosclerosis.⁽²¹⁾

Further studies, using more advanced conditional pDC deficient animal models, are required to better understand pDC functionality in mouse atherosclerosis. The use of depletion antibodies (120G8, mPDCA-1 or 927) targeting bone marrow stromal antigen 2 (BST2), has been shown to eliminate pDCs quite efficiently.⁽¹⁸⁾ BST-2 is restricted to pDCs in the steady-state, but is induced on other cell subsets upon type I IFN stimulation. Thus, the use of anti-BST2 antibodies potentially leads to the depletion of additional cell subsets during viral infections.⁽¹⁷⁾ In the context of atherosclerosis we demonstrated the specific depletion of pDCs by use of the 120G8 mAb.⁽²²⁾ Nevertheless, to corroborate the results we obtained with antibody-mediated depletion, we made use of the inducible pDC ablation model based on the transgenic diphtheria toxin receptor (DTR) expression under the pDC specific human BDCA-2 promoter (*Chapter 4*).⁽²³⁾ To dissect potential pDC-CD4⁺ T cell crosstalk in atherosclerosis, we additionally depleted CD4⁺ T cells in a subset of mice. We were able to confirm previous results, showing that administration of diphtheria toxin (DT) to BDCA-2 DTR transgenic mice resulted in an almost complete depletion of pDCs.⁽²⁴⁾ Nevertheless, under hyperlipidemic, potentially pro-inflammatory conditions in *Ldlr*^{-/-} mice, prolonged application of DT induced systemic toxicity possessed in gradual body weight loss, generalized infirmity complicated by sudden death, and acute pneumonia. The observations of our study led us to conclude that the BDCA-2 DTR model, while technically interesting in acute disease models (f.e. viral infections), is not applicable to study the impact of pDCs in a chronic disease as atherosclerosis, due to undesirable side effects (leaky expression) after repeated DT administration. The use of the diphtheria toxin A (DTA) subunit under the control of a pDC-specific promoter should be considered for conditional pDC depletion in chronic inflammatory disorders, such as atherosclerosis. Alternatively, mice expressing the Cre-recombinase under the control of a pDC-specific promoter could be crossed with mice expressing DTA under the control of a floxed neomycin resistance cassette from the ROSA26 locus to particularly deplete pDCs. This approach has been applied for CD11c-Cre mice, in which certain DC subsets (cDCs, pDCs and Langerhans cells) have been specifically depleted.⁽²⁵⁾

To summarize, the above findings (*Chapter 3*) warrant further examination of (triggered) pDCs as potential therapeutic targets in the treatment of atherosclerosis. However, appreciating pDC behaviour in human atherosclerosis will be challenging, since humans are exposed to infections throughout their whole life, which may affect immune responses of pDCs. Additionally, immuno-aging might interfere with pDC phenotype and functionality in patients with CVDs, thus impairing the feasible therapeutic potential of pDCs.⁽²⁶⁾

An immune response network associated with the presence of plasmacytoid dendritic cells in the human atherosclerotic plaque

By now it is well appreciated that pDCs are present in the human atherosclerotic plaque, although only scarcely, basically in regions of inflammation.^(1, 22) However, whether pDCs are functionally active in the local human plaque context has not been solved yet. During acute events, such as intraplaque hemorrhage, it might well be that pDCs act immunogenic by the release of type I IFNs, due to changes in their local microenvironment. Otherwise, pDCs may exert tolerogenic activity during processes of low-grade inflammation. No information is available on gene expression profiles, underlying biological pathways and functions of *in vivo* atherosclerotic pDCs. In *Chapter 7* we therefore addressed the question whether pDCs manipulate its cellular surroundings within the advanced human plaque. To dissolve this question we employed Laser Capture Microdissection (LCM) on pDC-enriched and pDC-deficient frozen human carotid endarterectomy tissue samples to isolate RNA, and subsequently perform genome-wide microarraying and bioinformatics analyses.

LCM has been commonly used in other fields, f.e. in cancer research,⁽²⁷⁾ but has manifested to be a cutting edge approach in atherosclerosis, due to the difficulty to extract sufficient, high quality RNA/ DNA from atherosclerotic tissue samples. Plaque RNA/ DNA degradation is prominent in regions of necrosis and apoptosis and consequently leads to decreased quality of the material. Additionally, the marginal number of pDCs present within the human plaque renders it even more challenging to obtain enough high quality material for microarray hybridization. Thanks to improvements in the RNA/ DNA amplification technique we overcame this issue. Furthermore, the LCM

technique afforded very precise excision of pDC-enriched and pDC-deficient control regions without prior modifications such as cell labeling or cell sorting, thereby avoiding inevitable changes in the gene expression.

Here, we primarily performed transcriptional profiling of pDC-enriched and pDC-deficient regions and subsequent pathway analysis within the human plaque to characterize a plaque-specific gene signature for pDCs. Transcriptional profiling lacked statistical significance, probably due to the small number of patient samples included, but also due to the scarce presence of pDCs within an enriched region. Additionally, inpatient heterogeneity, such as gender, age, CVD risk factors, and application of CVD-related medication, will influence the gene expression profile, thus introducing variability in the results. Nevertheless, seven overrepresented networks were identified in the pDC-enriched plaque regions, including activation of T cell receptor signaling pathway(s) and MAPK pathway(s) activation. This signature might reflect a more activated status of immune cells in the near presence of pDCs. Besides comparing gene expression patterns between pDC-enriched and pDC-deficient regions, we employed module trait correlation analysis to characterize clusters of gene modules, tightly co-expressed with macrophages, T cells and microvessels that are strongly associated with the presence of pDCs. Inspired by our own results (*Chapter 3*), but also by the findings of Weyand and coworkers,^(1, 2) we particularly focused on T-cell co-expressed modules. Interestingly, in our study we characterized a number of significantly overexpressed immune clusters in the T cell module, including positive regulation of Th1/ Th17 differentiation and regulation of leukocyte chemotaxis. These data potentially corroborate the findings of Weyand and coworkers, stressing the idea that pDCs exert immunogenic functions within the unstable lesion. Nevertheless, other DC subsets, including inflammatory DCs, might well be responsible to trigger Th1/ Th17 responses in the atherosclerotic plaque.⁽²⁸⁾

Additionally, we tagged two microRNAs that target the pDC- T cell gene cluster, microRNA-382 and microRNA-518a. Although our study suggests that these microRNA's are potentially being associated in T cell differentiation and/or activation, a role in pDC- T cell crosstalk/interplay needs to be further demonstrated. The employment of fluorescent in situ hybridization (FISH) is one approach to confirm the co-localization of the two microRNAs with T cells in the close neighbourhood of pDCs in the atherosclerotic lesion, thus

excluding their feasible expression by other immune cell subsets. Future approaches, specifically aiming these microRNAs or pathways they are involved in, will assist to elucidate a potential functional role and to find their targets.

To summarize, it will be of great importance to validate the aforementioned findings and identified candidates in pDC-related immune responses. Moreover, RNA/ DNA expression profiles are not always reflective of protein expression and cellular functionality.⁽²⁹⁾ Therefore, the performance of protein arrays and *in vitro/ in vivo* approaches will lead to a more comprehensive understanding in pDC biology in the human atherosclerotic plaque. Nevertheless, this study is the first to provide new insights and generates novel hypotheses on pDC actions on its local immune cell context in advanced human atherosclerosis, and led to the identification of potential central modifiers in pDC-mediated immune responses.

Modified lipoproteins critically affect human pDC functions

pDCs are crucial in the host defense against viral and bacterial antigens through the rapid release of large amounts of type I IFNs.⁽³⁰⁾ TLR7 and TLR9-mediated recognition of pathogenic antigens induces pDC maturation, and subsequent cytokine production.⁽³⁰⁾ Conditions that impact TLR signaling will have profound impact on pDC functionality in host defense. Hyperlipidemia has been shown to inhibit the activation of certain DC subsets, due to uncoupling of TLR-mediated signaling, thus compromising host resistance to microbial infections.⁽³¹⁾ Oxidized low-density lipoprotein (oxLDL) appeared to be the key player. Thus, impaired maturation and functionality after lipoprotein exposure seems to be critically dependent on DC-subtype and its environment.

Studies, which investigate the influence of hypercholesterolemia/ oxLDL on pDCs are lacking. Therefore, we have investigated in *Chapter 5* whether a hyperlipidemic environment impairs pDC functionality. We demonstrated that human pDCs are hypo-responsive after excessive lipoprotein exposure, despite the presence of the TLR9 agonist CpG-ODN, represented by the quenched ability to release type I IFNs, as well as other pro-inflammatory cytokines. In keeping, pDCs failed to induce T helper (Th) differentiation when pre-treated with oxLDL. As these experiments were

performed *in vitro*, it will be of importance to further ascertain whether a hyperlipidemic environment interferes with pDC responses to pathogen-derived signals *in vivo*. Interestingly, *in vitro* oxLDL treatment of murine pDCs did not lead to the increased expression of the maturation markers and major histocompatibility complex II (MHC II).⁽⁹⁾ OxLDL treatment resulted in elevated expression of the scavenger receptor CD36, one of the most important oxLDL uptake receptors.⁽³²⁾ Surprisingly, oxLDL-treated murine pDCs showed enhanced ability to trigger antigen-specific T cell proliferation *in vivo*.⁽⁹⁾ Summing up, duration of lipoprotein incubation possibly is decisive in the impairment of pDC activity. Short-term lipoprotein exposure may conserve pDC capacity to process and present antigens to T cells,⁽⁹⁾ whereas long-term pretreatment possibly interferes with pDC activation. Next to oxLDL, *in vivo* environmental factors may have triggered lipid-laden pDCs to stimulate T cell proliferation in the study of Doering *et al.* Thus, *in vitro* data have to be interpreted carefully, as they do not completely mimic the complex *in vivo* environment. Nevertheless, *in vitro* assays are a powerful tool to gain more insight into possible inter- and intracellular mechanisms in the context of hyperlipidemia that may occur *in vivo*. Notably, we performed our experiments in human pDCs, while Doering *et al.* used murine pDCs, rendering it more challenging to compare findings in these two studies.⁽⁹⁾ Although mouse and human pDCs share similar markers, it will be of importance to validate, how representative murine (*in vitro*) models are for human disease.⁽³³⁾

Altogether, our data reveal that a hyperlipidemic environment abrogates TLR9 agonist induced activation of human pDCs. It remains to be elusive how lipoproteins are engulfed, possibly by receptor-mediated endocytosis via specific surface receptors of the C-type lectin⁽³⁴⁾ or the siglec family.⁽³⁵⁾ Additionally, further studies are required to dissect which pathways and downstream lipid metabolites are responsible for the inhibitory effect of (ox)LDL, and the subsequent impact on disease/host defense.

Effects of hypercholesterolemia on DC (precursor) homeostasis and peripheral DC subset functionality

It has recently been shown that certain DC subsets are able to incorporate lipoproteins both *in vitro* and *in vivo*, which may induce their maturation and activation.⁽³⁶⁻³⁹⁾ A recent study observed the contribution of

vascular resident DCs to the onset of atherosclerosis by the rapid accumulation of lipoproteins, and ensuing foam cell formation during the first days of atherogenesis.⁽⁴⁰⁾ Interestingly, Gautier *et al.* revealed the cross-interaction between conventional DCs (cDCs) and cholesterol homeostasis under hyperlipidemic conditions, as specific cDC depletion led to increased plasma cholesterol levels. The authors suggest that DCs might favor lipoprotein uptake from the periphery. How/ whether (different stages of) hyperlipidemia impair DC homeostasis, including their development in the bone marrow, DC mobilization into the circulation, and migratory routes, remains to be addressed. Therefore, we investigated in *Chapter 6* the effects of early and later time points of dietary-induced hypercholesterolemia on DC (precursor) homeostasis, as well as the phenotype of lipid-laden DC subsets, and their T cell priming capability. For this purpose, we fed *Ldlr*^{-/-} mice either a normal standard fat diet, or a high fat diet for different time points, and analyzed cell composition, cell proliferation, and cell activation status in bone marrow and peripheral lymphoid organs. It is well appreciated that hypercholesterolemia induces monocytosis in *ApoE*^{-/-} mice, which may be partly associated with a defect in *ApoE*-dependent suppression of hematopoietic stem and multipotential progenitor cell proliferation in the bone marrow.⁽⁴¹⁻⁴³⁾ Thus, hypercholesterolemia likely impairs DC development as well. In our study we observed that dietary-induced hypercholesterolemia resulted in significantly increased myeloid and dendritic cell precursor (MDPs) numbers in bone marrow. However, this increase is not reflected in lymphoid organ DC subset numbers. Several options could be responsible for this. MDPs are the most differentiated clonogenic precursors that can give rise to classical DCs (cDCs and pDCs) and monocytes.^(44, 45) It might be that under dyslipidemic conditions MDPs preferentially differentiate into monocytes, as hyperlipidemia has been shown to be associated with monocytosis.⁽⁴⁶⁾ Alternatively, unaltered peripheral DC numbers could be explained by the direct migration of DC (precursors) to sites of inflammation (the inflamed artery), increased cell turnover or reduced release from bone marrow. We furthermore examined whether peripheral DC subsets, isolated from different lymphoid organs and small intestine, show features of lipoprotein accumulation, when exposed *in vivo* for different periods of time to a hyperlipidemic environment. We observed lipoprotein accumulation in a subset of splenic and lymph node cDCs in high fat diet fed *Ldlr*^{-/-} mice. Notably, lipid-laden cDCs retained an immature

phenotype, shown by the unaltered or even lowered expression of co-stimulatory molecules. Additionally, lipoprotein incorporation had functional consequences in that it impaired the ability of cDCs to stimulate naïve T cells. So far, whether and how cholesterol uptake and prolonged intracellular storage interferes with DC activation and their T cell priming capability remains subject to debate.^(31, 36, 37) Interestingly, Gabrilovich and coworkers demonstrated that lipid-laden DCs, isolated from tumor-bearing mice and cancer patients, were unable to effectively present tumor-associated antigens and to stimulate T cells.⁽⁴⁷⁾ These data most likely suggest that a hyperlipidemic environment interdicts DC activation, which consequently impairs their T cell stimulatory capacity. These data corroborate our findings showing that certain lipid-laden DC subsets were impaired in their functionality.

Conclusions and future perspectives

In this thesis we have explored the impact of certain DC subsets in hyperlipidemia-associated atherosclerosis both in animal models and in human. The involvement of different DC subsets (vascular resident DCs, cDCs, pDCs) in the development and progression of atherosclerosis has been studied quite extensively in recent years. Nevertheless, their actions in atherosclerosis remain poorly understood. We were the first to establish a critical role of pDC-dependent immune responses in murine atherosclerosis (*Chapter 3*). Furthermore, we addressed the question whether hyperlipidemia affects (p)DC functionality (in the periphery, but also within the local plaque context). We revealed that excessive lipoprotein exposure led to impaired functionality of human pDCs *in vitro* (*Chapter 5*). Such association between hyperlipidemia and pDC interferon response has profound implications for the host's pathogen defense capacity in hyperlipidemic patients. In addition, we demonstrated that peripheral murine cDC subsets were affected in their T cell priming capability upon lipoprotein uptake (*Chapter 6*), strengthening the idea that a cholesterol-rich environment potentially interferes with DC responses to host immunity. Future experiments will unravel how hyperlipidemia and lipoprotein exposure exactly quench (TLR-induced) activation of DCs. DC subset isolation (pDCs, cDCs, inflammatory DCs) from patients with familial hypercholesterolemia, and subsequent phenotypic characterization (cell sorting, genome-wide microarraying, functional assays) would certainly illuminate DC actions in a

cholesterol-rich environment. It should be critically addressed whether DC (subset) functions are affected systemically or just site-specific. In *Chapter 7* we revealed a direct cross-talk between pDCs and its local immune context within the advanced human atherosclerotic plaque, stressing the idea that pDCs, even though oxLDL-exposed, exert immunogenic functions. Conceivably, injury associated release of danger-associated molecular patterns (DAMPs) might trigger pDC activation and T cell priming ability at the inflamed locus, skewing the adaptive immune system towards Th1/17-like immune responses. These data emphasize that (p)DC activation/ functionality crucially depends on direct environmental signals, modifying DC phenotype.

Altogether, despite the enormous progress that has been made in recent years to appreciate DC biology in the context of hyperlipidemia-associated atherosclerosis, further studies are required to define molecular pathways that regulate DC differentiation, DC maturation, and their T cell priming ability in a disturbed lipoprotein environment- locally within the plaque and in the periphery.

DCs-based immunotherapy in atherosclerosis and side effects

Despite the current incomplete understanding of DC functionality in hyperlipidemia-associated atherosclerosis (as discussed above), DC-based immunotherapy may become a therapeutic option for atherosclerosis. DC vaccination has already been successfully used in clinical trials for cancer research.^(48, 49) Effective immuno-based vaccination strategies require the identification of tissue-specific epitopes, actual antigen presentation by specialized antigen-presenting cells (APCs), and T cell priming. DCs are specialized APCs, representing an ideal platform for vaccination, f.e. *ex vivo* pre-pulsing of DCs with individual tumor-bearing antigens induced specific CD8⁺ T cell responses *in vivo*.⁽⁵⁰⁾ This knowledge has encouraged several groups to ascertain the potential of DC-based vaccination in atherosclerosis. For instance, Hansson and coworkers illustrated recently that apolipoprotein B100 and IL-10 pre-pulsed DCs trigger an immunosuppressive state, thus effectively attenuating atherosclerosis.⁽⁵¹⁾ Beside, the use of immature pDCs, considered to be tolerogenic, hold promise as potential therapeutic tools in the treatment of atherosclerosis, as tolerized pDCs induced regulatory T cell development and prolonged vascularized cardiac graft survival in mice.⁽⁵²⁾ Important issues

regarding the immunological status of the patient, therapy timing, choice of the antigen, injection of fully matured versus immature/ pre-DCs and their trafficking routes, as well as possible side effects should be critically addressed. In murine models, DC-based vaccination proved to be successful during lesion development,^(51, 53) though, it is questioned how favorable DC immunotherapy affects symptomatic/manifest atherosclerosis in human, and which time point would be the most ideal to start vaccination since atherosclerosis is a chronic inflammatory disease, mostly affecting people of advanced age. It is known that CVDs are linked to the age-associated deterioration of the immune system.⁽⁵⁴⁻⁵⁷⁾ Therefore, it remains essential to explore whether immune cells, in particular DCs and T cells, are functionally impaired by aging in humans. Age-associated alterations of signal transduction pathways in DCs may lead to defective triggering of adaptive immune responses, thus decreasing the therapeutic potential of DCs. Further analyses are required to better understand age-related effects on innate and acquired immunity, which will in turn improve the efficiency in immunotherapy,⁽⁵⁷⁾ specifically in elderly subjects. Moreover, the hyperlipidemic context might interfere with DC functionality, as has been discussed in *Chapter 5 and 6* of this thesis.

The activation status of certain DC subsets can additionally be modified *in vivo* by administration of CVD-related medication, such as statins.⁽⁵⁸⁾ Statins, primarily introduced to block cholesterol synthesis and to lower cholesterol serum levels, have been shown to have some beneficial side effects, f.e. anti-inflammatory and anti-oxidant activities resulting in the reduction of cardiovascular risk, which might interfere DC-based vaccination efficiency. Finally, it is still of great importance to figure out the correct and/ or most efficient antigen for DC pulsing before transferring them *in vivo*.

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Summary

Summary

Atherosclerosis, the underlying pathological process of most cardiovascular diseases (CVDs), is a chronic inflammatory disease, which critically implies specialized immune cells such as monocytes/ macrophages, granulocytes, B and T cells in its pathomechanism. Activated immune cells elaborate a range of inflammatory processes, which tremendously account for disease onset and progression. In recent years another immune cell subset, dendritic cells (DCs), has been identified in the healthy and diseased vasculature. DCs, specialized antigen-presenting cells (APCs), are essential in linking innate and adaptive immune responses against pathogen-derived, but also modified self-antigens present at sites of injury, thus triggering T cell-mediated immunity. DC numbers were described to be elevated in advanced human atherosclerotic plaques, mainly present in inflammatory foci within the plaque shoulder. In this regard, a possible role for DCs as contributors to inflammatory processes in atherosclerosis is attaining more and more attention. Particular plaque-derived epitopes that might induce DC maturation and activation within their local context have not been identified yet. Additionally, the maintenance of antigen-presentation and T cell priming ability by DCs under hyperlipidemic conditions, even after excessive lipid-loading remains poorly understood, and studies addressing these issues are diverging. Consequently, this thesis focuses on elucidating the contribution of certain DC subsets in hyperlipidemia-associated atherosclerosis. Besides, it addresses the question whether a hypercholesterolemic environment interferes with DC activation and its ability to prime T cell immune responses.

Studying dendritic cell (DC) biology in a majority of cardiovascular diseases (CVDs) has gained intensive attention in the last years, due to their prominence to trigger adaptive immune responses. Nevertheless, the full scope of DC-driven effects in CVDs is still unclear. In **Chapter 2** we present an in-depth overview of the current knowledge on the impact of DC subsets on several CVDs in order to reveal underlying patterns, and to demonstrate gaps and shortcomings in the knowledge of DC function in CVDs. Besides, we highlight the therapeutic potential of DCs in cardiac transplantation which may provide an example for a future atherosclerosis vaccination strategy.

pDCs are thought to contribute to atherosclerotic lesion formation in human by a type I interferon (IFN) signature. In **Chapter 3** we demonstrated

the scarcely presence of pDCs within the human plaque, whereas they are almost completely absent in murine atherosclerotic lesions. Surprisingly, pDC ablation by systemic administration of the 120G monoclonal antibody was seen to aggravate lesion formation in low-density lipoprotein receptor deficient (*Ldlr*^{-/-}) mice, thus attributing pDCs an athero-protective role. In keeping, peripheral lymphoid organ pDCs from atherosclerotic mice dampened T cell activation and proliferation in an indoleamine 2,3- dioxygenase (IDO)-dependent manner. Altogether, our research identifies a tolerogenic role for pDCs in atherosclerosis, rendering them an interesting new target for future therapeutic approaches.

In **Chapter 4** we made additional use of the inducible pDC ablation model based on the transgenic diphtheria toxin receptor (DTR) expression under the pDC-specific human blood dendritic cell antigen 2 (BDCA-2) promoter. Administration of diphtheria toxin (DT) to BDCA-2 DTR transgenic *Ldlr*^{-/-} mice resulted in an almost complete pDC ablation. To explore pDC-induced CD4⁺ T cell immunity in atherosclerosis a subset of mice obtained a CD4⁺ T cell depletion antibody. Next to pDC depletion, prolonged DT administration induced unexpected systemic toxicity and immune cell modulations, shown by a dramatic increase in circulating granulocyte counts accompanied by tremendous immune cell infiltration in the lungs. We thus conclude that the BDCA-2 DTR model, while technically interesting in acute disease models, is not appropriate to study pDC immunity in a chronic disease as atherosclerosis, due to unrequested ectopic side-effects caused by repeated DT application.

pDCs are crucial in anti-viral defense mechanisms through the rapid release of huge amounts of type I IFNs. Factors in their environment, such as lipoproteins, might interfere with signaling pathways crucial for pDC-mediated immune responses. In **Chapter 5** we addressed the question whether a hyperlipidemic environment leads to impaired functionality of human pDCs. Indeed, we have demonstrated that exposure to excessive amounts of pro-atherogenic lipoproteins (LDL and oxLDL) completely ablated Toll-like receptor 9 (TLR9) agonist induced pDC activation *in vitro*, shown by the quenched production of pro-inflammatory cytokines, as well as the up-regulation of maturation markers. In keeping, oxLDL-induced abrogation of pDC stimulation profoundly impaired their ability to trigger CD4⁺ T helper (Th) cells. It remains to be elusive whether such an association between hyperlipidemia and pDC

IFN-response compromises the host's pathogen defense capacity in hyperlipidemic patients.

In **Chapter 6** we additionally addressed temporal effects of dietary-induced hypercholesterolemia on DC (precursor) homeostasis, and consequences of lipid accumulation on phenotype and T cell priming capability of lymphoid organ DC subsets in *Ldlr*^{-/-} mice. Congruent to results described in Chapter 5, lipid loading by peripheral conventional DC (cDC) subsets was accompanied by a sharply reduced capacity to stimulate naïve T cells, a finding that may have consequences for host defense mechanisms against infectious diseases.

In **Chapter 7** we addressed the question whether pDCs, rarely present in the human advanced plaque, impact their local context. Hereto, we applied genome-wide microarraying and bioinformatics analyses on pDC-enriched and pDC-deficient plaque fragments, isolated by laser capture microdissection from human carotid endarterectomy tissue samples. Network analysis and subsequent module-trait correlation analysis were performed to identify co-expressed gene module clusters for macrophages, T cells and microvessels, tightly linked to pDC presence within the plaque. Amongst others, we characterized immune clusters in the T cell module being involved in Th1/ Th17 differentiation, stressing the idea that pDCs exert immunogenic functions, even though exposed to modified lipoproteins. Conclusively, this study promotes the concept that pDCs interfere their immune cell context in the human plaque.

Finally, **Chapter 8** discusses the most relevant findings of this thesis and provides future perspectives. Altogether, this thesis affords novel insights into DC biology in hyperlipidemia-associated atherosclerosis both in animal models and in human, emphasizing an athero-protective role for murine pDCs. Otherwise, pDCs in the human advanced atherosclerotic plaque likely induce Th1/ Th17 responses within their local immune cell context. Moreover, we demonstrated that a cholesterol-rich environment impairs the functionality of peripheral lymphoid organ DC subsets, thus potentially interfering with the host's immunity against pathogen-derived signals.

Samenvatting

Samenvatting

Atherosclerose, het pathologische proces wat ten grondslag ligt aan de meeste cardiovasculaire ziekten (CVZ), is een chronisch inflammatoire aandoening. Hierbij spelen gespecialiseerde immuun cellen zoals monocysten/macrofagen, granulocyten, B en T cellen een grote rol. Geactiveerde immuun cellen zijn verantwoordelijk voor een aantal ontstekingsreacties, die bijdragen aan het ontstaan en de progressie van atherosclerose. Recent werd een ander immuun celtype gedetecteerd in normale en atherosclerotische slagaders, namelijk de dendritische cel (DC). DCs, gespecialiseerde antigeen presenterende cellen, zijn essentieel voor het koppelen van het aangeboren en het verworven immuun systeem. Ze kunnen een T cel immuun respons opwekken tegen exogene, maar ook gemodificeerde auto-antigenen aanwezig bij schade. Een verhoogd aantal DCs werd in gevorderde atherosclerotische plaques gevonden, voornamelijk in schouder regio's met veel inflammatoire loci. Er wordt dan ook meer en meer aandacht besteed aan de mogelijke bijdrage van DCs aan de ontstekingsprocessen in atherosclerose. Plaque-specifieke epitopen, die DC ontwikkeling en activering lokaal kunnen induceren, zijn tot nog toe niet geïdentificeerd. Verder is over het behoud van antigeen presentatie en T cel activering door DCs in hyperlipidemie, zelfs na overmatige vetophoping in de DC, nog maar weinig bekend en resultaten van reeds uitgevoerde studies zijn niet eenduidig. Dit proefschrift focust dan ook op de bijdrage van DC subtypes in hyperlipidemie-geassocieerde atherosclerose – lokaal in de plaque, maar ook in perifere lymfoïde organen. Daarnaast wordt de vraagstelling behandeld of hypercholesterolemie de activering van DC en de resulterende T cel immuun respons, beïnvloedt.

In de afgelopen jaren werd veel aandacht besteed aan het bestuderen van DC biologie in de meeste CVZ, omdat DC een adaptieve immuun respons opwekken. Toch is het volledige spectrum van DC-gemedieerde effecten in CVZ nog steeds niet bekend. In **Hoofdstuk 2** geven we een diepgaand overzicht van de huidige kennis over de rol van DC subtypes in CVZ. Hier proberen we ook onderliggende patronen te ontrafelen en tekortkomingen in de kennis over DC functie in CVZ in kaart te brengen. Daarnaast benadrukken we het mogelijk therapeutische potentieel van DCs voor harttransplantaties en als mogelijk vaccin tegen atherosclerose.

Plasmacytoïde DCs (pDC) kunnen bijdragen aan het ontstaan van atherosclerotische plaques via een type I interferon (IFN) respons. We tonen in **Hoofdstuk 3** dat pDCs nauwelijks voorkomen in humane plaques, en bovendien bijna helemaal afwezig zijn in atherosclerotische plaques van muizen. In tegenstelling tot verwacht, verergerde de antilichaam-gemedieerde depletie van pDCs de ontwikkeling van plaques in low-density lipoproteïne receptor deficiënte (*Ldlr*^{-/-}) muizen. Dit suggereert dat in de *Ldlr*^{-/-} muis pDCs een anti-atherogeen effect hebben. Bovendien veroorzaken pDCs uit perifere lymfoïde organen van atherosclerotische muizen een remming van T cel activering en proliferatie. Dit tolerogene effect werd gemedieerd via indoleamine 2,3- dioxygenase. De T-cel remming door pDCs verklaart waarschijnlijk het anti-atherogene effect van pDCs. Concluderend, onze studie identificeert een tolerogene rol voor pDCs in atherosclerose en maakt ze daarom een interessant doel voor toekomstige therapeutische strategieën.

In **hoofdstuk 4** gebruiken wij een induceerbaar pDC depletie model, waarin transgene expressie van de difterie toxine receptor (DTR) onder de controle staat van de pDC-specifieke humane dendritische cel antigen 2 (BDCA-2) promotor. Toediening van difterie toxine (DT) aan BDCA-2 DTR transgene *Ldlr*^{-/-} muizen resulteerde in een bijna complete pDC depletie. Om de pDC-geïnduceerde CD4⁺ T cel immuniteit in atherosclerose te bestuderen, werd een CD4⁺ T cel depleterend antilichaam aan een subgroep van muizen gegeven. Herhaalde DT toediening resulteerde echter niet alleen in pDC ablatie, maar induceerde ook onverwachte systemische toxiciteit en moduleerde immuun cellen. Deze bijwerkingen werden duidelijk door de waarneming van verhoogde aantallen circulerende granulocyten met gelijktijdige infiltratie van de long door immuun cellen. Wij kunnen dus concluderen, dat het BDCA-2 DTR model niet geschikt is om pDC immuniteit in de chronische ziekte atherosclerose te bestuderen, omdat de DT toediening ectopische bijwerkingen veroorzaakt. Aan de andere kant, zou het systeem wél technisch interessant zijn als model voor acute ziekten.

pDCs zijn belangrijk voor antivirale afweermechanismen door hun snelle en grote productie van type I IFNs. Factoren in de omgeving van pDCs, zoals lipoproteïnen, zouden de immuun signalering van pDCs kunnen beïnvloeden. In **hoofdstuk 5** hebben wij bestudeerd wat de effecten van hyperlipidemie zijn op de functie van humane pDCs. Inderdaad zagen wij *in vitro*, dat de activatie van pDCs, geïnduceerd door een Toll-like receptor 9

(TLR9) agonist, geremd wordt door een overmaat van de pro-atherogene lipoproteïnen LDL en oxLDL. Deze remming van pDC activatie werd zichtbaar door een verlaagde pro-inflammatoire cytokine productie en een verhoogde expressie van maturatie markers. In overeenstemming daarmee was de stimulatie van CD4⁺ T helper cellen door pDCs verlaagd in de aanwezigheid van oxLDL. Desondanks moet nog bevestigd worden of het remmende effect van hyperlipidemie op de IFN productie door pDCs, werkelijk het afweermechanisme van hyperlipidemische patiënten tegen pathogenen kan verminderen.

In **hoofdstuk 6** onderzochten wij het temporele effect van dieet-geïnduceerde hypercholesterolemie op de ontwikkeling en rijping van muis DCs. Verder bestudeerden wij de gevolgen van vetopname door muis pDCs, geïsoleerd uit perifere lymfoïde organen, op DC fenotype en DC-gemedieerde cel stimulatie. In overeenstemming met de pDC resultaten van hoofdstuk 5, veroorzaakt vetopname door perifere conventionele DC (cDC) ook een sterk verminderde stimulatie van naïve T cellen. Dit resultaat heeft mogelijk nadelige gevolgen voor de afweermechanismen tegen infectie ziekten.

In **hoofdstuk 7** bestudeerden wij of pDCs hun lokale omgeving kunnen beïnvloeden, ondanks het lage aantal pDCs in vergevorderde humane plaques. In operatief verwijderde humane arteria carotis plaques werden segmenten geïdentificeerd met hoge concentratie pDCs en afwezige pDCs. Na extractie van de segmenten met laser capture microdissectie, werd RNA geïsoleerd voor een gen expressie analyse van het hele genoom. Bio informatica analyses werden gebruikt om gen clusters te identificeren specifiek voor macrofagen, T cellen en microvaten, die een sterk verband vertoonden met de aanwezigheid van pDCs. Met deze techniek werd een T-cel gen cluster geïdentificeerd, wat de aanwezigheid van pDCs relateerde aan Th1/Th17 differentiatie. Dit suggereert dat pDCs toch in staat zijn een immuun respons te veroorzaken, ondanks blootstelling aan lipoproteïnen in de plaque. Deze studie ondersteunt het concept dat pDCs de immuun cellen in humane plaques kunnen beïnvloeden.

Tenslotte werden in **hoofdstuk 8** de meest opmerkelijke bevindingen van deze thesis bediscussieerd en toekomstverwachtingen aangegeven. Samenvattend, geven de resultaten in deze thesis nieuwe inzichten over DC biologie in hyperlipidemie-geassocieerde atherosclerose in dier modellen en in de humane situatie. In het bijzonder, ondersteunen de resultaten een anti-

atherogene rol voor pDCs in de muis. In tegenstelling daarmee, lijken pDCs in gevorderde humane plaques juist in staat om een Th1/Th17, dus pro-inflammatoire reactie in hun lokale immuun cel omgeving te stimuleren. Bovendien hebben wij aangetoond, dat een cholesterol-rijke omgeving de functie van perifere pDCs en cDCs kan remmen. Cholesterol zou dus mogelijk ook het afweermecanisme tegen exogene pathogenen kunnen remmen.

Zusammenfassung

Zusammenfassung

Arteriosklerose, der zugrunde liegende pathologische Prozess der meisten kardiovaskulären Erkrankungen, ist eine chronisch entzündliche Gefäßerkrankung, in deren Pathomechanismus spezifische Immunzellen, unter anderem Monozyten/ Makrophagen, Granulozyten, B- und T-Zellen, impliziert sind. Aktivierte Immunzellen führen eine Anzahl an entzündlichen Prozessen durch, welche entscheidend zum Beginn und dem Voranschreiten der Krankheit beitragen. In den letzten Jahren wurde ein weiterer Immunzell-Subtyp in gesunden und erkrankten Blutgefäßen identifiziert – dendritische Zellen. Dendritische Zellen sind spezialisierte Antigen-präsentierende Zellen, welche eine zentrale Rolle in der angeborenen und erworbenen Immunantwort spielen, indem sie Antigene von Krankheitserregern oder modifizierte körpereigene Antigene naiven T-Zellen präsentieren und somit die T-Zell vermittelte Immunität stimulieren. Mehrere Studien haben eine Zunahme von dendritischen Zellen in humanen vorangeschrittenen arteriosklerotischen Plaques beschrieben, hauptsächlich in entzündlichen Herden in der Schulterregion der Plaques. Diese Ergebnisse haben dazu beigetragen, dendritischen Zellen eine Rolle in entzündlichen Prozessen in der Arterioskleroseentstehung zuzuschreiben. Spezifische, in den Plaques vorkommende Epitope, welche die Reifung und Aktivierung der dendritischen Zellen induzieren könnten, wurden bisher nicht identifiziert. Zudem ist es bis jetzt unklar, ob dendritische Zellen in einer hyperlipidämischen Umgebung – nach übermäßiger intrazellulärer Lipidanreicherung - ihre Funktion als Antigen-präsentierende Zellen beibehalten und T-Zellen in gleichem Maße stimulieren können. Folglich fokussiert sich diese Doktorarbeit darauf, die Funktion verschiedener dendritischer Zell-Subtypen in der hyperlipidämischen-assoziierten Arteriosklerose aufzuklären. Zudem, wird in dieser Thesis untersucht, ob eine hypercholesterolämische Umgebung die Aktivierung dendritischer Zellen und deren Fähigkeit T-Zell Immunantworten zu schärfen beeinträchtigt.

Da dendritischer Zellen die erworbene Immunantwort triggern, hat die Untersuchung ihrer Immunobiologie in einer Mehrzahl von kardiovaskulären Erkrankungen in den letzten Jahren intensive Aufmerksamkeit erlangt. Nichtsdestotrotz ist der volle Umfang der Auswirkungen, welche durch dendritische Zellen angetrieben werden, bisher unklar. In **Kapitel 2**

präsentieren wir eine fundamentale Übersicht über das momentane Wissen des Einflusses dendritischer Zell-Subtypen in verschiedenen kardiovaskulären Erkrankungen, um grundlegende Muster zu zeigen, und Lücken und Unzulänglichkeiten über die Funktion von dendritischen Zellen aufzudecken. Des Weiteren heben wir das therapeutische Potential dendritischer Zellen in der Herztransplantation hervor, welches als Beispiel für zukünftige Impfungsstrategien in der Arterioskleroseforschung herangezogen werden könnte.

Es wird vermutet, dass plasmazytoide dendritische Zellen durch das Freisetzen von Typ I Interferonen zur Ausbildung von arteriosklerotischen Plaques beitragen. In **Kapitel 3** demonstrieren wir die spärliche Anwesenheit plasmazytoider dendritischer Zellen in humanen Plaques, wohingegen sie in murinen arteriosklerotischen Läsionen nicht vorzufinden sind. Die Entfernung plasmazytoider dendritischer Zellen durch die systemische Verabreichung des monoklonalen Antikörpers 120G8 in *low-density* Lipoprotein-Rezeptor defizienten Mäusen hat unerwarteterweise eine Verschärfung der arteriosklerotischen Plaques hervorgerufen, welches auf eine protektive Rolle plasmazytoider dendritischer Zellen in der Arteriosklerose hindeutet. Im Einklang mit diesem Resultat konnten wir zeigen, dass plasmazytoide dendritische Zellen, welche aus den peripheren lymphoiden Organen arteriosklerotischer Mäuse isoliert wurden, die T-Zellaktivierung und deren Proliferation in einer *indoleamine 2,3- dioxygenase* (IDO)-abhängigen Art gedämpft haben. Zusammengefasst zeigen unsere Daten, dass plasmazytoide dendritische Zellen eine tolerogene Funktion in der Arteriosklerose übernehmen, welches sie zu einem interessanten Zielobjekt für zukünftige therapeutische Ansätze macht.

In **Kapitel 4** haben wir Gebrauch von einem weiteren induzierbaren plasmazytoiden dendritischen Zell Ablationsmodell gemacht, basierend auf der Expression des transgenen Diphtheria Toxin Rezeptors unter dem plasmazytoiden dendritischen Zell-spezifischen Promoter BDCA-2 (*blood dendritic cell antigen 2*). Die Verabreichung von Diphtheria Toxin zu BDCA-2 transgenen *low-density* Lipoprotein-Rezeptor defizienten Mäusen resultierte in der fast vollständigen Depletion plasmazytoider dendritischer Zellen. Um die plasmazytoide dendritische Zell-induzierte CD4⁺ T-Zell Immunität in der Arteriosklerose zu untersuchen, wurde einem Teil der Mäuse zusätzlich ein CD4⁺ T-Zell Depletionsantikörper eingespritzt. Neben der Entfernung

plasmazytoider dendritischer Zellen hat die anhaltende Verabreichung von Diphtheria Toxin jedoch unerwartete Nebeneffekte hervorgerufen. Unter anderem haben wir eine systemische Toxizität und Immunzellmodulationen festgestellt, verdeutlicht durch die dramatische Zunahme zirkulierender Granulozyten, und begleitet durch die enorme Einwanderung von Immunzellen in die Lunge. Hierdurch schlussfolgern wir, dass das BDCA-2 Diphtheria Toxin Rezeptor Modell, welches in der Untersuchung akuter Krankheitsmodelle technisch interessant ist, aufgrund der unerwünschten Nebeneffekte nicht anwendbar ist, um die Immunbiologie plasmazytoider dendritischer Zellen in chronischen Erkrankungen wie der Arteriosklerose zu untersuchen.

Plasmazytoide dendritische Zellen sind durch die schnelle Freisetzung großer Mengen von Typ I Interferonen unentbehrlich in der antiviralen Abwehr. Faktoren in ihrer direkten Umgebung, wie zum Beispiel Lipoproteine, könnten Signalwege, welche wichtig für plasmazytoide dendritische Zell-vermittelte Immunantworten sind, behindern. In **Kapitel 5** haben wir untersucht, ob eine hyperlipidämische Umgebung zu einer verminderten Funktionalität humaner plasmazytoider dendritischer Zellen führt. Wir konnten in der Tat zeigen, dass die *Toll-like* Rezeptor 9 Agonist induzierte Aktivierung plasmazytoider dendritischer Zellen nach Aussetzen in eine hyperlipidämische Umgebung *in vitro* komplett gehemmt wurde. Dies wurde durch die signifikant verminderte Freisetzung pro-entzündlicher Zytokine, sowie die gedämpfte Expression von Maturierungsmarkern bewiesen. In Übereinstimmung mit diesen Resultaten konnten wir zeigen, dass die Fähigkeit plasmazytoider dendritischer Zellen CD4⁺ T-Helfer Zellen zu aktivieren, in der Gegenwart von oxLDL schwer beeinträchtigt wurde. Es bleibt zu untersuchen, ob die Assoziierung zwischen Hyperlipidämie und plasmazytoider dendritischer Zell-vermittelter Interferon-Antwort die Abwehrkapazität gegen Krankheitserreger in hyperlipidämischen Patienten gefährdet.

In **Kapitel 6** haben wir die zeitlichen Effekte einer Diät-induzierten Hypercholesterolämie in *low-density* Lipoprotein-Rezeptor defizienten Mäusen auf die Homeostase dendritischer (Vorläufer-) Zellen untersucht. Zudem haben wir erforscht, ob die Lipidanreicherung in dendritischen Zell-Subtypen, isoliert aus lymphoiden Organen, deren Phänotyp verändert und ihre Fähigkeit T-Zellen zu stimulieren, beeinträchtigt. Übereinstimmend mit unseren Resultaten in Kapitel 5 konnten wir hier zeigen, dass die intrazelluläre Lipidanreicherung in peripheren konventionellen dendritischen Zell-Subtypen

zu einer Verminderung ihrer Kapazität, naive T-Zellen zu stimulieren, führte; eine Erkenntnis, welche Konsequenzen für die Immunabwehr gegen infektiöse Erreger haben könnte.

In **Kapitel 7** haben wir erforscht, ob plasmazytoide dendritische Zellen, welche spärlich in vorangeschrittenen humanen Plaques vorzufinden sind, einen Einfluss auf ihr lokales Umfeld ausüben. Hierzu haben wir Plaque Regionen aus humanen Karotis-Gewebeproben mit Hilfe der *Laser Capture* Mikroskopie isoliert, welche plasmazytoide dendritische Zellen enthielten oder negativ für diese Zellen waren. Anschließend wurde ein Genom-weites *Microarraying* mit bioinformatischer Analyse auf diese Proben appliziert. *Network* und nachfolgende *module-trait* Korrelationsanalysen wurden durchgeführt, um Ko-expressions *Cluster*-Module für Makrophagen, T-Zellen und Mikrogefäße zu identifizieren, welche mit der Anwesenheit plasmazytoider dendritischer Zellen in den humanen arteriosklerotischen Plaques verknüpft werden können. Wir konnten unter anderem Immun-Cluster im T-Zellmodul charakterisieren, welche in die T-Helfer 1/ T-Helfer 17 Differenzierung involviert sind. Diese Resultate unterstützen die Hypothese, dass plasmazytoide dendritische Zellen immunogene Funktionen in humanen Plaques ausüben, obwohl sie einer Lipid-reichen Umgebung ausgesetzt sind. Zusammenfassend avanciert diese Studie das Konzept, dass plasmazytoide dendritische Zellen in humanen arteriosklerotischen Läsionen einen Einfluss auf ihren Immunzell-Kontext ausüben.

Letzlich diskutiert **Kapitel 8** die wichtigsten Erkenntnisse dieser Doktorarbeit und bietet Zukunftsperspektiven. Zusammenfassend gibt diese These einen neuen Einblick in die Biologie dendritischer Zellen in der hyperlipidämischen-assoziierten Arteriosklerose. Wir konnten eine protektive Rolle für plasmazytoide dendritische Zellen in der Maus-Arteriosklerose zeigen. Andererseits sind plasmazytoide dendritische Zellen wahrscheinlich in die Induktion von inflammatorischen T-Helferzellen in humanen arteriosklerotischen Plaques involviert. Des Weiteren haben wir demonstriert, dass eine Cholesterol-reiche Umgebung die Funktionalität peripherer dendritischer Zell-Subtypen beeinträchtigt, welches enorme Konsequenzen für die Immunabwehr gegen infektiöse Erreger haben könnte.

Curriculum Vitae

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07/2010	Poster Award at the European Society of Cardiology conference – Frontiers in Cardiovascular Science; Berlin, D Title: <i>‘Presence of dendritic cell subsets in human advanced stable and vulnerable plaques’</i>
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List of Abbreviations

(N) STEMI	(non) ST-segment elevation myocardial infarction
1-MT	1-methyl-tryptophan
ABC	ATP-binding cassette transporter
ACE	Angiotensin-converting enzyme
ACI-S	asymptomatic cerebral infarction stenosis
AEC	3-amino-9-ethylcarbazole
AHS	acute haemorrhagic stroke
AIS	acute ischemic stroke
AMI	Acute myocardial infarction
Ang	angiotensin
APC	Antigen-presenting cell
APC	Allophycocyanin
ApoE ^{-/-}	Apolipoprotein deficient
ASMA	A-smooth muscle actin
Batf3	basic leucine zipper transcription factor, ATF-like 3
BCA	bicinchoninic acid
BDCA	Blood dendritic cell antigen
BSA	Bovine serum albumine
BST-2	Bone marrow stromal antigen-2
CAD	Coronary artery disease
CBA	Cytometric bead array
cDC	Conventional dendritic cell
CDP	Common dendritic cell precursor
CEA	Carotid endarterectomy
CLI	critical limb ischemia
CLP	Common lymphoid progenitor
CLP	Common lymphoid precursor
CLR	C-type lectin receptor
CMP	Common myeloid progenitor
CTL	Cytotoxic T cell
CTLA-4	Cytotoxic T-lymphocyte antigen 4
Ctr	Control
CVD	Cardiovascular disease
DAMP	Danger-associated molecular pattern
DC	Dendritic cell
DCF	2', 7' -dichlorofluorescein

DCFDA	2',7'-dichlorofluorescein diacetate
DCIR	Dendritic cell immuno receptor
Dil	1,1'-dioctadecyl-3,3,3',3'- tetramethylindocarbocyanine perchlorate
DMSO	Dimethyl sulfoxide
DT (A)	Diphtheria toxin (A)
DTM	Diphtheria toxin mutant
DTR	Diphtheria toxin receptor
E4BP4	E4 promoter-binding protein 4
EC	Endothelial cell
EDTA	ethylenediaminetetraacetic acid
EGFP	Enhanced green fluorescent protein
FACS	Fluorescence activated cell sorter
FGF	Fibroblast growth factor
FITC	Fluorescein isothiocyanate
Flt3L	FMS-like tyrosine kinase 3 ligand
GM-CSF(R)	granulocyte– macrophage colony-stimulating factor (receptor)
GO	Gene ontology
GTC	Guanidine thiocyanate
GvHD	Graft versus host disease
HE	Hematoxylin and eosin
HF	Heart failure
HFD	High fat diet
hGH	Human growth hormone
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HMGB-1	High-mobility group box 1
HSC	Hematopoietic stem cell
HSP	Heat shock protein
HT	Heart transplantation
i.p.	Intraperitoneal
i.v.	intravenous
IC	intermittent claudication
ICAM	Intracellular adhesion molecule
ICOS-L	Inducible costimulator ligand
ID2	inhibitor of DNA binding 2
IDO	indoleamine-2,3-dioxygenase
IFN	Interferon
IL	Interleukin

ILT7	Immunoglobulin-like receptor 7
IPH	Intraplaque hemorrhage
IRAK-4	Interleukin-1 associated kinase-4
IRF	IFN regulatory factor
IRI	Ischemia and reperfusion injury
L. major	Leishmania major
LAL	Limulus amebocyte lysate
LCM	Laser capture microdissection
LDL	Low density lipoprotein
Ldlr ^{-/-}	Low-density lipoprotein receptor deficient
LMPP	lymphoid-primed multipotent progenitor
LN	Lymph node
mAB	Monoclonal antibody
M-CSF(R)	macrophage colony-stimulating factor (receptor)
MDA-Ldl	malondialdehyde modified LDL
MDP	Myeloid dendritic cell progenitor
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
MLN	Mesenteric lymph node
MLR	Mixed leukocyte reaction
MMP	Matrix metalloproteinase
MPD	Monocyte and dendritic cell precursor
MPO	Myeloperoxidase
NET	Neutrophil extracellular traps
NK	Natural killer
NKT cell	Natural killer T cell
NYHA	New York Heart Association
ORO	Oil-Red O staining
oxLDL	Oxidized low density lipoprotein
oxPL	Oxidized phospholipids
PAD	Peripheral arterial disease
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PD-1	Programmed death-1
pDC	Plasmacytoid dendritic cell
PDGF	Platelet-derived growth factor
PD-L1	Programmed death ligand 1

PE	Phycoerythrin
PFA	Paraformaldehyde
PRR	Pattern recognition receptor
RMA	Robust multiarray average
ROS	Reactive oxygen species
SAP	Stable angina pectoris
SEB	Staphylococcus Enterotoxin B
SFD	Standard fat diet
SLE	Systemic lupus erythematosus
SSC	Side scatter
T2D	Type II diabetes
T-bet	T-box transcription factor TBX21
TBS	Tris buffered saline
TCR	T cell receptor
TGF- β	Tumor growth factor- β
Th	T helper
TIA	transient ischemic attack
TkFCA	Thick fibrous cap atheroma
TLR	Toll-like receptor
TnFCA	Thin fibrous cap atheroma
TNF- α	Tumor necrosis factor- α
TRAIL	Tumor necrosis factor-related apoptosis-inducing ligand
Treg	Regulatory T cell
UAP	Unstable angina pectoris
VCAM	Vascular cell adhesion molecule
vSMC	Vascular smooth muscle cell

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