The potential use of dendritic cells in mouse models of atherosclerosis

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Chapter 8

General discussion & Perspectives

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Introduction

Over the past decades, a tremendous effort has been put into the research to develop new therapeutic approaches for the treatment of atherosclerosis, the main underlying pathology of cardiovascular disease (CVD). However, standard treatment of CVD still focuses on the reduction of risk factors by lipid lowering or anti-thrombotic drugs in combination with instructions to improve life style and CVD. Therefore it is of the utmost importance that the processes that contribute to lesion progression are well understood in order to explore this knowledge to develop new, effective and preventive treatments. Atherosclerosis is a chronic inflammatory disease of the vasculature in which both a disturbed lipid metabolism and inflammatory immune responses against several self-antigens are involved. Because of the prominent role of the immune response, especially in the initial stages of lesion development, this thesis focused on the possibility to manipulate the immune response in murine models for both atherosclerosis as abdominal aortic aneurysm formation.
In **chapter two to four** dendritic cell (DCs) based therapies have been evaluated in different stages of atherosclerotic lesion development. In **chapter five** we assessed the importance of TGF-β producing DCs in atherosclerosis. TGF-β plays several atheroprotective roles such as reducing T cell and macrophage activation and stabilization of lesions through the induction of collagen production. Next, we analyzed the effects of foam cell formation on the antigen presenting and processing capacities of macrophages and DCs in **chapter six**. Finally we attempted to elucidate the role of regulatory T cells in aneurysm formation in **chapter seven**.

Dendritic Cells in atherosclerosis

Antigen presenting cells (APCs) play a prominent role in immune responses. APCs recognize both endogenous and foreign pathogens via their Pattern Recognition Receptors such as Toll Like Receptors (TLRs) and Nod Like Receptors (NLRs). After internalization of these pathogens, antigenic peptides will be presented to specific T and B cells to induce an immune response. Dendritic cells (DCs) are the most potent antigen presenting cells. They have the unique capacity to activate naïve cells and thereby form a bridge between the innate and adaptive immune response.[1, 2] Low numbers of DCs reside in the healthy vessels but their number increases during lesion progression.[3, 4] Moreover, hyperlipidemia can disturb the migration of DCs towards the lymphoid organs.[5] This may lead to a diminished presentation of atherosclerotic antigens and subsequently a disturbed immune response. Because of their capacity to activate all kinds of immune cells such as T cells, NKT cells and even B cells, DCs form an interesting opportunity to manipulate the immune response.

Vaccination strategy using oxLDL-pulsed mDCs

In **chapter two** we investigated the potential of pulsed mature DCs as a vaccination protocol. For this we treated LDLr$^{-/-}$ mice prior to atherosclerosis induction by means of collar placement and subsequent western type diet feeding. Oxidized Low-Density Lipoprotein (oxLDL) is a promising target for immunotherapy as several studies have shown the relevance of oxLDL-immunization leading to 40-70 % reduction in lesion size.[6-8] However, oxLDL is a very complex particle containing many different epitopes that can potentially act pro-atherogenic or atheroprotective. The vaccination strategies described in the literature focus on inducing an immune response to a single epitope or a limited number of epitopes found in (ox)LDL. In addition, most immunization protocols require the presence of adjuvants, which may cause undesired side-effects. In contrast, DC-based therapies do not require the addition of adjuvants and DCs will present a broad spectrum of epitopes after internalization and processing of oxLDL and can therefore activate a wide range of oxLDL-specific T cells. Treatment of LDLr$^{-/-}$ mice with oxLDL-pulsed mDCs resulted in attenuation of lesion development. In addition, a decreased number of macrophages and increased collagen content was observed, contributing to a more stable plaque phenotype. Vaccination with oxLDL-pulsed mDCs also resulted in the induction of oxLDL-specific IgG and moreover, the IgG2c/IgG1 ratio was reduced, suggestive of a reduced Th1 response. In addition, we observed an induction of oxLDL-specific T cells as determined by *ex vivo* proliferation which, when stimulated with ConA, produced less IFN-γ while the secretion of IL-4 and IL-10 was not affected. We hypothesize that in the current setup, injection of oxLDL-pulsed mDCs leads to a migration towards the lymphoid tissues where it can activate or generate oxLDL-specific T cells that provide help towards B cells. When hyperlipidemia becomes effective by western type diet feeding, oxLDL-specific antibodies and T cells can then be rapidly generated and can prevent the accumulation of oxLDL in the vasculature. At current, clinical studies showed that treatment with anti-ApoB100 antibodies may induce inhibition of plaque progression, indicating that the induction of specific antibodies may be very effective.[9]

DC-based immunotherapy to target NKT cells

Presentation of lipid antigens by antigen presenting cells (APCs) is not mediated by MHC class I or II molecules but by a MHC class I like molecule CD1d. Lipid antigens are therefore recognized by a specialized subset of T cells, the Natural Killer T (NKT) cells. In atherosclerotic lesions, NKT cells are found in close vicinity of DCs in the shoulder region and may have an important role in plaque destabilization.[10] The exact role of NKT cells in atherosclerosis remains however unclear. Data in literature are contradictive with an atherogenic function in ApoE$^{-/-}$ mice while in LDLr$^{-/-}$ mice the activation of NKT cells prior to atherosclerosis induction is protective. Because αGalCer mainly produces TH1 cytokines, we used an analogue of αGalCer, OCH which is known to induce a more TH2-phenotype in NKT cells.[11, 12] Subsequently, we investigated the potential of OCH as a therapeutic agent in atherosclerosis. In **chapter three** we observed that while *i.p.* injections

of OCH did not affect lesion development, adminstration of OCH-pulsed mDCs dramatically reduced lesion size. In addition, we showed that OCH-pulsed mDCs treatment in Jα281$^{-/-}$LDLr$^{-/-}$ KO mice had no effect on lesion progression, indicating that the observed effects are NKT cell dependent. We showed that treatment with OCH-pulsed mDCs resulted in an increase of NKT cells in blood and liver. Moreover, after restimulation *ex vivo*, NKT cells produced more IL-10 while IFN-γ production was not affected. The increased levels of IL-10 may explain the reduced cholesterol levels and the more beneficial lipoprotein profile as observed at sacrifice since systemic IL-10 administration has been shown to reduce VLDL and LDL cholesterol levels in LDLr$^{-/-}$ mice.[13]

DC-based therapy in established atherosclerosis

Clinical symptoms of atherosclerosis only appear at late stages of the disease. Also, vaccination strategies using DCs are likely to be used for the treatment of patients with ongoing atherosclerosis. In **chapter four** we investigated whether we could implement DC-based therapy in a model of established atherosclerosis. We have treated LDLr$^{-/-}$ with pre-formed lesions with immature, unpulsed DCs (ImDCs) because this type of DCs renders a higher protection in collagen induced arthritis then collagen-pulsed ImDCs.[14] We have observed an inhibition of plaque growth in mice treated with ImDCs. Moreover, we observed a clear induction of tolerogenic CD4^{+}DX5^{+} cells in liver and spleen but adoptive transfer of these cells in mice with established atherosclerosis, did not affect lesion size. ImDCs treatment also induced a decrease in cholesterol levels and a more beneficial lipoprotein profile which may be explained by a rapid induction of ABCA1, ABCG1, HL and LPL mRNA and a reduced expression of LIGHT and lymphotoxin-α mRNA expression in the liver.[15] Mice treated with ImDCs also exhibited higher numbers of regulatory T cells in blood three weeks after injection and this increase was still present ten weeks after injection. Induction of Tregs may reflect the induction of tolerance, which is confirmed by the increased peripheral to liver CD4/CD8 ratio which is also used as a measurement of tolerance. In addition we could not detect an increase in IgM or IgG levels suggesting that we have not induced a humoral response. Consequently, these data showed the beneficial effect of treatment with ImDCs in a mouse model of established atherosclerosis. Taking into account our previous work on the use of pulsed mDCs as a vaccination strategy, we thus provide additional evidence for the potential use of DC-based immunotherapies in cardiovascular diseases.

Deficiency of TGF-beta signaling in DCs

In **chapter five** we evaluated the effects of a disturbed TGF-β signaling in DCs and the subsequent effects on atherosclerosis. TGF-β exerts several crucial anti-inflammatory functions such as the inhibition of T cell proliferation and activation of macrophages and reduces the expression of adhesion molecules and chemokines required for the recruitment of leukocytes.[16, 17] Furthermore, TGF-β enhances plaque stability by stimulating collagen production by smooth muscle cells. Treatment with inhibitory antibodies for TGF-β has atherogenic effects while

overexpression of TGF-β inhibited lesion progression.[18-20] These effects could be attributed to TGF-β signaling in T-cells as mice with deficient TGF-β signaling in $CD4^+$ T-cells have larger and more unstable plaques.[21] There is however little known on the effect of a disturbed TGF-β signaling in DCs. Therefore we have used $ApoE^{-/-}$ mice which have a dysfunctional TGF-β Receptor II under the CD11c promoter. We observed an increased lesion progression with a more inflammatory phenotype as indicated by the enhanced influx of $CD3^+$, $CD4^+$ and $CD8^+$ T cells in the lesions. Also plaque stability was reduced as indicated by a lower collagen content. Splenic $CD4^+$ and $CD8^+$ T cells exhibited a more differentiated phenotype as indicated by an increase of activation markers in T cells of $ApoE^{-/-}$CD11cDNR mice. Moreover, T cells from these mice produced increased amounts of both TH1, TH2 cytokines as well as IL-17, while T cell proliferation was not altered. We also observed that deficiency in TGF-β signaling does not affect DC maturation *in vivo* but DC from $ApoE^{-/-}$CD11cDNR mice do produce lower amounts of TGF-β and IL-12 which may affect T cell differentiation and activation. In conclusion, we showed that TGF-β signaling in DCs is necessary to control T cell differentiation towards the effector T-cell phenotype. Loss of TGF-β signalling in DCs will increase the number of activated T cells that show an increased migration into atherosclerotic lesions and subsequently enhance the inflammatory state of the plaque. This, accompanied by a loss of collagen, may lead to a more vulnerable phenotype.

Effects of foam cell formation on APC functions

Key processes in the development of atherosclerosis are monocyte recruitment into the arterial wall, subsequent uptake of cholesterol and the formation of foam cells.[22] More recently there is accumulating evidence that macrophage foam cell formation induces a dendritic cell-like phenotype with increased CD11c and MHC-II expression.[23-25] However, at present it is unclear whether the induction of a dendritic cell-like phenotype in macrophages affects their antigen presentation or processing capacities. Therefore we assessed the effect of foam-cell formation on macrophages and DCs in **chapter six**. By means of flow cytometry we determined that bone-marrow derived macrophages (MΦ) express upon foam cell formation higher levels of CD11c, MHC class I and class II and co-stimulatory molecules suggesting that foam cell formation enhances the antigen presenting capacities of MΦ. Indeed we have observed higher MHC class I mediated presentation of ovalbumin peptide in both DCs and MΦ upon foam cell formation. In addition, we also demonstrated that not only presentation of peptides is enhanced, also MHC class I processing of ovalbumin was increased in foam-cell MΦ. In contrast MHC class II presentation and processing was not affected by foam cell formation. These data were confirmed *in vivo* were hypercholesteremia induced a DCs-like phenotype of MΦ with increased CD11c and CD86 expression. This may have major implications for the inflammatory process in atherosclerotic lesions as CD11c is an integrin and enhances the firm arrest of monocytes to the endothelial layer.[25] However, further extensive research on the exact role of hypercholesteremia on antigen presentation and processing by antigen-presenting cells still needs to be performed.

Potential role of regulatory T cells in Aortic Aneurysms

Regulatory T cells (Tregs) potently suppress the proliferation and cytokine production of effector $CD4^+$ (TH1 and TH2) and $CD8^+$ T cells and deficiency of Tregs leads to autoimmunity in both murine experimental models as in humans.[26, 27] In atherosclerosis, Tregs have been shown to play a protective role. In contrast, Tregs inhibit postischemic neovascularization by controlling the effector immune cell response.[28-31] However, the exact role of Tregs in AAA has not been elucidated yet. Therefore we sought out to determine the importance of Tregs in a mouse model of AAA formation in **chapter seven**. We used $ApoE^{-/-}$ mice that were infused with angiotensin II (AngII) to induce aneurysm formation. Depletion of Tregs using an anti-CD25 antibody immediately prior to AngII infusion reduced survival due to acute rupture of the aorta. Already after nine days, the survival rate dropped to 60% while in the control group no mice died. When we performed micro-array analysis on the aortas of the control group and the Treg depletion group we observed that genes related to the disease process aneurysm were highly regulated. Interestingly, the expression of genes involved in energy metabolism and especially genes of the Krebs cycle are down regulated in mice treated with anti-CD25. Next we attempted to induce Treg numbers by anti-CD3 treatment. Although we induced the required T cell death, we could not observe an increase in Tregs after treatment. In conclusion, the manipulation of the numbers of Tregs *in vivo* still needs further optimization. For example injection of IL-2 murine antibody complexes could provide us with an effective method to induce Tregs *in vivo*[35] and to elucidate the precise role of Tregs in aneurysm formation or rupture.

Perspectives

In this thesis we have described different strategies concerning the use of dendritic cells as an immunotherapy for atherosclerosis. It is tempting to speculate that we can translate the outcome of our preclinical studies into a clinical application. DC-based immunotherapies are already used in clinical trails for cancer research where DCs are pulsed with tumor antigens which induce a tumor specific $CD8^+$ T cell response. Yet, it has been observed that some patients are non-responsive to the treatment. This could partially be explained by the fact that these patients have induced numbers of Tregs that counteract the DC therapy.[36, 37] Therefore it is of the outmost importance that the immunological status of the patients is characterized prior to DC treatment and that the correct antigen is being used to pulse the DCs. Another possible way to use DC-based therapy in the future is the targeting of DCs *in vivo* through DC-specific receptors like for example CD205, the mannose receptor or DC-SIGN instead of culturing them *ex vivo*. By discrimination of markers like DC-SIGN, Langherin and MGL that are unique to certain types of DCs, it should be possible to target specific DC subsets. C-type lectins, like DC-SIGN or MGL recognize carbohydrate or glycan structures so glycosylation of antigens can therefore modify antigens for DC-targeting. Also the use of antigen-containing liposomes, which are coupled to antibodies recognizing DC specific subsets, may be used for *in vivo* targeting.

However the techniques described above rely on the use of a disease specific antigen. The choice of the correct antigen in the field of atherosclerosis is less evident as the specific antigen is still unknown. OxLDL forms an interesting target as we have shown that vaccination strategy, in which an oxLDL-specific immune is induced, is atheroprotective. However further activation of the immune system in patients could have adverse effects. As we have shown in chapter seven, defective TGF-β signaling in DCs (these DCs are not capable of becoming tolerogenic DCs) induced an enhanced lesion progression with a more inflammatory phenotype. Furthermore, an increasing number of preclinical studies are focusing on the use of immature DCs to silence immunity by either inducing T cell anergy or by expanding regulatory T cells. Therefore, the use of the unpulsed immature DCs (**chapter four**) forms an attractive approach to implement in patients. DCs are cultured from the patient's blood or bone marrow and can subsequently be transferred back to the patient without the need of adjuvants and further pulsing with an atherospecific antigen. Also the induction of tolerogenic $CD4^+DX5^+$ cells after treatment with unpulsed ImDCs remains an interesting finding as it has been shown that these cells are protective in other auto-immune diseases.[14, 41-43] Therefore we would like to implement $CD4^+DX5^+$ adoptive transfer at different stages of the disease and determine in which stage of atherosclerosis these cells could be protective.

We have also observed that foam cell formation or hyperlipidemia induces a DC-like phenotype in macrophages and moreover, influences the antigen presentation and processing capacities *in vitro*. Therefore we would like to investigate the role of foam cell formation *in vivo* on APCs function and the possible effect thereof

on local $CD8^+$ activation and lesion development. $LDLr^{-/-}$ mice that receive a bone-marrow transplantation of ABCA1/SRBI double knock-out mice exhibit an extreme foam cell phenotype in the periphery. Hence, these mice provide us with an ideal model to study the influences of foam cell formation on the activation of an immune response during hyperlipidemia and cardiovascular disease. We have shown that cholesterol loading affects APC function but on the other hand we have seen that DCs could also influence cholesterol levels. However, the underlying mechanism remains elusive. PPARγ is a transcriptional regulator of CD36 and the oxysterol receptor LXRα and is highly expressed on DCs where it is a regulator of DC function by altering antigen uptake, maturation, activation, migration, cytokine production and lipid antigen presentation.[38, 39] In addition, LXR decreases LDL and tissue cholesterol by facilitating cholesterol excretion in the gallbladder, reducing cholesterol absorption in the intestine and promoting cholesterol efflux from macrophages by increasing ABCA1, ABCG1 and ApoE.[40] Therefore we would like to extend our research about the potential LXR activating properties of DCs and the possible effect on cholesterol levels. With regards to our studies about the role of Tregs in aneurysm formation, we are planning to increase the number of Tregs by injection of mouse IL-2 immune complexes.

In conclusion, we have shown the high potential of DC-based therapy in atherosclerosis. Furthermore, the progress being made in the many clinical trails will further enhance our knowledge of DC-based therapy. Therefore, it seems likely that in the future it is save to implement DC-based therapy as a new strategy in the battle against atherosclerosis.

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