Single-cell immune profiling of atherosclerosis: from omics to therapeutics

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Chapter 9

General discussion

Summary

Cardiovascular disease (CVD) remains the principal cause of mortality by comprising 32% of all deaths worldwide.[1,2] The majority of these cardiovascular deaths are attributed to myocardial infarction and stroke, with atherosclerosis as underlying cause of disease.[3] Atherosclerosis is characterized by excessive lipid accumulation in the arterial wall and a subsequent ongoing local inflammation. This chronic inflammatory disease induces continuous intimal growth of medium to large arteries resulting in the formation of atherosclerotic plaques.[4] With disease progression, these plaques further develop, become unstable and eventually rupture, leading to thrombus formation, ischaemia and subsequent clinical events. Revascularisation by means of anti-thrombotic treatment and/or surgical procedures is the primary therapeutic strategy upon a cardiovascular event. However, these surgical interventions, which include percutaneous transluminal coronary angioplasty, bypass surgery and carotid endarterectomy surgery, are highly invasive and may require repetition due to restenosis of the arteries.[5–8] Patients with either a history of CVD, or with familiar hypercholesterolemia and thus a high risk for disease development, are mainly treated with lipid-lowering therapeutics, such as statins and PCSK9 inhibitors. Although statins effectively lower circulating LDL cholesterol levels, recurrent events occur in over 20% of the symptomatic patients on high-dose statin treatment.[9,10] Considering that a large body of (experimental) studies have proven that the immune system plays a pivotal role in atherosclerosis development and progression, recent advances have been made to therapeutically target the ongoing chronic inflammation in CVD patients. Multiple clinical trials have provided proof-of-principle that targeting inflammation could alleviate CVD burden. The CANTOS trial was the first to prove this as treatment with a monoclonal antibody against IL-1β reduced the risk on recurrent cardiovascular events.[11] This was followed up by several other trials that applied immunosuppressive or anti-inflammatory agents, such as anti-TNF, methotrexate and colchicine, of which only the latter effectively reduced risk of ischemic cardiovascular events.[12–15] However, adverse effects caused by general immune suppression have also been described in these trials. As immunotherapy could also affect the host-defence response, the next challenge lies in the identification of more specific therapeutic targets that particularly target plaque-residing immune cells.

In the past decade, the rapid development of single-cell transcriptomics has revolutionized the bio-medical research field. Single-cell RNA sequencing (scRNA-seq) provides the full transcriptome on a per cell basis, thereby allowing a completely unbiased analysis of cellular phenotypes in heterogenous samples.[16] Single-cell RNA sequencing was first applied in 2009 by Tang *et al.*, who measured the whole-

transcriptome of a single cell.[17] By now, the technology has developed to such an extent that by using plate-based and microfluidic-based methods over thousands of single cells can be measured simultaneously.[18,19] The advantage of this technique is that it generates data at a much higher resolution, which results in a more detailed description of the cells and the possibility to detect rare cell populations that might become undetectable when applying more conventional techniques such as qPCR, microarray and bulk RNA sequencing.[20] Other approaches that measure on a single cell-level, such as immunohistochemistry, flow cytometry and cytometry by time-of-flight (CYTOF), are restricted by the use of a limited number of pre-defined targets that can be included for analysis.[20-22] Still, it remains of vital importance to keep using these techniques complementary to scRNA-seq analyses, especially as gene expression does not always correlate with protein levels. The field of single-cell transcriptomics has improved in such an extent, that currently the field moves towards single-cell multi-omics. This provides the opportunity to additionally identify the single-cell epigenome by measuring open chromatin regions (scATAC-seq), to assess clonality of respectively T and B cells by single-cell TCR and BCR sequencing and the opportunity to add an, in theory, indefinite number of monoclonal antibodies to simultaneously profile cell phenotype on protein level.[23] Altogether, single-cell multi-omics has proven to be a valuable tool in the understanding of disease development and in the identification of potential druggable targets.

This thesis

In this thesis, we applied single-cell multi-omics to generate a detailed cellular atlas of human atherosclerosis. We aimed to better characterize the cells that accumulate in advanced disease and subsequently employed this data set to define cells and genes that could be of therapeutic interest.

In the field of cardiovascular research, scRNA-seq has rapidly accelerated our understanding of vascular pathologies, including atherosclerosis and aortic aneurysm (AA). AA is the second aortic disease leading to cardiovascular death.[24,25] Due to progressive loss of vascular smooth muscle cells and extracellular matrix degradation the vascular wall becomes prone to rupture which results in severe clinical complications. In **chapter 2** we have provided an overview of the studies that have applied scRNA-seq to examine the cellular content of both diseases and reflect on potential similarities and differences between both diseases. Whereas AA is generally considered to be prominently affected by dysfunctional non-immune cells, including vascular smooth muscle cells, endothelial cells and fibroblasts,

scRNA-seq studies have strikingly described an established immune component to this disease as well. Both macrophages and T cells have been described in AA pathology, of which amongst others pro-inflammatory *IL1B*$^{+}$ macrophages and regulatory T cells were described with a similar phenotype in atherosclerosis. In AA, there has been more attention for a detailed description of fibroblasts compared to atherosclerosis, indicating the importance of these cells for this pathology. However, more recently, scRNA-seq has been applied to highlight the importance of fibroblasts in atherosclerosis as well.[26] Both in atherosclerosis and in AA, phenotypic modulation of smooth muscle cells is prominently described, often characterized by expression of genes involved in extracellular matrix regulation. In atherosclerosis, smooth muscle cell to macrophage transition is commonly described, whereas in AA the increased stress response leading to the loss of these cells in this disease is more pronounced. Endothelial dysfunction as consequence of inflammation was observed in both pathologies, however endothelial to mesenchymal transition was only observed in atherosclerosis studies. In both pathologies, the majority of the cellular communication pathways were between non-immune cells and macrophages, in which chemotaxis-related pathways were most commonly found. Yet, the only overlapping ligand-receptor pair between both diseases was the *C3-C3AR1* complement pathway. Although one could suggest that by targeting these pathways two birds are hit with one stone, this should be carefully interpreted as these cell subsets and pathways could have different functional properties in both diseases. Overall, this review highlights the extensive increased knowledge in vascular pathology that is obtained by scRNA-seq. The cellular content of both diseases has been shown to be much more diverse and dynamic than previously thought. ScRNA-seq will therefore be instrumental in further defining disease mechanisms and candidate targets for drug development.

Cellular atlas of the human atherosclerotic plaque by single cell transcriptomics

Atherosclerotic plaques are characterized by their heterogeneity in cellular content. To generate an unbiased immune cell atlas of murine atherosclerosis, in 2018, scRNA-seq was for the first time applied in atherosclerosis.[27,28] This showed the enormous potential of this technique as it led to the identification of amongst others TREM2^{+} macrophages, which by now have been proven to play a prominent role in atherosclerosis.[29,30] Subsequently, the immune cell content of human atherosclerotic plaques was first investigated by Fernandez *et al*. using a combination of CYTOF and scRNA-seq.[31] Yet, a full description of the cellular landscape of human atherosclerosis, including non-immune cells, was still lacking. Therefore, in **chapter 3**, we performed scRNA-seq and scATAC-seq on advanced human carotid plaques of a cohort of 18

patients and provide an overview of the various cell (sub)types present. We revealed 14 main cell populations and described their activation status, cellular plasticity and we examined potential pathways of intercellular communication. Finally, we integrated our data with existing cardiovascular GWAS data to map susceptibility genes to our identified cell populations.

Interestingly, we detected that the intraplaque leukocyte population predominantly consisted of T cells, encompassing over 50% of the analysed cells. Although the proportions of the cells present in the plaque could be affected by tissue dissociation[32], we could confirm by histological analysis that T cells indeed outnumbered myeloid cells in the human carotid atherosclerotic plaques. We showed that both $CD4^+$ and $CD8^+$ T cell subsets were mainly distinguished based on their activation status, rather than the conventional transcription factors that are classically used. Specifically of interest was the population of cytotoxic $CD4^+$ T cells that lacked *CD28* expression but did express *PRF1* and multiple granzymes, including Granzyme B. These cells had been previously found in the circulation of coronary artery disease patients [33,34], and now we could confirm their presence in the plaque on a cellular level as well. Whereas we were unable to detect cytokine expression, we did detect open chromatin regions of *IFNG* in this population.

Within the myeloid compartment, we observed one *CD1C* expressing dendritic cell cluster and three macrophage subsets, of which two proinflammatory and one anti-inflammatory foam cell-like cluster. The *IL1B*$^+$ macrophages express genes that are associated with inflammasome and caspase activity.[35,36] Likely, this subset could have been a target in the CANTOS trial, as well as in the LoDoCo trials, since colchicine has been shown to reduce NLRP3 activation.[11,13,15,37,38] The second pro-inflammatory subset was characterized by expression of *TNF* and *TLR4*, but also showed an enrichment for motifs from IFN-induced transcription factors. We hypothesized that the IFN-γ secreted by the $CD4^+CD28^{null}$ T cells could, in part, induce this pro-inflammatory macrophage phenotype. In line, scATAC-seq also revealed gene activation of *IL12* in the dendritic cell cluster, further confirming that the previously described IFN-γ-IL-12 loop could contribute to the local pro-inflammatory environment in the human plaque.[39–41] The last macrophage subset was characterized as a foam cell-like subset due to expression of *TREM2* and other lipid accumulation-associated genes, such as *ABCA1* and *ABCG1* and enrichment for lipid-associated LXR_RXR transcription factor motifs.[42,43] Whereas for long foam cells were considered pro-inflammatory, both *in vivo* and single-cell studies have shown that these cells are of anti-inflammatory nature instead.[29,44]

Due to the high resolution obtained with single-cell technologies, subtle phenotypic differences, such as cellular plasticity, can be detected. In this chapter, we also provided evidence for trans-differentiation of cells in the human plaque. Within the *TREM2* macrophage cluster, we detected expression of smooth-muscle cell associated genes. Lineage tracing studies in mice have reported that smooth muscle cells are also capable of lipid uptake and of differentiation into a macrophage-like cell.[45-47] Within the endothelial cells, we observed a similar phenomenon. Apart from angiogenesis-related gene signatures, we found a subset of *ACTA2*$^{+}$ endothelial cells, indicative of endothelial cells that underwent endothelial-to-mesenchymal transition, which occurs in inflammatory conditions.[48,49]

Apart from an extensive characterization of the cellular content of the plaque, we envisioned this data set as tool to simultaneously aid research into novel drug targets. Hereto, we predicted intercellular communication routes within the plaque. We mainly identified interactions involved in chemotaxis and extravasation of myeloid cells between endothelial cells and smooth muscle cells. Moreover, we predicted pathways that could be involved in t cell recruitment and activation. Finally, we used this data set to map candidate genes that have common variants in coronary artery disease (CAD) GWAS susceptibility loci on a single cell level. Even though GWAS has provided many genes of interest that could be causally related to cardiovascular disease, it remains challenging to identify which candidate genes have clinical potential and how to target them. By mapping them on a single cell level, direct functional tests can be performed to assess patient-driven druggable targets. We observed significant enrichment of these CAD GWAS hits in the macrophages, endothelial cells and smooth muscle cells. Future pre-clinical studies using both *in vitro* and *in vivo* models will be required to examine the function of the mapped CAD target genes in these cells to address how they contribute to disease progression.

Altogether, we have defined the microanatomy of the human atherosclerotic plaque by single cell technologies and provided two examples of how this data can be employed in the future. Throughout the rest of the chapters of this thesis, we have used this data set as basis for the definition of cells of interest and specific target finding and have validated these subsequently.

An autoimmune-like component in atherosclerosis

The role of T cells in atherosclerosis has been studied extensively. Within experimental studies, both atherogenic and atheroprotective mechanisms have been described for the different subsets.[50-53] As described in **chapter 3** and by Fernandez *et al.*[31], a large number of T cells can be found in human atherosclerotic plaques. Yet, what drives these T cells to migrate to the plaque and if they undergo antigen-specific activation remained elusive.

For this reason, in **chapter 4**, we performed single-cell TCR sequencing (scTCR-seq) on matched PBMC and human advanced carotid plaques investigate the extent of TCR clonality in the plaque and to assess the activation status of these antigen-specific T cells. In line with previous work, we observed that $CD8^+$ T cells were considerably more expanded in the plaque compared to $CD4^+$ T cells.[54] However, by using matched PBMC samples we showed that the proportion of clonally expanded $CD8^+$ T cells was equally represented or even overrepresented in the circulation, whereas we did observe an increased fraction of clonally expanded $CD4^+$ T cells in the plaque compared to PBMC. We identified one specific plaque-enriched clonally expanded $CD4^+$ T cell subset, that had a gene signature indicative of recent antigen-induced activation. Apart from this effector subset, we also detected a clear regulatory T cell (T_{reg}) subset with an antigen activation signature. Nevertheless, we only detected a small proportion of clonally expanded T_{regs}, which were furthermore detected in similar proportions in both environments. Interestingly, by applying lineage tracing we detected a clear path from the *CCR4*$^+$*CCR10*$^+$ migratory subset in the PBMC towards the plaque-enriched effector $CD4^+$ T cells. This suggests that these migratory cells could be a circulating precursor, which was further supported by the multiple overlapping TCR clones between both cell types of which a considerable amount was enriched in the plaque. Subsequently, we were interested to see whether we could identify interactions between these effector $CD4^+$ T cells and local antigen presenting cells in the plaque. There is contrasting evidence regarding how antigen-presentation in the lesion contributes to disease progression. The current hypothesis is that the location of the antigen-presentation is essential for the outcome of the T cell activation, in which the plaque environment should induce an atheroprotective T cell response.[55,56] Yet, we showed that pathogenic interactions may also occur in the lesion, as we predicted a co-stimulatory interaction through the CD40-CD40L pathway between lesional *TREM2*$^+$ myeloid cells and the plaque-enriched effector $CD4^+$ T cells. By showing an accumulation of antigen-specific T cells in the plaque, we hypothesized that there may be an autoimmune component to atherosclerosis. We therefore compared the transcriptome of the $CD4^+$ T cells to that in the synovial fluid of patients with the autoimmune disease psoriatic arthritis and found substantial similarities which supported this theory. In line with our results, scTCR-seq of atherosclerotic plaques, artery tertiary lymphoid organs (ATLOs) and draining lymph nodes of aged *apoE*$^{-/-}$ displayed similar clonal expansion of $CD4^+$ T cells in the plaque and ATLOs of these mice and revealed a predominantly pro-inflammatory phenotype of dendritic cells in ATLOs as well.[57] Altogether, these data support the notion that the pathophysiology of atherosclerosis has an autoimmune component, highlighting its potential for the development of novel therapeutic targets.

Mast cell activation and migration in advanced atherosclerosis

Whereas the largest population of immune cells was ascribed to T cells in **chapter 3**, the smallest population was a distinct cluster of mast cells. Albeit in low numbers, mast cells have been proven to significantly contribute to atherosclerosis progression. In experimental models for atherosclerosis, mast cells promote plaque vulnerability amongst others due to the proteases they secrete.[58–61] Moreover, the number of intraplaque mast cells was positively associated with future cardiovascular events independent of the main CVD risk factors.[62] Here, we aimed to further characterize human intraplaque mast cells, examine whether we could detect age-related changes and investigated whether we could inhibit mast cell migration towards the plaque.

In **chapter 5**, we set up a flow-cytometry based approach to better characterize mast cell numbers and phenotype in a cohort of plaques obtained from both carotid and femoral endarterectomy surgery. First, we established that approximately 1% of all $CD45^+$ leukocytes consists of mast cells, as identified by high expression of classical mast cell markers CD117 and FcεRI. Subsequently, we investigated mast cell activation status. Whereas mast cell activation in human atherosclerosis was generally assessed with immunohistochemical tryptase staining to visualize degranulation, we applied flow cytometry to address mast cell activation status. Notably, the majority of the intraplaque mast cells were found to be activated as measured by CD63 expression, a tetraspanin upregulated upon mast cell degranulation.[63] The most prominent route of mast cell activation is the FcεRI-IgE pathway.[64–67] Circulating IgE has been positively correlated to acute cardiovascular events[68,69], however whether IgE was also bound to the activated mast cells was yet to be determined. Therefore, we subsequently measured IgE on the intraplaque mast cells. Indeed, the majority of the activated mast cells had IgE bound to their surface. As in experimental models of atherosclerosis mast cell stabilization has been proven to moderate atherosclerosis development[58], a potential therapeutic strategy could be to limit circulating IgE thereby aiming for reduced intraplaque mast cell activation. In addition, we also observed a population of IgE^-CD63^+ mast cells, which have likely been activated through other pathways. It remains to be determined which pathways underlie this activation. Finally, we showed by flow cytometry that not all mast cells are positive for tryptase, indicating that immunohistochemical staining may not include all mast cells present in atherosclerosis. Therefore, we believe that this flow cytometry approach is a valid method to examine mast cells in human atherosclerosis. Future studies in larger cohorts could shed light on how certain activation patterns of mast cells may be correlated to clinical outcomes, thereby paving the way for new therapeutic approaches to target mast cells in atherosclerosis.

Aging is an independent risk factor for atherosclerosis. This is in part mediated by the low-grade chronic inflammation, termed inflammaging, that occurs with age. The pro-inflammatory conditions significantly affect the plaque-residing immune cells. Recent work has described multiple age-associated changes in intraplaque immune cells and confirmed these findings in human CVD patients.[70] Mast cells function has also been shown to alter with age[71–74], however it remained unknown whether they also undergo age-associated changes in atherosclerosis. Therefore, in **chapter 6**, we examined the effect of aging on mast cell phenotype and activation status. We examined mast cell populations in young and old *Ldlr*$^{-/-}$ mice and show an increased accumulation of mast cells in the aged atherosclerotic aorta and peritoneum. There is some disparity between tissues as to whether aging induces or inhibits mast cell activation.[72,74] Here, we observed that with age, mast cells exert a predominantly activated phenotype. Moreover, we observed increased serum IgE levels in aged *Ldlr*$^{-/-}$ mice, suggesting that the IgE-FcεRI pathway is involved in this increased activation. Congruently, we observed a similar mast cell phenotype in **chapter 5** in human plaques, which are generally obtained from elderly patients. Interestingly, we detected intrinsic mast cell activation in aged bone-marrow derived mast cells, which was accompanied by increased CCL2 secretion upon stimulation. The low-grade activation phenotype was not sustained upon adoptive transfer of these aged cells into young mast-cell deficient *apoE*$^{-/-}$*Kit*$^{W\text{-}sh/W\text{-}sh}$ mice and no differences were observed in myeloid cell recruitment as well. This can in part be explained by the fact that mast cell phenotype is largely dependent on the inflammatory environment in which the mast cell resides.[75–79] These results therefore highlight an instrumental role of the microenvironment for mast cell phenotype in atherosclerosis.

Although mast cell activation and degranulation are the most prominent line of investigation, mast cells have also been described to act as atypical antigen-presenting cell.[80] Indeed, mast cells have been found in close proximity to CD4$^+$ T cells in the skin of psoriasis patients and mast cell depletion resulted in reduced CD4$^+$ T cell infiltration and activation in a murine model of multiple sclerosis.[81,82] Furthermore, previous work from our lab has shown that hypercholesterolemia significantly upregulates MHC-II expression on mast cells.[83] Moreover, these mast cells were proven to be capable of functional antigen-presentation to CD4$^+$ T cells *in vivo* and mast cell depletion reduced CD4$^+$ T cell numbers in the atherosclerotic aorta. Considering the in **chapter 3** and **4** described importance of CD4$^+$ T cells in human atherosclerosis, we were particularly interested to examine whether aging also affects these antigen-presenting capacities of mast cells. Increased numbers of MHC-II$^+$ mast cells were retrieved in the aged atherosclerotic aorta compared to young. Notably, we reported increased CD4$^+$ T cell proliferation upon incubation with aged MHC-II$^+$ mast cells versus their young

counterparts, indicating that aged mast cells are capable of antigen internalization and direct presentation to $CD4^+$ T cells. However, similar to the activation profile of the mast cells, MHC-II upregulation is likely also influenced by the microenvironment as this was also lost upon reintroduction in a young microenvironment. Apart from direct antigen presentation, mast cells have also been shown to affect $CD4^+$ T cell polarization by the cytokines secreted upon activation.[84] So, we hypothesized that, although local antigen-presentation by mast cells could take place in the plaque, the aged mast cell secretome is likely to play a more important role in $CD4^+$ T cell differentiation. Indeed, we observed that treatment of aged *Ldlr*$^{-/-}$ mice with the mast cell stabilizer DSCG significantly reduced systemic and intraplaque effector $CD4^+$ T cells, in particular $FoxP3^+$ and $T\text{-}bet^+$ $CD4^+$ T cells. Conclusively, we show that aging and the inflammaging microenvironment significantly contributes to mast cell phenotype and function in atherosclerosis. This affects both their activation and degranulation as well as the subsequent changes on $CD4^+$ T cell phenotype in the plaque. It is therefore essential to consider age as important factor when further investigating mast cells for novel therapeutic interventions for atherosclerosis.

Because of the increased accumulation and activation of mast cells in advanced atherosclerosis [85], a promising therapeutic strategy could be to inhibit mast cell recruitment towards the atherosclerotic lesion to prevent plaque destabilization. One way of targeting this migration is through lipid mediators.[86,87] Leukotriene B4 (LTB_4) is a pro-inflammatory lipid mediator which has the leukotriene B4 receptor (BLT1) as its most potent receptor.[88] LTB_4 is synthesized as a result of the enzymatic reaction of arachidonic acid by 5-lipoxygenase (5-LOX), 5-lipoxygenase activating protein (FLAP) and LTA4 hydrolase (LTA4H).[89] The BLT1-LTB_4 axis has been shown to play a role in the recruitment of multiple immune cells, including myeloid cells and mast cell progenitors.[90–92] This chemotactic pathway has already been extensively researched in the context of atherosclerosis. Both genetic deletion and pharmacological inhibition of BLT1 or 5-LOX have been shown to reduce initial atherosclerosis development, in part due to reduced macrophage content in the plaque.[93–96] Yet, whether this lipid mediator also contributes to immune cell, and in particular mast cell, recruitment in pre-existing atherosclerosis remained elusive. Therefore, in **chapter 7** we examined whether BLT1-inhibition could reduce mast cell migration in advanced atherosclerosis. We first examined expression of the previously mentioned genes involved in the biosynthesis of LTB_4 were expressed in our single-cell human plaque atlas (**chapter 3**). Indeed, we detected expression of all genes in the myeloid compartment, but particularly high expression in the mast cells. Subsequently, we treated *Ldlr*$^{-/-}$ with established atherosclerosis with the BL1-antagonist CP105,696. We observed significantly reduced splenic myeloid cells, which is could be a direct effect of BLT1

inhibition, but is also congruent with the notion that upon LTB_4 binding, monocytes can actively synthesize monocyte-chemoattractant protein 1 (MCP-1) to enhance their recruitment.[97,98] We did not observe any changes in plaque size and morphology upon treatment with CP105,696. Likely, this treatment is less sufficient in more advanced atherosclerosis, which is in line with a previous study from Aiello *et al.*, who described the most pronounced effect of BLT1 antagonism in early lesions.[94] Finally, we did not observe any changes in the numbers of circulating mast cell progenitors nor the number of mast cells in the atherosclerotic aortic root. This suggests that in advanced atherosclerosis the BLT1-LTB_4 axis is not involved in mast cell recruitment to the site of disease. Of note, the low affinity BLT2 receptor could also induce mast cell migration.[92] As CP105,696 is BLT1-specific, we cannot exclude potential BLT2-induced mast cell migration in this study and this will need further investigation. Although BLT1-antagonism is apparently not the right approach to intervene with mast cell migration in advanced atherosclerosis, we did establish local LTB_4 production by mast cells in the human atherosclerotic plaque, which consequently could contribute to the intraplaque (immune) cell content. Overall, we are convinced that targeting mast cell migration, albeit through a different pathway, remains a promising strategy to intervene with atherosclerosis progression.

Single cell atlas as tool for the identification of druggable targets

Many potential targets have already been investigated for atherosclerosis, yet unfortunately the translation to the clinic is often unsuccessful. As previously mentioned, we generated the cellular atlas in **chapter 3** to improve the understanding of the pathophysiology of atherosclerosis and to aid in the definition of druggable targets. In **chapter 8** we employed this tool and found specific expression of *IL4I1* within the *TREM2*$^+$ macrophages. Interleukin-4-induced gene-1 (IL4I1) is an enzyme which is primarily produced by antigen-presenting cells and it induces the conversion of its substrate L-phenyl alanine into phenyl pyruvate, ammonia and hydrogen peroxide.[99,100] IL4I1 specifically accumulates in the immune synapse and subsequently interferes with T cell proliferation and promotes differentiation towards T_{regs}.[101] IL4I1 was originally discovered in different types of cancer and inhibition of this enzyme has been shown to significantly reduce tumor growth by increasing the cytotoxic $CD8^+$ T cell response.[102] Recently, a specific small-molecule IL4I1-inhibitor, CB-668, was reported to reduce tumor growth by interference with the $CD8^+$ T cell response.[103] Although cancer and atherosclerosis share mutual inflammatory pathways, the favorable response is often contradictory. Especially with immune checkpoint therapies, increased risk for cardiovascular disease has been reported repeatedly.[104,105] We therefore examined whether IL4I1-inhibition with CB-668 affected atherosclerosis progression in *Ldlr*$^{-/-}$ mice. In line

with the previously highlighted results, we observed a shift from naive towards effector and central memory T cells in the spleen. Furthermore, we observed an increase in splenic cytotoxic $CD8^+$ T cells and a trend towards an increase in the atherosclerotic aorta. Within the draining lymph node, we also observed an increase in pro-inflammatory T_{h1} $CD4^+$ T cells, altogether emphasizing the pro-inflammatory response induced upon CB-668 treatment. Nevertheless, we did not observe any changes in atherosclerotic lesion size and composition. Here, we examined IL4I1 inhibition in initial atherosclerosis, whereas more pronounced effects could occur in more advanced stages of the disease. So, even though our data demonstrated that IL4I1 antagonism does not affect initial atherosclerosis development, future studies will have to pinpoint if the induced pro-inflammatory microenvironment will further promote disease progression in advanced stages.

Future perspectives

Recent advances in the field have proven the enormous potential for targeting the immune system to treat atherosclerosis. Multiple trials targeting the immune system in atherosclerotic cardiovascular disease patients have proven beneficial cardiovascular outcomes. However, not all immunotherapies were successful in ameliorating disease burden. The heterogenous and plastic immune cells in the atherosclerotic plaque harbor both atherogenic and atheroprotective populations, each affecting disease progression differently. It is therefore essential to provide a more tailored approach to identify specific processes that can be targeted to restore the immunological balance within the lesion. The rapidly evolving single-cell technologies will become of vital importance when advancing towards precision medicine for atherosclerosis.

In the past decade, the datasets generated by single-cell multi-omics have provided an important tool for future therapeutic targeting of atherosclerosis. These studies have highlighted the delicate changes in cellular phenotype and provided crucial new insights in the disease. Apart from a detailed description of the transcriptome of the cells present in the plaque, advanced computational analyses of this data have also elucidated trajectories of cellular differentiation. For instance, in this thesis we describe a circulating precursor for the intraplaque antigen-specific effector $CD4^+$ T cell subset and recent work has extensively described macrophage plasticity in atherosclerosis which revealed that the anti-inflammatory *TREM2*$^+$ macrophages could also further differentiate into a pro-inflammatory lipid-associate macrophage phenotype.[106] It will be also of interest to correlate specific cell populations or gene signatures to clinical parameters to identify causal relationships. Importantly,

recent work has applied single-cell data as tool for drug repurposing to identify new therapeutic strategies for atherosclerosis.[107] By using a systems-immunology based approach, inflammatory signatures were screened for existing drugs that may target prominent pathways. This targeted approach allows for more rapid pre-clinical testing and accelerates the transition to the clinic.

Another common practice in single-cell analysis, is the prediction of intercellular communication to highlight important pathways for intervention.[31,108] Although this provides a proper idea on what cells could potentially interact, it also touches upon one of the limitations of single-cell technologies. As a single-cell suspension is needed, tissue dissociation is required. This could not only slightly affect the ratio of cell types present in the plaque, but you also lose the spatial architecture of the tissue.[32] Spatial transcriptomics have been introduced to overcome this issue.[109] In the context of this thesis it will be very valuable to apply this technique to examine interactions between antigen-presenting cells, including mast cells, and T cells to further define whether indeed the CD40-CD40L pathway is as important for antigen-specific activation of plaque $CD4^+$ T cells or if there are other more pronounced pathways involved in this process.

Now that we have established the accumulation of plaque-enriched antigen-specific T cells in the plaque, this immediately raises the question which antigen(s) initiate this T cell response. Several candidate antigens have been examined in atherosclerosis, including heat shock proteins, plasma protein β2-glycoprotein Ib and fibronectin.[110] Yet, T cell responses against peptides originating from Apolipoprotein B100 (ApoB100) have been investigated most prominently. Since vaccination with LDL and ox-LDL were shown to be atheroprotective[111–113], several methods have been applied to further define the exact antigens involved. This includes epitope-binding assays as well as prediction models that calculate MHC-II binding affinity.[114,115] Altogether these discovered auto-antigens have been proven to have therapeutic potential in experimental models of atherosclerosis.[116–118] However, with the advance of single-cell TCR sequencing and the identification of clonally expanded T cells in the tissue itself, there is a need to bridge the gap from TCR to peptide. Bioinformatic tools such as GLIPH2 and GIANA have been applied in atherosclerosis previously,[54,119,120] Although theses computational approaches have high potential, in our data this did not give the desired accuracy yet. An upcoming different approach to examine auto-antigen is by the use of immunopeptidomics. This technique allows the measurement of peptides presented on MHC molecules at the site of disease.[121] It will be of great importance to apply this technique to human atherosclerosis and to define its immunopeptidome, which may be a great tool for future vaccine development.

Finally, we provide evidence that targeting mast cells in atherosclerosis could be an effective therapeutic strategy. Either through inhibiting their highly activated state or by targeting mast cell recruitment to the plaque, atherosclerotic burden can be ameliorated. As our data demonstrate that IgE could significantly contribute to intraplaque mast cell activation, it will be of interest to treat patients with IgE-blocking antibodies, such as Omalizumab[122], to see how this affects atherosclerosis progression. Even though we were not yet able to define the prominent pathways involved in mast cell migration to the atherosclerotic plaque, we believe it is crucial to further examine this in the future.

Apart from drug target finding and validation, single-cell technologies could also be applied for diagnostic approaches. Currently, early detection of atherosclerotic cardiovascular disease is a prominent focus point in the research field. Therefore, it is essential to define new biomarkers for this disease. As mentioned before, if clinical parameters could be associated to certain pathological cell subsets, this could improve risk prediction. Additionally, by examining whether the cell subsets detected in the plaque also occur in the circulation or if we can find pre-cursors of pathogenic subsets, these could potentially be used as biomarker. Based on the work in this thesis, it could be of interest to further define if the $CCR4^{+}CCR10^{+}$ precursor $CD4^{+}$ T cells could be associated to future cardiovascular outcomes. Furthermore, investigating if circulating $CD4^{+}CD28^{null}$ cells could also be correlated to their intraplaque counterparts could be very relevant. In addition, measuring mast cell progenitors in the circulation could potentially also be a tool to predict plaque stability. Lastly, there lies great potential in the use of single-cell technologies for patient stratification and to predict drug response by the presence or absence of therapeutically relevant markers. In cancer, this has already been applied to define patients that are more likely to be a good responder to chemo- or immunotherapy.[20,123] In atherosclerosis, differences between immune cells of symptomatic and asymptomatic patients have been described previously on a single cell level[31], but gene signatures predicting response to cardiovascular therapeutics are yet to be determined. To aid in the challenges described above, it is of importance to assess whether there is similarity in cellular content between all vascular beds, or even to pinpoint significant differences. Currently, scRNA-seq is applied in human plaques originating from either carotid or coronary arteries. In a recent study, an integrated analysis has been performed on mononuclear phagocytes from two studies that used respectively carotid and coronary plaques.[31,124,125] This showed that, apart from some interpatient variability in the proportions, all myeloid subclusters were conserved in both tissues. To define proper biomarkers for all atherosclerosis patients, it will be valuable to further increase this meta-analysis by increasing the number of patients and studies and to elaborate on all cells detected within both vascular beds.

In conclusion, the work in this thesis shows that single-cell transcriptomics is a valuable tool to examine the pathophysiology of atherosclerosis in detail and to identify novel targets for intervention. It will be intriguing to see how the untapped potential of single-cell technologies will aid in tackling the challenges that lie ahead towards a tailored therapy for atherosclerosis.

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