Atherosclerosis & inflammation: Macrophage heterogeneity in focus
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Chapter 9

General discussion
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Monocyte-derived macrophages are fundamental to the multifaceted pathogenesis of atherosclerosis and its clinical correlates \(^1,2\). These cells accumulate in the subendothelium of arteries in response to local lipoprotein retention and fuel a chronic inflammatory response that is detrimental to plaque stability over time \(^3-5\). However, like other mononuclear phagocytes, macrophages comprise a heterogeneous population with profound phenotypical diversity that mirrors the micro-environment \(^6,7\). While the initial classification recognised M1 and M2 macrophages (in response to incubation with IFN-\(\gamma\)/ IL-4 or IL-10, respectively) \(^8,9\), subsets induced by other triggers have been integrated into this framework more recently \(^10-12\). Together, these cells represent an array of macrophage phenotypes with distinct and often opposing effector functions in health and disease.

The main findings presented in this thesis are that:

1. In humans, M1 and M2 macrophages are present throughout atherosclerotic plaque development, but localize to different morphological compartments of the vessel wall.
2. M1 macrophages allocate to rupture-prone shoulder area of the plaque.
3. Defective IL-10R1 signalling in myeloid cells reduces plaque size and severity in LDLR\(^{-/-}\) mice by decreasing hypercholesterolaemia and myeloid cell accumulation, while enhancing plaque apoptotic cell content.
4. Myeloid IL-10R1 deficiency causes changes in intestinal cholesterol fluxes and reduces hypercholesterolaemia.
5. Helminth-derived soluble egg-antigens (SEA) treatment protects from atherosclerosis in LDLR\(^{-/-}\) mice by restraining myeloid inflammatory responses.
6. Glatiramere acetate (Copaxone\(^{®}\)) does not limit plaque development in hyperlipidaemic LDLR\(^{-/-}\) mice.

Macrophage heterogeneity in human atherosclerotic disease

Macrophage heterogeneity is an established concept in atherosclerosis \(^5,13,14\). Works by Bouhlel et al. \(^15\) and Boyle et al. \(^1,2,16\) were among the first to identify the presence of M2 macrophages in human atherosclerotic plaques, respectively linking this alternative activation to PPAR\(\gamma\) signalling and intraplaque haemorrhage. These subsets were demonstrated to express resistance to cholesterol loading and repress inflammation through autocrine IL-10 signalling \(^3-5,17,18\), before being unified by findings of Finn et al. characterising a haemoglobin-induced CD163\(^+\)MR\(^+\) macrophage subset with an aptitude for RCT \(^6,7,10\).
In Chapter 4 we are able to reaffirm and expand on some of these observations. By examining the presence and spatial distribution of markers for polarized macrophages in different stages of human plaque development, we identified enhanced gene expression for both M1 and M2 macrophages in ruptured vs. stable plaque segments. Ensuing histopathological analysis confirmed a gradual accumulation of M1 and M2 macrophages with lesion progression. Here, foam cells expressed an ambivalent repertoire of M1 and M2 markers, whereas adventitial macrophages were profoundly skewed to an M2 phenotype. More importantly however, M1 macrophages were found to be the largest subset in the rupture-prone shoulder area of the plaque, whereas such a predominance did not occur in fibrous cap regions. Thereby our findings do not only show M1 and M2 macrophages localize to distinct morphological compartments of the vessel wall, but also provide the first evidence to strengthen the prevailing hypothesis that M1 macrophages are deleterious to lesion stability. This presents an important novel mechanistic insight into plaque aetiology and might be the basis for continued efforts to target this inflammatory subset.

Recently, some have raised concern about the validity of certain macrophage markers and the degree to which they truly represent a phenotype and its effector functions \(^8,^9,^19\). We acknowledge this as a valid point of debate. Evidently, functional macrophage phenotypes are as complex as the micro-environment that fosters them. Atherosclerotic plaques are particularly heterogeneous and offer plaque macrophages unique challenges (e.g. modified LDL) in addition to more conventional triggers. Therefore, expression of a single surface protein is often part of, not equivalent to a polarized state. More so, a specific marker in atherosclerosis need not have the same characteristics in other disease states, as atherogenic lipids also modulate macrophage function \(^20\). Although this issue can be partially addressed by employing panels of M1/M2-associated markers, as demonstrated by us and others \(^5,^13,^14,^21\), this poses limitations on throughput and applicability. Thus, as described in Chapter 3, more work is required to identify signalling pathways, transcription factors and epigenetic regulators that can more accurately distinguish macrophage phenotypes in atherosclerosis and other inflammatory conditions alike. A major field to watch here is that concerning epigenetics, which is shown with increasing frequency to be instrumental for appropriate regulation of macrophage phenotype \(^19,^22,^23\).

**Myeloid cell-specific involvement of IL-10R signalling in murine atherogenesis and cholesterol metabolism**

IL-10 is a pleiotropic cytokine with established atheroprotective qualities \(^15,^24-^27\). IL-10 affects plaque development mainly by negatively regulating inflammatory responses in the vessel wall \(^28-^31\), but also influences foam cell formation \(^32,^33\), cell survival \(^34\) and susceptibility to thrombus formation \(^35\). To exert these effects, IL-10 requires its canonical receptor complex
that features a tetramer of two ligand-binding components and two accessory signalling subunits, called IL-10R1 and IL-10R2 respectively \(^{24,36}\). Although IL-10R2 is ubiquitously expressed and shared by a number of other type II cytokines \(^{37}\), the more limited expression pattern of IL-10R1 restricts cellular IL-10 responsiveness to mainly hematopoietic cells.

In this thesis, we investigated the hypothesis that responsiveness of macrophages and neutrophils to IL-10 is paramount to its protective function in atherosclerosis (Chapter 5). By transplanting myeloid IL-10R1-deficient (IL-10R1\(^{\text{del}}\)) bone marrow to lethally irradiated LDLR\(^{-/}\) mice, we observed that loss of IL-10R signalling in myeloid cells significantly reduces the size and severity of aortic atherosclerotic plaques upon 6 or 10 weeks of high-cholesterol feeding. This robust phenotype was composite in nature, involving marked reduction of plasma cholesterol and plaque neutral lipid accumulation, reduced myeloid cell content and increased apoptotic cells counts. Hence, IL-10 requires vascular (immune) cells other than macrophages and neutrophils for its effects in atherogenesis. By showing that IL-10 acts on different target cells in the vessel wall to exert contrasting effects on the atherosclerotic process, our data provide key new insight into the complexity of IL-10 function.

In chapter 2 we advocated investigating the functional relevance of macrophage subsets in atherosclerosis through cell-specific knock-out of appropriate cytokine receptors. As such, the IL-10R1\(^{\text{del}}\) mice discussed in this thesis could in principle serve as a model lacking M2c macrophages. Although the current hypothesis that M2 macrophages are anti-inflammatory in nature \(^{38}\) holds true for our in vitro findings, myeloid IL-10R1 deficiency in LDLR\(^{-/}\) mice fails to translate into a pro-inflammatory and pro-atherogenic phenotype in vivo. This would indicate that M2c macrophages promote atherogenesis. Alternatively, we would like to propose the current approach does more than abrogate myeloid sensitivity to IL-10. In fact, it exchanges regular macrophages for cells that behave differently in various processes key to plaque development. A cleaner approach to the matter at hand would be to evaluate plaque burden in wildtype or IL-10R\(^{-/}\) atherogenic mice after adoptive transfer of ex vivo IL-10-polarized macrophages. Although such a setup would be of great interest, it might be subject to issues concerning cell directionality and macrophage plasticity.

Next, as IL-10R1\(^{\text{del}-\text{tp}}\) LDLR\(^{-/}\) mice demonstrated a profound reduction in circulating VLDL and LDL cholesterol, we set out to identify the mechanisms underlying this resistance to diet-induced hypercholesterolaemia (Chapter 6). By quantifying intake, de novo synthesis, excretion and hepatobiliary output of cholesterol in myeloid IL-10R1-deficiency, we could demonstrate these mice feature impaired intestinal cholesterol absorption and shunt large quantities of neutral sterols towards the faeces through bile-independent pathways. This in turn resulted in a very robust upregulation of cholesterol biosynthesis in IL-10R1\(^{\text{del}}\) mice. Not co-incidentally, this phenotype closely resembles that of a model of hepatic ACAT2 depletion yielding augmented non-biliary cholesterol efflux \(^{39}\). In our studies, we found a small but significant decrease in hepatic ACAT2 expression in myeloid IL-10R1 deficiency that accommodated diminished quantities of cholesteryl esters in liver tissue and apoB-
lipoproteins. Despite previous research not showing a preference for either cholesteryl esters or free cholesterol in non-biliary cholesterol efflux⁴⁰, we postulated that altered composition of VLDL and LDL might preferentially direct these particles towards transintestinal excretion.

Although the close relationship between cholesterol metabolism and macrophages in atherosclerosis development was exemplified previously by reports showing that administration of the macrophage growth factor M-CSF negatively regulates plasma cholesterol levels in animal studies⁴¹,⁴² and humans alike⁴³, we cannot yet explain in what ways our myeloid-specific gene defect influences hepatocyte function to alter cholesterol homeostasis. In this light, we considered a feedback loop between lack of IL-10 responsiveness and IL-10 production, but found no consistent data to support such a theory. Thus, future research should focus on identifying a macrophage-derived soluble factor that interacts with hepatocytes to account for these effects.

In all, myeloid IL-10R signalling forms a potent and in some ways counterintuitive aspect of systemic IL-10 function. Since IL-10 is widely acknowledged as a stereotypical atheroprotective cytokine, our current observations emphasize the advantages of employing cell-specific approaches to gain insight into immune function and showcase the interdependence of IL-10-based immune cell interactions in atherosclerosis. Moreover, we are the first to demonstrate such a pronounced effect of IL-10 on systemic cholesterol balance.

**Targeting macrophage-mediated inflammation to reduce atherosclerosis development**

Previous work in mouse models deficient for macrophage-colony stimulating factor (M-CSF) has shown that mononuclear phagocytes are indispensable to atherosclerotic plaque formation⁴⁴-⁴⁶. Although macrophages can be selectively removed from plaques, the outcome (and applicability) of such an approach depends on the timing of intervention and the stage of plaque development⁴⁷-⁵⁰. Thus, rather than deleting macrophages from plaques entirely, it might be advantageous to capitalise on the plasticity of these cells⁵¹,⁵² and skew macrophages to a more favourable phenotype. However, this might prove challenging, as Bouhlel et al. demonstrated PPARγ-activation is able to program circulating mononuclear precursor cells but not plaque macrophages towards an M2 phenotype in vivo⁵³.

In this thesis, we explored two experimental immunomodulatory approaches for their potential to alter macrophage phenotype in atherosclerosis. First, in Chapter 7, we show that treatment of hyperlipidaemic LDLR⁻⁻/⁻ mice with *Schistosoma mansoni*-derived soluble
egg antigens (SEA) strongly protects from atherosclerosis development. Importantly, these effects occurred largely independently from reduced plasma cholesterol levels or T-cell responses. Instead, SEA treatment of bone marrow-derived and peritoneal macrophages elicits an anti-inflammatory phenotype characterized by enhanced IL-10 secretion. Accordingly, SEA decreased the amount of circulating myeloid cells, most notably the inflammatory Ly6C\text{high} subset, while also impeding plaque inflammation related to adherence and accumulation of macrophages and granulocytes in atherosclerotic lesions. Thereby, our current findings provide solid evidence that helminth-derived antigens are capable of impairing plaque development and diet-induced hypercholesterolaemia.

Looking forward, two important issues regarding SEA as a therapeutic agent remain to be resolved. First, future research will need to identify which components of the heterogeneous SEA-mixture correlate with certain beneficial properties. This way, the composition of efficacious antigens can be fine-tuned for maximum therapeutic impact. Secondly, pinpointing the surface receptors (e.g. TLRs, C-type lectins) and signalling pathways \textsuperscript{53} responsible for transforming the extrinsic SEA stimulus into improved outcome will certainly contribute to a more conscious application of SEA in chronic inflammatory disease. Thus, once optimised, SEA treatment could provide physicians with a new therapeutic options in the management of human atherosclerotic disease.

Next, in Chapter 8, we investigated Glatiramer acetate (GA or Copaxone\textregistered, formerly copolymer 1) treatment as a means of altering macrophage function and inhibiting atherosclerotic plaque formation. This random copolymer of four amino acids is FDA-approved for the treatment of relapsing-remitting multiple sclerosis \textsuperscript{54,55}, but was also evaluated in other (neuro)inflammatory settings \textsuperscript{56-59}. Interestingly, besides affecting adaptive immune responses \textsuperscript{60}, GA was more recently shown to influence effector functions of monocytes and dendritic cells \textsuperscript{61-63}. In the present thesis, we were able to confirm previous findings in rats \textsuperscript{64} by showing that GA strongly augments expression and secretion of IL-10 in primary murine macrophages. Yet, this induction of IL-10 was offset by amplified pro-inflammatory cytokine responses (e.g. TNF, IL-6) upon TLR-stimulation, which is at odds with earlier observations in related cell types \textsuperscript{65,66}. Moreover, in hyperlipidaemic LDLR\textsuperscript{-/-} mice, atherosclerotic lesion size, severity and inflammatory burden of established plaques were equal between treatment groups upon subcutaneous administration of GA, despite significantly reduced plasma cholesterol levels in GA-treated mice. Remarkably, plasma cytokine levels and ex vivo cytokine production by peritoneal macrophages were similarly unaffected between GA-treated and PBS-injected mice. Although previous pilot data had revealed a dose-dependent relative decrease in Ly6C\text{high}-monocytes upon GA treatment, no significant differences in circulating myeloid cell types were detectable by flow cytometry under hyperlipidaemic conditions. Thus, our current treatment regimen does not evoke substantial anti-inflammatory changes or altered atherosclerosis development in hyperlipidaemic LDLR\textsuperscript{-/-} mice. Although we assume it possible that variations in timing,
frequency and dosage of treatment, as well as the experimental mouse model used could yield different results, the introduction of hyperlipidaemia into the equation is likely a critical difference with previous reports successfully demonstrating GA’s immuno-modulatory potential.

Concluding remarks and future perspectives

Taken together, our findings highlight the authority of mononuclear phagocytes - and macrophages in particular - over cellular processes in atherogenesis. Research is currently moving away from merely identifying macrophage heterogeneity in favour of approaches that illustrate its impact on pathology. By associating M1 macrophages with unstable plaque regions, we hope to encourage continued investigation into their impact on the local microenvironment and development of novel approaches to address either their presence in general or the unwanted aspects of their functionality. In this regard, SEA treatment can serve as proof-of-principle. Although clarifying the functional contribution of macrophage subsets in atherosclerosis through cell-specific knock-outs of associated cytokine receptors might prove less straight-forward than originally anticipated, we are confident that identification and modulation of selected signalling pathways and epigenetic signatures can in time provide us with reliable ways to influence macrophage phenotype and function. To this end, we must keep in mind that while the intricacies of the macrophage heterogeneity paradigm challenge us in more ways than one, this very complexity also represents its greatest strength by creating myriad opportunity for tailoring diagnostic and therapeutic modalities in chronic vascular inflammation.
Chapter 9

References