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Chapter 6

General Conclusions

As a chronic inflammatory disease and metabolic disorder, atherosclerosis is driven by hyperlipidemia, altered vascular leukocyte homeostasis and pro-inflammatory immune responses. Atherosclerotic lesions progress as result of a shift in homeostasis towards the progressive accumulation of monocyte derived macrophages driven by increased circulatory monocyte recruitment, secretion of proinflammatory cytokines, reduced macrophage efflux and increased macrophage apoptosis with reduced disposal of death cells and secondary necrosis driven inflammation.

The studies presented in this thesis describe experiments designed to manipulate processes controlling vascular macrophage homeostasis; in order to study their influence in atherosclerotic lesion progression and phenotype in mice. The first part (Chapters 2 and 3) deals with disruption of monocyte extravasation and macrophage differentiation in atherosclerotic mice either by deficiency of Hck and Fgr (Chapter 2) or lentiviral mediated over-expression of NAMPT in hematopoietic cells (Chapter 3). The second part targets two subsets of lesion macrophages (CD115$^+$ and CD169$^+$) for systemic and local induction of apoptosis in advanced mouse atherosclerosis. The effect of these treatments was assessed in terms of atherosclerotic lesion phenotype as well as vascular and extravascular leukocyte homeostasis.

6.1 Modulation of Monocyte Migration and Macrophage Differentiation in Atherosclerosis

Extravasation is a multi-step process critical for leukocyte recruitment and accumulation of macrophages in atherosclerosis. It includes selectin mediated rolling on the endothelium, integrin dependent arrest and binding of monocytes to endothelial cells, and diapedesis (Figure 1.1, page 2). Hck and Fgr are two Src tyrosine kinases critical for cell migration, that display hematopoietic specific co-expression and have overlapping molecular functions not observable in single Hck or Fgr deficient leukocytes, due to redundancy [80].

Specific subsets of circulatory monocytes, have been demonstrated to extravasate and accumulate differentially into atherosclerotic lesions. These subsets are characterized by the display of molecular markers and genes that are differentially expressed
as consequence of the activation of gene networks, that control biological processes not shared among subsets (for more details see page 9).

Two monocyte subsets identified in hyperlipidemia-mediated atherosclerotic mice have been characterized by their differential expression of chemokine receptors (CCR2 and CX3CR1) and Ly6c, a cell surface protein involved in leukocyte migration and integrin clustering and activation [280–282].

The first subset of circulatory monocytes, commonly referred to as “resident” and denoted as CX3CR1hiCCR2−Ly6ch, is found in resting and inflamed tissues and display high expression of CX3CR1 and two integrins: LFA-1 and VLA-4. These monocytes, referred to as Ly6clo, downregulate the expression of Ly6c, CCR2 and L-selectin [62, 283].

The second subset, denoted as CX3CR1loCCR2+Ly6ch monocytes and denominated “inflammatory”, are characterized by the upregulation of Ly6c (Gr1), the chemokine receptor CCR2 and the adhesion molecule L-selectin [62, 284]. Circulatory Ly6ch monocytes are expanded in hyperlipidemic mice and are preferentially recruited to inflamed arteries, leading to the expansion of atherosclerotic lesions [58]. Ly6ch monocytes either intravasate from the bone marrow or originate from bone marrow hematopoietic stem cell precursors that intravasate in higher quantities in hyperlipidemia [64], to home in the spleen were they differentiate into Ly6ch inflammatory monocytes that emigrate to the circulation, becoming the major source of pro-inflammatory monocytes that infiltrate atherosclerotic lesions [66].

Once in the lesion, ingestion of oxLDL by inflammatory monocytes promotes their maturation into macrophages [285], and induces downregulation of CCR2 with simultaneous upregulation of the intercellular adhesion chemokine receptor CX3CR1. This switch in chemokine receptor expression induces their adhesion to various cell types that express CX3CL1, leading to macrophage trapping and skewing macrophage homeostasis towards accumulation in atherosclerotic lesions.

Deficiency of Hck and Fgr in LDLr− mice caused reduced accumulation of macrophages in atherosclerotic lesions as consequence of reduced monocyte adhesion to the endothelium and diapedesis. This, although favorable in terms of lesion size, was surprisingly accompanied by features of lesion instability such as enlarged necrotic cores and reduced collagen deposition and SMC accumulation, that suggested reduced accumulation of monocyte derived fibrocytes and/or the involvement of Hck and/or Fgr in leukocyte functions beyond cell migration. Interestingly, Hck/Fgr deficiency caused a higher Ly6ch/Ly6cl ratio in circulatory monocytes, suggesting a higher availability and infiltration of pro-inflammatory monocyte precursors in atherosclerotic lesions of Hck/Fgr double deficient chimeras, compared to control mice.

Hematopoietic upregulation of iNAMPT in contrast, caused reduced levels of circulatory Ly6ch monocytes due to poor intravasation out of the bone marrow. This concurred with reduced atherosclerotic burden and lower lesion macrophage accumulation, suggesting delayed lesion progression due to reduced availability of inflammatory monocytes.

Lesion macrophages activate specific gene networks in response to differentiation signals present in atherosclerotic lesions. Similar to monocytes, two macrophage subsets, commonly known as M1 and M2 macrophages, have been defined in–vitro based on the differential activation of antagonistic biological processes: M2 or alter-
native activated macrophages (AAM), induce processes that promote wound repair and inflammation smoldering in response to mediators such as IL–4 and IL–10. One such a process is the induction of cholesterol efflux by upregulation of PPAR\(_\gamma\) and ATP binding cassette transporters ABCA1 and ABCG1; which delay the progress of atherosclerosis by reducing foam cell formation and inflammation. Other important processes performed by M2 macrophages are the stimulation of smooth muscle cells to synthesize collagen and extracellular matrix proteins [286], the negative regulation of inflammation and inhibition of extracellular matrix degradation, by secretion of mediators such as TGF\(_\beta\) and IL–10 [287]. M2 macrophages, or their equivalent in–vivo are expected to induce the cessation of inflammation and promote the formation of fibrous caps that keep the thrombogenic contents of atherosclerotic lesions isolated from the circulation, thereby preventing the formation of thrombus that cause arterial occlusion and clinical manifestations of atherosclerosis, such as myocardial infarction, stroke and sudden death.

M1 or classically activated macrophages (CAM), deemed to be activated by proinflammatory mediators present in atherosclerosis such as IFN\(_\gamma\) and oxidized lipids [288], are in contrast detrimental for lesion stability by expression of proteases that degrade the extracellular matrix and secretion of immune mediators such as IFN\(_\gamma\) and TNFs that induce accelerated recruitment of pro-inflammatory leukocytes.

M1 and M2 macrophage markers are detectable in human atherosclerotic lesions [166,289], but M2 macrophages are predominant in early lesions while their M1 counterparts prevail in advanced atherosclerosis [290], which suggest that M2 macrophages might limit the progression of atherosclerosis in the initial stages, while M1 macrophages cause lesion expansion and vulnerability in advanced atherosclerosis, either upon differentiation from Ly6c\(_{hi}\) monocyte precursors, or after transdifferentiation from M2 macrophages [117].

Analysis of the effect of Hck/Fgr deficiency on mouse macrophage function, demonstrated that besides impairing leukocyte migration, and increasing the levels of Ly6c\(_{hi}\) monocytes in the circulation, it caused skewed M1 macrophage differentiation and reduced collagen deposition by smooth muscle cells. In agreement with these results, transcriptome analysis of gene expression in human macrophages allowed the clustering of Hck and Fgr in gene networks with different expression profiles in response to immune modulators. Interestingly, Hck clustered with genes overrepresenting cytokine-cytokine receptor interaction and chemokine signaling pathways, suggesting its preferential involvement in leukocyte extravasation and migration. Fgr in contrast, clustered with genes characteristic of macrophage M2 differentiation.

Collectively, these results suggest that although Hck and Fgr play overlapping molecular functions critical for leukocyte migration, which makes necessary the use of double deficiency or inhibition approaches to avoid compensation [80, 80], they might be differentially required for activation of macrophage M1 and M2 responses. Disrupted M2 type macrophage responses in double deficient Hck\(^{-/-}\)Fgr\(^{-/-}\)LDLr\(^{-/-}\) mouse chimeras likely caused the vulnerable lesion phenotype observed.

In remarkable contrast, over–expression of NAMPT led to features of lesion stabilization in LDLr\(^{-/-}\) atherosclerotic mice by skewed M2 differentiation, with concomitant downregulation of M1 proinflammatory cytokines, reduced intracellular accumulation of cholesterol esters and free cholesterol and increased expression of ATP binding cassette transporters ABCA1 and ABCG1. In fact, NAMPT inhibited
the proinflammatory activation of macrophages in atherosclerosis and positively regulated their alternative polarization by upregulation and activation of PPARγ, SIRT1, ABCA1 and ABCG1, which are known to modulate macrophage differentiation.

In addition, over-expression of NAMPT acted cytoprotective in response to ox-LDL pro-apoptotic stimuli, indicating that over-expression of NAMPT in mouse atherosclerosis has pleiotropic anti-atherosclerotic effects targeting different molecular pathways.

Taken together, the results obtained in atherosclerotic mice chimeras deficient in Hck/Fgr or over-expressing iNAMPT, complement to indicate that reducing Ly6c\textsuperscript{hi} to Ly6c\textsuperscript{lo} subset balance and skewing macrophage differentiation towards an anti-inflammatory and wound healing phenotype might be promising to reduce and stabilize atherosclerotic lesions. However, the crucial role of inflammatory monocytes and CAM in host defense imposes that these strategies are designed without interfering with systemic innate immunity and host defense.

6.2 Induction of Macrophage Apoptosis and Its Effect on Atherosclerosis

An increasing body of evidence from both animal models and human studies suggests that programmed cell death in the form of apoptosis or necrosis [49], are major events in the pathophysiology of atherosclerosis, and determinant of the phenotype adopted by lesion macrophages. In fact, macrophages accumulate in the vicinity of ruptured lesions and correlate with the formation of necrotic cores [291] and the progression of lesions towards vulnerability [292, 293].

In addition to increased apoptosis inside atherosclerotic lesions, M1 and M2 macrophages display reduced chemotaxis in response to modified lipids. Then, extracellular environment rich in oxidized cholesterol and proinflammatory cytokines present in atherosclerotic lesions, not only promotes monocyte recruitment and differentiation into M1 macrophages but also limits their exit out of lesions [68], induces their death and impairs their phagocytic capacity, skewing all their responses towards their accumulation, the degradation of their extracellular matrix and the induction of lesion rupture, ultimately leading to thrombus formation and arterial occlusion.

Deficiency of anti-apoptotic genes during atherosclerosis onset and progression has a biphasic effect in macrophage homeostasis in atherosclerosis, promoting cell turnover and reduced atherosclerosis when coupled to prompt efferocytosis, mainly in early lesions [248, 294]; but inducing accelerated lesion expansion and instability when non phagocytosed apoptotic cells loose the integrity of their membrane, leaking proinflammatory, lytic and thrombogenic intracellular contents that induce monocyte recruitment, fibrous cap degradation and thrombus formation in advanced atherosclerosis [252–254, 295]. Conversely, protection from apoptosis in atherosclerosis causes early lesion expansion [250, 251] but reduces atherosclerosis progression into advance stages [250, 251, 255, 296].

Collectively, these results have led to the generalized idea that macrophage apoptosis has a biphasic effect in atherosclerosis, which suggest that skewing the balance of prosurvival and proapoptotic genes in atherosclerosis would be inappropriate to treat patients normally having lesions of all stages at any given time [297]. However, pharmacological (reviewed by Martinet et al. [256]) and suicide gene mediated induction
of macrophage apoptosis [25, 225] have afforded contradictory results regarding the impact of macrophage ablation on atherosclerosis, likely due to site and cell related off target effects.

In fact, systemic induction of CD11b$^+$ myelocyte apoptosis has no effect on lesion size and composition, despite inducing intra-lesion macrophage apoptosis. The reason for this paradoxical result is that induction of CD11b$^+$ myelocyte apoptosis causes massive depletion of circulatory monocytes thereby reducing the impact of intra-lesion macrophage apoptosis on vascular inflammation and lesion expansion. Systemic induction of CD11c$^+$ myelocyte apoptosis in contrast, does not deplete circulatory monocytes and leads to lesion expansion and higher counts of lesion apoptotic cells in atherosclerotic mice. However, no reduction in CD68$^+$ lesion macrophage contents is observed [25] upon induction of CD11c$^+$ myelocyte apoptosis in atherosclerotic mice, which however display increased plasma cholesterol levels in response to dendritic cell ablation, making difficult to discriminate the contribution of macrophage apoptosis to the effects observed [23, 25].

In remarkable contrast with these two studies, induction of macrophage apoptosis using suicide genes expressed in CD169$^+$ or CD115$^+$ cells, as described in this thesis (Chapter 4 and 5) permitted the ablation of lesion macrophages without off target effects, either because the promoter used was highly specific for macrophages as in the case of CD169–DTR–LDLr$^{−/−}$ chimeras (Chapter, 4) or because the induction of CD115$^+$ myelocyte apoptosis was restricted to atherosclerotic lesions by local delivery of an inducer of apoptosis (Chapter 5).

Analysis of atherosclerotic lesions following apoptosis induction and after a recovery period, permitted the evaluation of the impact of macrophage ablation on atherosclerosis and its dynamics after cessation of treatment. In addition, by comparison with the local experimental setup, systemic induction of CD115$^+$ myelocyte apoptosis permitted discrimination of the effect of induction of myelocyte apoptosis on peripheral leukocyte homeostasis, and its influence back in atherosclerotic lesions bearing higher CD115$^+$ myelocyte apoptosis.

Systemic induction of macrophage apoptosis using both CD169–DTR–LDLr$^{−/−}$ chimeras and ApoE$^{−/−}$ MaFIA mice, caused reduced macrophage contents with concomitant increase in lesion apoptosis. In addition, lesion confined CD115$^+$ myelocyte apoptosis in ApoE$^{−/−}$ MaFIA$^{+/−}$ mice (Chapter 5), and systemic induction of CD169$^+$ macrophage apoptosis (Chapter 4) in LDLr$^{−/−}$ chimeras, caused atherosclerotic plaque expansion likely due to local proliferation or increased inflammation under conditions of augmented intra-lesion macrophage apoptosis. Furthermore, induction of CD169$^+$ macrophage apoptosis in CD169–DTR–LDLr$^{−/−}$ mice led to necrotic core expansion; likely due to overload of the efferocytosis capacity of remaining phagocytes. However, necrotic cores from ApoE$^{−/−}$ MaFIA$^{+/−}$ mice, used for induction of CD115$^+$ macrophage apoptosis, were not expanded, compared to ApoE$^{−/−}$ MaFIA$^{−/−}$ controls.

Importantly, no changes in lesion composition nor features of lesion vulnerability were observed upon local induction of CD115$^+$ myelocyte apoptosis. In contrast, systemic induction of CD115$^+$ myelocyte apoptosis not only caused macrophage turnover and increased apoptosis in atherosclerotic lesions but also induced features of lesion vulnerability not observed in the local application. Simultaneously, systemic induction of CD115$^+$ myelocyte apoptosis in ApoE$^{−/−}$, atherosclerosis prone mice, caused en-
hanced extramedullary myelopoiesis, proinflammatory Ly6c$^{hi}$ monocytosis, depletion of spleen dendritic cells and skewed pro-inflammatory macrophage differentiation.

Systemic induction of CD169$^+$ macrophage apoptosis caused lesion vulnerability, however a remarkable recovery was observed two weeks after cessation of apoptosis induction, when no differences in lesion size and necrotic core contents were observed between mice receiving diphtheria toxin to induce CD169$^+$ macrophage ablation, and diphtheria-toxin-mutant-receiving controls. In addition, induction of apoptosis in CD169–DTR–LDLr$^{-/-}$ mice caused transient elevation of pro-inflammatory plasma cytokines and Ly6c$^{hi}$ monocytosis, that normalized giving way to lesion stabilization, higher contents of VSMC and increased deposition of collagen and thicker fibrous caps.

Taken together, the experiments presented in Chapter 4 and 5 are the first to address the dynamic response that follows induction of macrophage apoptosis in atherosclerosis and segregate local from systemic effects of this intervention.

By comparison of the results obtained upon systemic and local induction of CD115$^+$ myelocyte apoptosis, and considering the dynamics of recovery following induction of CD169$^+$ macrophage apoptosis, the results presented in this section indicate that simultaneous manipulation of systemic leukocyte homeostasis and acute induction of macrophage apoptosis, might be used to achieve macrophage turnover with concurrent atherosclerotic lesion stabilization.

### 6.3 Concluding Remarks and Future Perspectives

The onset and progression of atherosclerosis is the result of the interplay of thousands of molecules in multiple interacting cells types, that contribute differently in various organs and tissues throughout the body.

This thesis addressed multiple mechanisms that control intra and extravascular leukocyte homeostasis, ultimately dictating the fate of atherosclerotic lesions, at the balance between macrophage accumulation and lesion vulnerability versus resolution of inflammation and wound healing.

The first part of this thesis demonstrated that targeting a single gene, or a combination of redundant genes, with identified molecular function, resulted in multiple disturbances to different processes important in atherosclerosis. As such, hematopoietic deficiency of Hck and Fgr was demonstrated to reduce monocyte diapedesis and skew monocyte and macrophage differentiation towards inflammation. Over-expression of iNAMPT in turn caused impaired intravasation of inflammatory monocytes, thereby reducing their availability to migrate into atherosclerotic lesions, while simultaneously activating multiple pathways that promoted macrophage survival, resolution of inflammation and cholesterol efflux.

The second part of this thesis indicated that the impact of acute induction of macrophage apoptosis in atherosclerosis is highly dynamic and dependent on peripheral as well as intra-lesion leukocyte homeostasis. As such, induction of CD169$^+$ macrophage apoptosis exerts a response characterized by transient extramedullary myelopoiesis, monocytosis and atherosclerotic lesions that change their phenotype over time, from lesion vulnerability upon induction of apoptosis, to stabilization upon recovery. Similarly, systemic and local induction of CD115$^+$ myelocyte apoptosis cause accumulation of apoptotic cells and macrophage turnover in atherosclerotic lesions. However,
systemic induction of CD115$^+$ cell apoptosis leads to features of lesion vulnerability, extramedullar myelopoiesis and altered circulatory and spleen leukocyte homeostasis, not observed in the local application.

As a whole, these results highlight the interplay of different immune system compartments in atherosclerosis and place emphasis on the participation of genes in pleiotropic and antagonistic biological processes due to their ability to cooperate their molecular functions in networks, whose spatio-temporal regulation and function proceed beyond single molecular functions of particular genes.

The future direction of the research in atherosclerosis must therefore integrate data from different levels of complexity and take advantage of the latest technologies to develop improved diagnostics and therapeutic solutions for atherosclerosis, the number one cause of death in the world.