Neutrophils: emerging role in the immunopathology of atherosclerosis
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General discussion
CHAPTER 8
Neutrophils are professional phagocytes and play an important role in innate immunity as the first line-defense during infection. By the release of their granule proteins and ROS, they mediate both anti-microbial and immunopathological activity by communicating with cells of the adaptive immune system. Under normal conditions, neutrophils undergo apoptosis after performing their action. These apoptotic neutrophils are ingested by macrophages and this process leads to a switch from a pro- to an anti-inflammatory macrophage phenotype promoting resolution of inflammation. But if it comes to a dysfunction in the regulation of neutrophil production, release, apoptosis and clearance it can lead to exacerbated chronic inflammation and tissue damage. Atherosclerosis for instance is known as an unresolved inflammation, lacking the switch from pro- to anti-inflammatory status (1). This disease is characterized by an unbalanced lipid metabolism and an enhanced infiltration of immune cells throughout all stages. Monocytes and macrophages have been demonstrated to play a crucial role in atherosclerosis progression and over the preceding years also neutrophils have gained importance in atherosclerosis.

Given the expansion of neutrophils in myeloproliferative disease, we analyzed the lesion formation in interferon regulatory factor 8 (Irf8\(^{-/-}\)) deficient mice (Chapter 2). IRF8 is a transcription factor, which is exclusively expressed in hematopoietic cells and has been demonstrated to be involved in granulocytic differentiation and maturation (2-4). Besides the striking expansion of neutrophils, these mice exhibit reduced monocyte counts in the bone marrow and circulation, which gave us the opportunity to primarily analyze the impact of neutrophils. We could demonstrate that the expansion in plaque size was due to the increased amount of functionally intact neutrophils as the effect could be abolished by specific neutrophil depletion. Neutrophils act as accelerators of early monocyte recruitment to the arterial wall via release of various granule proteins. For instance, the release of azurocidin, which is depositioned on the endothelium promotes firm monocyte adhesion (5, 6). Furthermore, the discharge of proteinase-3 and \(\alpha\)-defensins stimulate the endothelial cells to express CCL2, which amplifies the monocyte recruitment (7). This exacerbated efficiency in monocyte recruitment was demonstrated also in this study (Chapter 2). Although the monocyte counts were decreased in Irf8\(^{-/-}\) mice, the macrophage amount within the plaque did not differ compared to the controls demonstrating the highly efficient recruitment of monocytes by neutrophils.
Besides neutrophilia, hypercholesterolemia also induces monocytosis and has been described as a crucial step in the development of atherosclerosis (8). Monocytes display at least two functionally different subsets in human and mice termed classical (inflammatory, Gr1+) and non-classical (resident, Gr1-) monocytes. Although this heterogeneity has been extensively described, the dissection of the role of either subset in atherosclerosis remains unclear, which and is mainly due to technical limitations. While for instance neutrophils can be specifically depleted, this approach is not feasible for monocyte subsets. Here, for the first time we propose an elegant design to investigate the role of each subset (Chapter 3). To distinguish between the two types, absolute leukopenia was induced in Apoε−/− mice by the cytostatic drug cyclophosphamide. Thereafter, reconstitution with either classical or non-classical monocytes was performed to separate between the impact of either monocyte subset. It was clearly shown that Gr1+ rather than Gr1- monocytes were key players in the progression of atherosclerosis. Gr1+ monocytes accumulate at inflammatory sites where they undergo differentiation to M1 macrophages characterized by enhanced secretion of pro-inflammatory cytokines and mediators (IFN-γ and TNF). In contrast, M2 macrophages have been suggested to be involved in resolution of inflammation (summarized in (9)). The recruitment of monocytes is regulated by chemokine interaction with their respective receptor. Thus we were interested in the better understanding of the chemokine-driven mechanism underlying monocytes entry into atherosclerotic lesions (Chapter 3). As first analysis, lesion formation was assessed in mice deficient for the receptors CCR1, CCR2, CCR5 and CX3CXR1, which have been described in the context with atherosclerosis (10). Additionally, counts of aortic Gr1+ monocytes and peripheral inflammatory cell content were taken into account. The results of this study clearly demonstrated the significant involvement of CCR1 and CCR5 but not CCR2 or CX3CXR1 during arterial accumulation (Chapter 3). It seems more likely that CCR2 has an impact on monocyte mobilization under inflammatory conditions (11, 12). On the other hand, CX3CXR1 has an influence on the survival of lesional macrophages (13, 14).

As monocytes undergo differentiation into macrophages, these macrophages may become foam cells as a consequence of the uptake of oxLDL (15). Neutrophil-borne α-defensins can promote this process. In this context it has been shown that macrophages produce nitrotyrosine in presence of α-defensins, which contributes to oxidization of LDL and in addition it
upregulates the expression of the scavenger receptors CD36 and CD68 (6). In line, a reduced uptake of oxLDL by functionally impaired macrophages and increased accumulation of extracellular lipid deposits in plaques have been detected in the Irf8-/- mice (Chapter 2). Besides granule proteins, neutrophils produce and release a large amount of ROS via MPO, lipoxygenases and NADPH oxidase. The interaction of the neutrophil derived MPO and the mannose receptor of macrophages leads to release of ROS as well as pro-inflammatory cytokines (e.g. TNF-α, IL-1, IL-6, IL-8, GM-CSF) contributing to increased inflammation within the lesion. Furthermore, ROS release by neutrophils or activated macrophages promotes oxidization of LDL promoting foam cell formation (16). These foam cells induce additional recruitment of inflammatory cells via secretion of inflammatory mediators leading to formation of fatty streaks.

The progression from fatty streaks to a more complex lesional phenotype involves the migration of SMCs from the media to the intima. SMCs receive cytokines and growth factors from the endothelium to migrate and proliferate (17). SMCs start to synthesize ECM macromolecules and begin to cover the fatty streak, leading to the formation of the FC covering the necrotic core (NC), which consists of necrotic cells. Neutrophils are considered as short-lived phagocytes undergoing rapid apoptosis. For late stages of atherosclerosis it has been demonstrated that efferocytosis of apoptotic cells via macrophages is impaired (18). If not cleared, apoptotic neutrophils undergo secondary necrosis and release their cell content. This leads to tissue damage and contributes to the necrotic core enlargement and increased inflammation in the plaque. The apoptosis rate of blood or migrated neutrophils was not different in the Irf8-/- mice compared to the control mice (Chapter 2). However, due to the impaired efferocytosis capacity of the macrophages with numerous dying neutrophils in Irf8-/-Apoe-/- mice, the plaque phenotype was more pro-inflammatory and displayed enhanced NCs.

Neutrophils have been described to undergo different cell death pathways such as NETosis, autophagy, apoptosis and necrosis. Döring et al. demonstrated the presence of Cramp/NET complexes in mouse plaque and showed that these trigger further activation of pDC and hereby driving atherogenesis in mice (19, 20). On the other hand, autophagy is looked upon as an anti-inflammatory cell death pathway and considered to maintain cell homeostasis (21). Interestingly, recently it has been shown that autophagy may promote NETosis (22). Our study demonstrates for the first time an analysis of the different cell deaths
neutrophils may undergo within atherosclerotic lesions (Chapter 4). In human carotid endarterectomy samples an increase of NETting neutrophils was detected with advanced stage of atherosclerosis. Although increased autophagy positive neutrophils were found, a correlation between neutrophils undergoing autophagy and NETosis could not be identified (Chapter 4). Of note, the majority of the lesional human neutrophils was not positive for any of the tested cell death pathways and was considered as living. Given the short life span of the neutrophils this would point towards the high turnover of these cells with frequent de novo recruitment into the lesion. Thus, despite the low presence of neutrophils within the plaque, living or dead neutrophils could shape plaque destabilization.

Rupture-prone plaques are characterized by high inflammatory cell content, an enlarged NC covered by a thin FC. Lesional neutrophil counts were strongly associated with histopathologic features of rupture-prone plaques (23). Interestingly, in the here presented study only counts of dead neutrophils seem to correlate with the FC thinning (Chapter 4). Numerous factors promote the mechanical weakening of the FC by degradation of the ECM. Thus, neutrophils may contribute with various components as a result of controlled release or due to necrosis. Besides macrophages neutrophils are an important source of MMPs, which have been shown to promote SMC apoptosis due to their degradation of the ECM and the cell-to-cell interaction (24). In addition, neutrophil-derived MMPs cleave CXCL1, CXCL5, CXCL6 and CXCL8 to enhance their chemotactic activity, leading to enhanced recruitment of immune cells (25, 26).

Structural damage in the FC results in the exposure of thrombogenic material of the plaque to the blood, leading to thrombus formation and occlusion of the artery (27). Albeit the clinical relevance, the understanding of the process underlying the conversion from a stable into an unstable plaque remains unclear. Various mouse models of generating vulnerable plaques have been proposed based on diverse strategies such as genetic modifications, surgical techniques or combination of both in genetically-modified hypercholesterolemic mouse models (reviewed in (28)). These models describe the formation of advanced atheromas representing different signs of plaque instability as for instance NC expansion, FC thinning and an inflammatory phenotype (29).

Additionally, destabilizing features such as neoangiogenesis or intraplaque hemorrhages (30-32) are eventually detected while others such as plaque disruption with superimposed thrombosis can hardly be identified (31,
Indeed, it is well accepted that mouse atherosclerotic plaques only partly resemble the characteristics observed in human advanced lesions. However, a consensus model of plaque destabilization is still lacking. Hence, we performed a comparative study where analyzed different surgical approaches to induce vulnerable plaques in mice. Thus, we applied two models in Apoe/− mice of local shear stress alteration based on the partial ligation of and/or the implantation of a shear stress modifier device (cast) in the left common carotid artery (Chapter 5). Additionally, we induced endogenous renovascular hypertension through partial ligation of the left renal artery and furthermore examined the effect of hypercholesterolemia on plaque development. It is worth mentioning, that all models displayed different instability traits with human vulnerable atherosclerotic plaques as previously described. Collectively, we observed that the model based on the cast placement under hypercholesterolemia exhibited the highest incidence of vulnerable plaques with less variability between the analyzed specimens, although no intraplaque hemorrhages were detected.

Plaque rupture is the final event how atherosclerosis may cause acute vascular complications such as myocardial infarction and stroke. In this context, involvement of platelets has been demonstrated as they as well as the coagulation cascade become activated and contribute to thrombus formation (35, 36). The contribution of platelets in the progression of atherosclerosis is however not limited to the late stages - they are in fact important throughout all phases as summarized in (37). Furthermore, delayed neutrophil apoptosis has been evidenced in patients with acute myocardial infarction (38, 39) and hence may be a mechanism of facilitated neutrophil accumulation. Thus, we were interested in the interaction of activated platelets and neutrophils. Here, we identified that the platelet-released chemokine PF4 (platelet factor 4) is crucial in regulating neutrophil apoptosis (Chapter 6). Activated platelets release proteins from their α-granules (e.g. CCL5, serotonin, TGFβ), of which only PF4 could delay neutrophil apoptosis in vitro. This effect was confirmed in vivo performing a hind limb ischemia as arterial occlusion model in combination with or without platelet depletion. In this setup, the absence of platelets increased neutrophil. To identify the specific influence of PF4 on the delayed neutrophil apoptosis, we injected an anti-PF4 antibody prior to ligation of the femoral artery. In comparison to the control a significant increase of apoptotic neutrophils in the circulation was observed. These findings demonstrate the delaying effect of platelets on neutrophil cell death.
Apoptosis of neutrophils represents a control mechanism limiting the damaging potential of these cells. In fact, induction of neutrophil apoptosis has been proven to be beneficial in acute inflammation in various mouse models (28). Besides interference with neutrophil apoptosis targeting of neutrophil activation and recruitment might be a possibility of therapeutic intervention.

**Overall conclusion**

This thesis demonstrates the crucial role of neutrophils in the onset and progression of atherosclerosis. Although neutrophils represent just a small population within the plaque when compared to macrophages, they have to be considered as important promoters of lesion formation. The work presented here demonstrates that increased neutrophil production correlates with the pro-inflammatory phenotype and enlargement of lesions. Future studies may be important in controlling neutrophil mobilization and recruitment as interference in the chemokine-receptor axes would be a possibility.

Neutrophil death is central to resolution. However, the lifespan of neutrophils during atherosclerosis is delayed, which we identified is mediated by the chemokine PF4 released by activated platelets. On this note, strategies aiming at the promotion of neutrophil apoptosis could be of high interest. R-roscovitine has been demonstrated to promote neutrophil apoptosis, leading to resolution in a wide range of inflammatory models (41-44). Furthermore, Knight et al. described that the PAD4 inhibitor Cl-amidine prevents NETosis (45). However, it has to be considered that inducing neutrophil apoptosis for a long period may lead to innate immune response. Possible solution would be eventually to choose specific molecules inducing the delay of neutrophil cell death.

Finally we establish a mouse model that allows studying the impact of neutrophils on vulnerable plaque progression. Thus, the comparative study of the vulnerable plaque mouse models can set the basis to study neutrophil targeted treatment, such as inhibition of neutrophil production, recruitment, and degranulation. Hence, findings from this thesis may open new strategies and therapeutic approaches for the future.
References


