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## **Modulation of leukocyte homeostasis in atherosclerosis**

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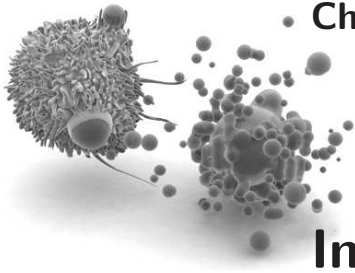


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## • Chapter 1

# Introduction

## 1.1 Atherosclerosis: Mortality, Morbidity and Costs

With improved public health and increasing life spans, cardio-vascular disease (CVD) has become an important problem and burden to the society as it is the number one cause of death in the world [1]. In 2008, 17 million people died from CVD, accounting for 30% of global deaths [1]. CVD is projected to remain the single most important cause of global death by 2030, when 25 million people are expected to die from it [1].

Without exception, CVD is the leading cause of death in the United States [2] and Europe [3]. In the Netherlands, CVD is the second most important cause of death, comprising 29.2% of total deaths, which is exceeded only by diverse types of cancer that together account for 31% of the national mortality [4].

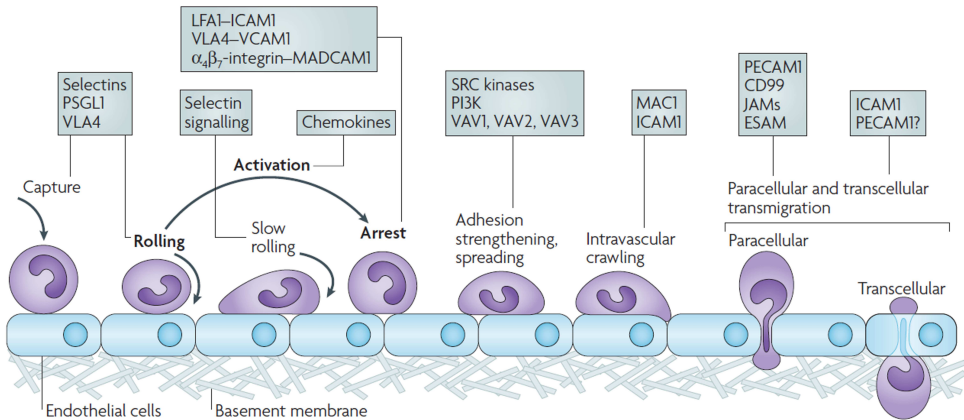
Besides causing a high mortality, CVD also represents a major economic burden on health care systems in terms of direct (hospitalizations, rehabilitation services, medications) and indirect (losses of productivity due to premature mortality and short- or long-term disability) costs that account for more expenditures than any other major diagnostic group in the United States [2]. In fact, the costs linked to heart failure in the United States are expected to more than double within the next two decades going from 21 to 53 billion dollars per year, as the population ages and treatments help patients live longer. If indirect costs related to heart failure, such as lost productivity and wages, are included in this projection, the total costs will go from 31 to 70 billion [5].

## 1.2 Atherosclerosis: Onset and Development

Atherosclerosis is the underlying cause of CVD [2] and therefore the principal target for therapeutic intervention. Atherosclerosis is a chronic inflammatory disease and metabolic disorder characterized by hyperlipidemia and altered leukocyte homeostasis.

During the asymptomatic stages of atherosclerosis, dysfunctional and activated endothelial cells allow the formation of fat deposits in the vasculature upon diffusion of cholesterol lipids to the subendothelial space. This leads them to secrete chemotactic cytokines that attract monocytes to extravasate in a multi-step process that

starts with integrin dependent tight adhesion and rolling on the endothelium, followed by transmigration across the endothelial lining and adhesion to other cells and the extracellular matrix present in the vascular interstitium (Fig. 1.1).



**Figure. 1.1.** *Leukocyte extravasation in atherosclerosis. Schematic representation of the diapedesis process that starts with leukocyte capture on the endothelial surface, followed by selectin mediated slow rolling, chemokine dependent activation and integrin arrest. Activation of Src kinases allows the cell stronger adhesion to the endothelium in anticipation to intra-luminal crawling, transmigration across the endothelial cells and passage across the basement membrane. Key molecules involved in each step are indicated in boxes. ESAM, Endothelial-cell Selective Adhesion Molecule; ICAM1, Inter-Cellular Adhesion Molecule-1; JAM, Junctional Adhesion Molecule; LFA1, Lymphocyte Function Associated Antigen-1 (also known as  $\alpha$ -1 $\beta$ -2-Integrin); MAC1, Macrophage Antigen-1; MADCAM1, Mucosal Vascular Addressin Cell Adhesion Molecule-1; PSGL1, P-Selectin Glycoprotein Ligand-1; PECAM1, Platelet/Endothelial Cell Adhesion Molecule-1; PI3K, Phosphoinositide-3-Kinase; VCAM1, Vascular Cell Adhesion Molecule-1; VLA4, Very Late Antigen-4 (also known as  $\alpha$ -4 $\beta$ -1-Integrin). Adapted from [6].*

### 1.3 Altered Leukocyte Homeostasis in Atherosclerosis

Monocyte extravasation and differentiation drive a slow accumulation of monocyte derived macrophages that scavenge and ingest oxidized cholesterol (Fig. 1.2), becoming the most prominent cell type at all stages of the disease, although multiple leukocyte subsets are recruited to the lesion and have been demonstrated to contribute to atherosclerosis onset and development [7].

The development and progression of atherosclerosis not only proceeds locally, inside lesions, but is also characterized by altered peripheral leukocyte homeostasis. In fact, a higher prevalence of leukocytosis has been shown to occur in patients with chronic coronary artery disease (CAD) [8]. In addition, evidence is accumulating that hyperlipidemia induces monocytosis and neutrophilia [9–11] and primes circulating

neutrophils to infiltrate early atherosclerotic lesions to subsequently recruit and modulate the function of inflammatory monocytes [12]. As result, a positive correlation exist between peripheral blood PMN cells count and acute coronary events in atherosclerotic patients [13,14]. Similarly, clinical data has shown that human monocytosis is an independent risk factor for coronary artery disease [8].

Dendritic cells (DC) also display altered peripheral homeostasis in atherosclerosis. In fact, reduced counts of circulating myeloid and plasmacytoid dendritic cells (mDC and pDC, respectively) have been observed in patients with atherosclerotic CAD [15,16]; which correlates with its clinical manifestations [17–19]. Furthermore, a stage-dependent decrease in the number of circulating mDCs and pDC precursors is an independent predictor of the requirement for percutaneous coronary intervention and artery bypass graft in patients with stable coronary artery disease [20]. But beyond the inverse correlation of circulatory dendritic cells counts in CAD, these cells have the potential to modulate T and B cell dependent adaptive immune responses to modified lipids and self-antigens present in atherosclerotic lesions [21,22], and have been demonstrated to regulate plasma cholesterol levels [23] and lipid metabolism [24]. Hence, induction of CD11c<sup>+</sup> dendritic cell apoptosis results in increased atherosclerotic lesion burden [25].

Recent studies indicate that the function of DC in atherosclerosis depends on their origin, maturation stage and differentiation phenotype. In particular, immature DC that lack sufficient exposure to co-stimulatory cytokines acquire tolerogenic properties and induce Treg activation, which in turn inhibits effector T cells and therefore suppresses adaptive immune responses [26]. Hence, interfering with TLR activation of conventional DC in CD11c–MyD88 deficient mice [27] and interrupting monocyte-independent Flt3L driven DC maturation [28] leads to increased atherosclerosis and monocyte extravasation by loss of Treg mediated suppression of inflammation. Conversely, oral administration of calcitriol [29] or injection of DC previously pulsed with atherosclerotic antigens such as oxidized Low Density Lipoproteins (oxLDL) [30,31] or immunoregulatory cytokines such as IL-10 in combination with the protein moiety of LDL, apolipoprotein B100 (ApoB100) [32], causes reduced atherosclerotic lesion burden and macrophage accumulation by dendritic cell mediated induction of immune regulation, highlighting the importance of dendritic cells for monocyte extravasation and macrophage homeostasis in atherosclerosis.

Simultaneously, studies where pro-inflammatory DC functions have been disabled turned out to decrease atherosclerosis, suggesting a major role of these cells in activation of proatherogenic effector T cells [33–35]. In addition, blockage of dendritic cell and T lymphocyte migration to secondary lymph nodes causes their accumulation in atherosclerotic lesions, thereby reducing macrophage accumulation and delaying atherosclerotic lesion expansion [36]; which indicates that the egress of DC and T cells from inflamed vascular lesions into the draining lymphatics is essential for the generation of adaptive immune responses that promote lesion progression [36].

The role of macrophages in atherosclerosis is highly dependent on the extracellular environment present in atherosclerotic lesions, that evolve through stages from fatty streaks with few leukocytes, to advanced plaques rich in macrophages and characterized by the accumulation of apoptotic and necrotic cells surrounding lipid pools filled with necrotic debris. As such, the main function of macrophages during the earlier stages of atherosclerosis is the ingestion of modified cholesterol. This, although

temporally beneficial, inhibits their migration [37] and causes the upregulation of adhesion molecules, pro-inflammatory cytokines and growth factors. Atherosclerotic lesion macrophages become therefore trapped in an extra-cellular environment that promotes their proliferation and hinders their getaway out of lesions [38].

## 1.4 Vascular Remodeling and Atherosclerotic Lesion Destabilization

As the lesion progresses, the accumulation of cytotoxic molecules induces macrophage death in a vicious circle that promotes lesion expansion by further recruitment of monocytes and accumulation of monocyte derived macrophages. Tertiary lymphoid organs form outside the lesions [39] and Vascular Smooth Muscle Cells (VSMC) proliferate and migrate from the media to the intima in response to macrophage signals (Fig. 1.3). Once in the intima, lesion smooth muscle cells (SMC) change their contractile phenotype into a synthetic one, active in extracellular matrix protein deposition and formation of fibrous caps that stabilize the lesion keeping its contents isolated from the blood stream (Fig. 1.3).

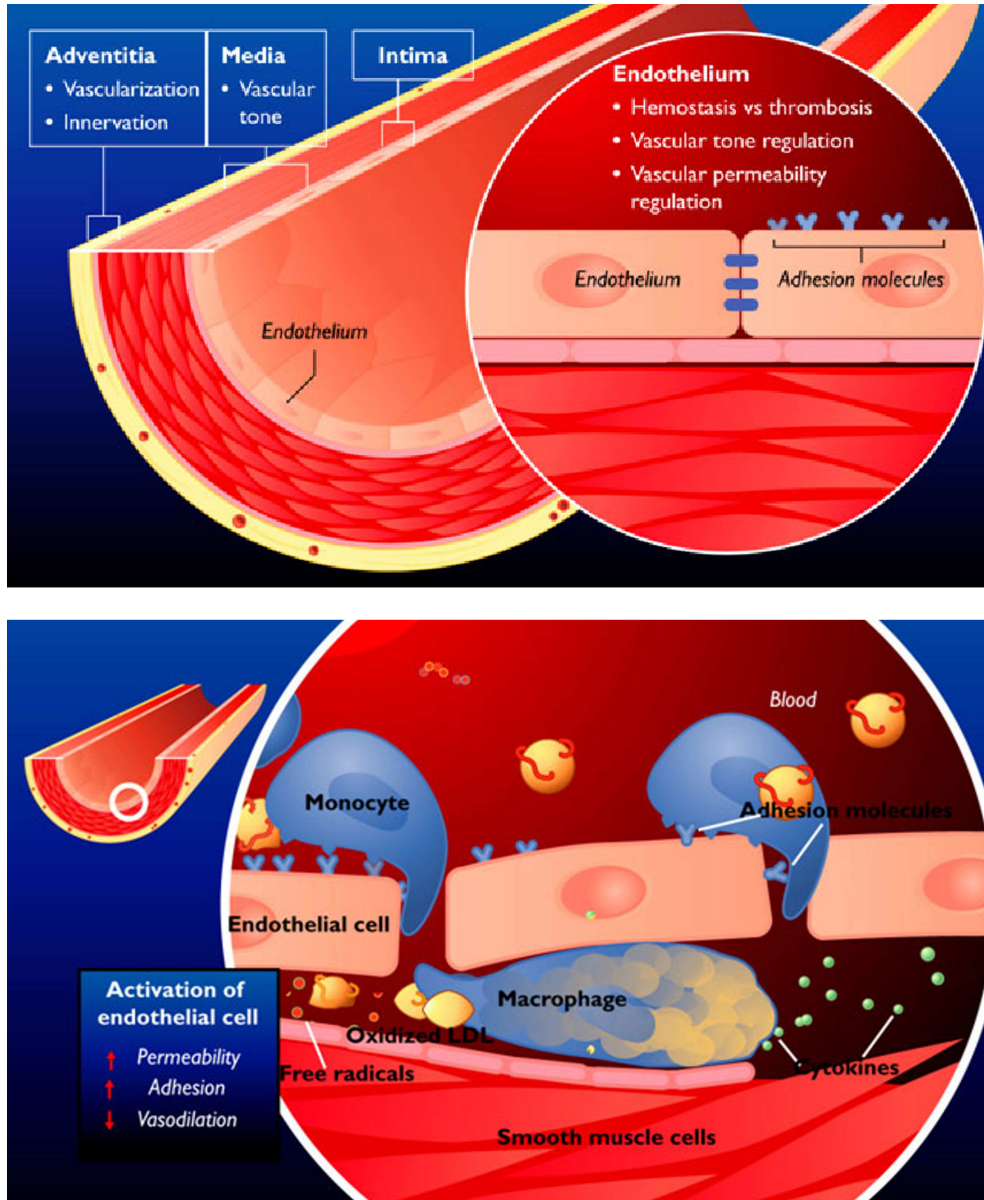
Arterial enlargement and fibrous cap formation prevent early detection and treatment of atherosclerosis, keeping it silent for decades [41, 42]. The progressive accumulation of macrophages along with the formation of fibrous caps, is accompanied by outward expansion of the artery to accommodate the growing plaque and normalize the flow of blood, that would otherwise present disturbed fluid dynamics, causing excessive shear stress on the endothelium. This natural compensation is initially beneficial, as it prevents stenosis and ischemia, keeping the disease asymptomatic up to its end stage [43].

Arterial remodeling however contributes to high mortality rates in asymptomatic individuals; in fact fifty percent of men and 64% of women who die suddenly of CAD in the United States had no previous symptoms of atherosclerosis [2]. Those percentages are in the Netherlands 55% and 52% for men and women, respectively [4]. Atherosclerosis is therefore considered a silent killer that starts early in life and progresses over time.

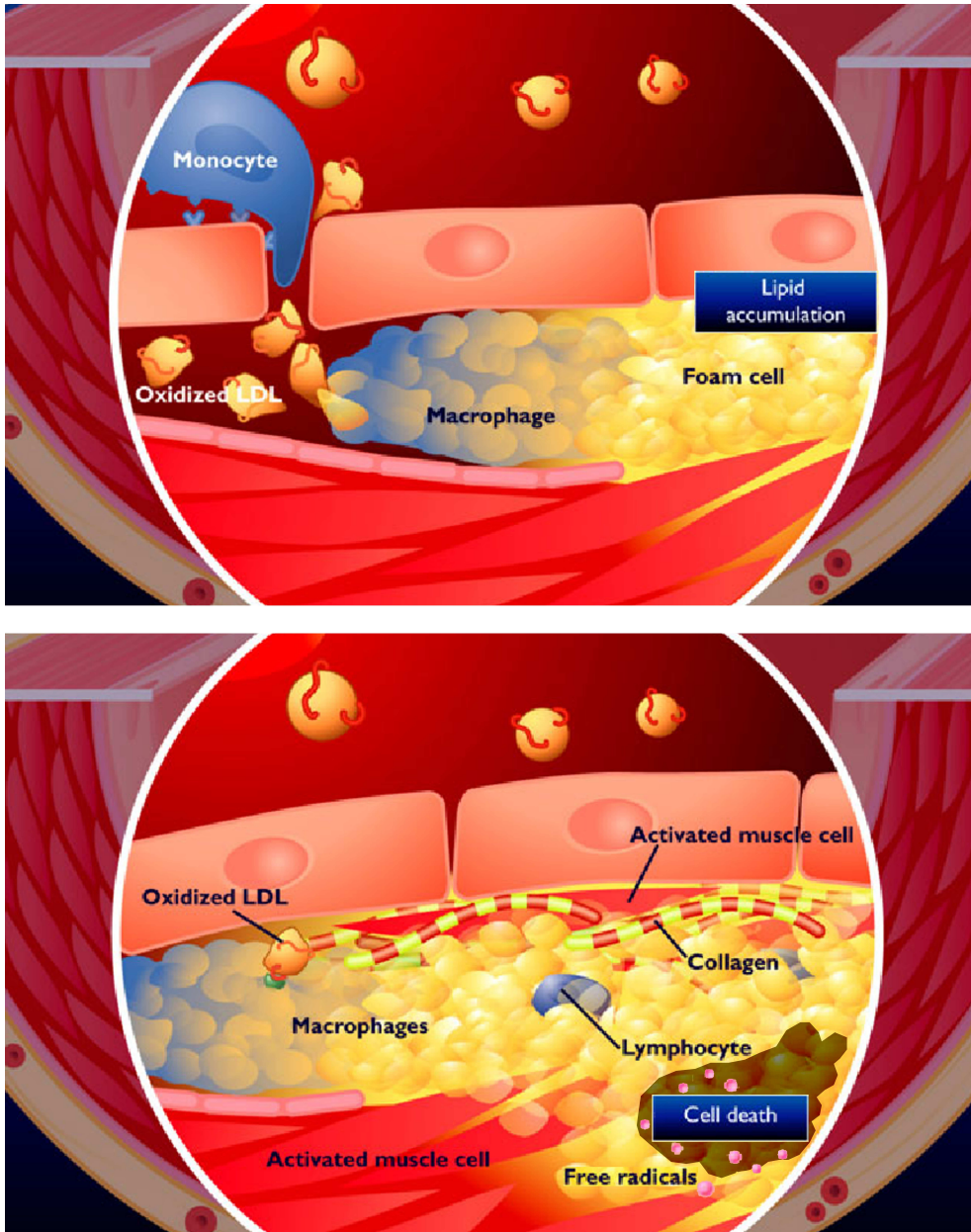
## 1.5 Macrophage Apoptosis and Lesion Destabilization

The progression from stable to vulnerable and clinically relevant atherosclerotic lesions is characterized by accumulation of apoptotic macrophages and formation of necrotic cores that cause erosion and rupture of fibrous caps [44] (Fig. 1.4). In fact, macrophage apoptosis is significantly higher at sites of plaque rupture and thrombosis in patients with sudden coronary death [45]. In addition, macrophage apoptosis causes secondary necrosis [46] which together with non-post-apoptotic programmed necrosis [47, 48] cause the formation of necrotic cores [49, 50], characteristic of advanced human atherosclerotic lesions [51].

The accumulation of apoptotic macrophages and formation of necrotic cores has three important consequences for macrophage homeostasis and atherosclerotic lesion vulnerability: First is the induction of lesion macrophage differentiation towards a proinflammatory phenotype active in inducing lesion vulnerability (Fig. 1.5). Second, the increase in lesion burden by recruitment of circulatory monocytes and

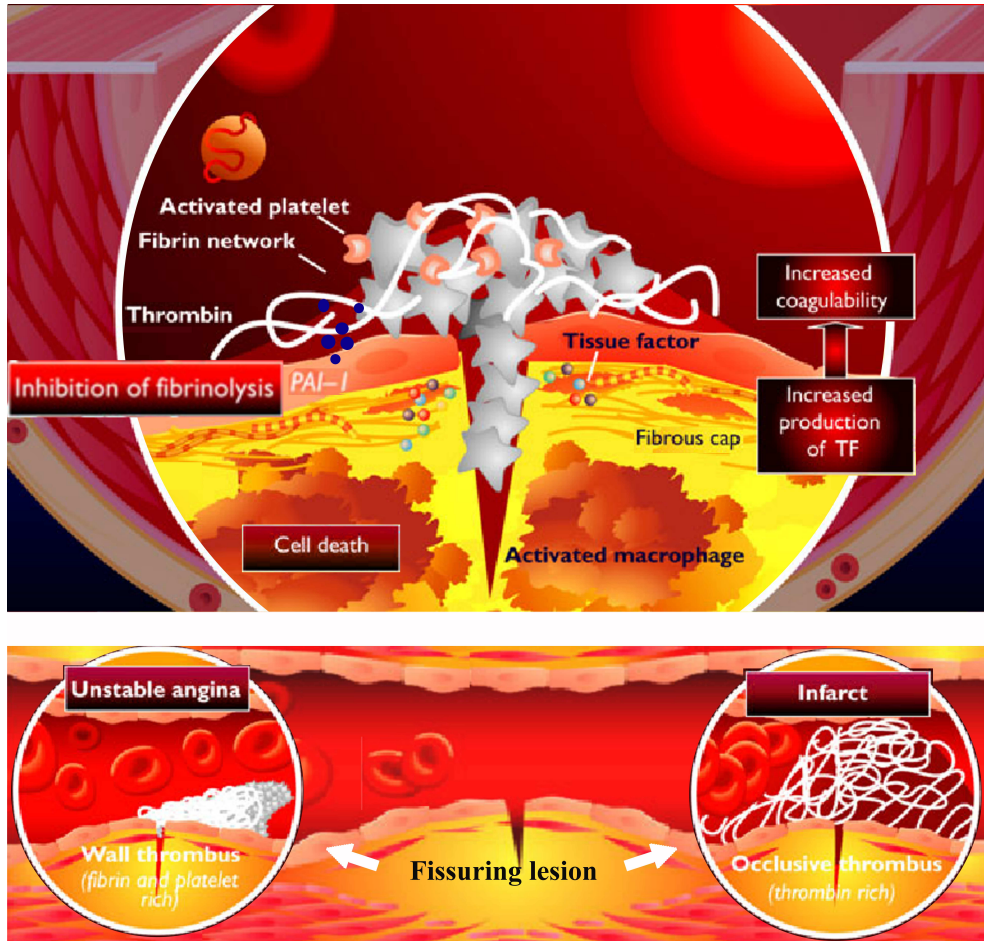


**Figure. 1.2.** *Atherosclerosis onset. Schematic representation of healthy arteries, depicting their intima media and adventitia. The endothelial layer provides a barrier that provides vascular tone and regulates immune cell permeability, (top panel). Upon the onset of atherosclerosis, endothelial cells secrete proinflammatory cytokines and display dysfunctional permeability. Fat deposits form and circulatory monocytes extravasate to scavenge and ingest oxidized cholesterol becoming foam cells (bottom panel). The progressive accumulation of intra and extra-cellular cholesterol marks the formation of fatty streaks. Adapted with permission from [40]*



**Figure. 1.3.** *Progression of Atherosclerosis. The growing fatty streak eventually forms a lipid core (top panel), that gets isolated by the progressive formation of a fibrous cap (bottom panel) that contains collagen, proteoglycans and activated smooth muscle cells. The sturdier the cap, the less likelihood there is of plaque rupture. Lipid accumulation and cell stressors such as free radicals cause cell death and necrosis (bottom panel). Adapted with permission from [40]*





**Figure. 1.4.** *Atherosclerotic Lesion Rupture.* Oxidized LDL activates macrophages and stimulates the expression of tissue factor (TF), a major inflammatory cytokine, pro-coagulant and trigger of thrombosis. *ox*LDL also stimulates increased production of Plasminogen Activator Inhibitor type 1 (PAI-1), which inhibits fibrinolysis. The end result is a state of hyper-coagulability around the plaque. Activated macrophages weaken the fibrous cap by releasing metalloproteases that also degrade the connective tissue within the lesion. Uncontrolled necrosis of macrophages contributes to fibrous cap erosion and lesion destabilization by leakage of intracellular lytic enzymes. The erosion of the fibrous cap causes lesion rupture (top panel) with subsequent formation of thrombus and arterial occlusions that interrupt the flow of blood (bottom panel). The occlusions may be either partial, upon the formation of fibrin and platelet rich wall thrombus (bottom panel left), or complete (bottom panel, right). Partial occlusions are prone to embolization and, as such, can give rise to distal occlusions. Total occlusions in contrast are thrombin rich and cause ischemia leading to clinical manifestations such as infarct, stroke and death. Adapted with permission from [40]

other leukocytes that migrate in response to secondary necrosis derived inflammatory clues [48] (Fig. 1.5). Finally, the third and most threatening consequence of lesion necrosis is the erosion and rupture of fibrous caps by lytic enzymes arising either from intracellular contents of necrotic cells, or released by proinflammatory macrophages (Fig. 1.5). Fibrous cap rupture cause leakage of thrombogenic contents from the lesion to the circulation, leading to thrombus formation, acute blood vessel occlusion, cerebral or myocardial infarction and sudden death, as first clinical manifestation of atherosclerosis (Fig. 1.4).

The impact of macrophage apoptosis on atherosclerosis is however dependent on the cause of cell death and stage of the disease. In fact, macrophage cell turnover by apoptosis coupled to prompt phagocytosis of apoptotic bodies, delays the expansion of early atherosclerotic lesions and causes phagocytes to secrete anti-inflammatory signals [25, 52]. But, cholesterol-induced macrophage apoptosis, as occurs in atherosclerosis, triggers inflammation in phagocytes [53]. In addition, when the clearance capacity of lesion phagocytes is impaired or overwhelmed, or viable phagocytes are depleted [54]; apoptotic cells that are not removed loose the integrity of their membrane leading to secondary necrosis and the formation of necrotic pools rich in pro-apoptotic, pro-inflammatory and thrombogenic intracellular contents, characteristic of lesion destabilization (Fig. 1.5).

Phagocytosis of death cells is therefore an important process that prevents plaque progression and induces its stability by triggering intracellular signal transduction pathways that result in the release of anti-inflammatory factors while preventing the leakage of apoptotic cell contents that would otherwise lead to autoimmunity, necrotic core expansion, plaque destabilization and thrombus formation.

## 1.6 Myelocyte Differentiation as Determinant of Lesion Stability

A further determinant of lesion instability is the skewed differentiation of monocytes into pro-inflammatory and lytic macrophages prompt to inducing inflammation and fibrous cap erosion and rupture. These macrophages [55], usually found in Type I diseases, are commonly denoted as M1, to follow a nomenclature similar to that used for T helper cells.

Infiltrating monocytes, homing into atherosclerotic lesions are deemed to preferentially differentiate into proinflammatory M1 macrophages that secrete immune mediators in response to apoptotic cells and other stressors found inside atherosclerotic lesions. Cytokines secreted by M1 macrophages such as  $\text{IFN}\gamma$ ,  $\text{TNF}\alpha$ ,  $\text{IL-12}$  and  $\text{IL-18}$  induce pro-inflammatory responses in T and B lymphocytes and promote inflammation and newly recruited monocyte differentiation into M1 macrophages, in a vicious cycle that progressively reduce the contents of tissue repairing and immunoregulatory M2 (again, following the T helper dichotomy) alternatively activated macrophages (AAM). In addition, proinflammatory M1 macrophages, produce proteases and chemokines that degrade the components of the fibrous cap [54, 56] leading to lesion vulnerability (Fig. 1.5).

The skewed differentiation of extravasated monocytes into pro-inflammatory macrophages is deemed to occur in response to the local extracellular environment present in atherosclerotic lesions. However, recent evidence indicates that  $\text{CX}_3\text{CR1}^{lo}$

CCR2<sup>+</sup>Ly6c<sup>hi</sup> circulatory monocytes might be precursors of inflammatory macrophages in mice; while their CX<sub>3</sub>CR1<sup>hi</sup>CCR2<sup>-</sup>Ly6c<sup>lo</sup> counterparts might give rise to wound healing and immunoregulatory macrophages. This has led to the hypothesis that the progression of atherosclerotic lesions towards vulnerability to rupture and thrombus formation might be predetermined already in the circulation. In fact, blood Ly6c<sup>hi</sup> monocytes are increased in hyperlipidemic mice, where they have been demonstrated to selectively accumulate [57, 58] and destabilize atherosclerotic lesions by secretion of proteases and inflammatory cytokines [59–61]. Circulatory Ly6c<sup>lo</sup> monocytes are in contrast reduced in atherosclerotic mice, which likely contribute to lesion vulnerability, as this subset is deemed to differentiate into M2 macrophages that induce collagen secretion, wound healing and reduced inflammation [59, 61].

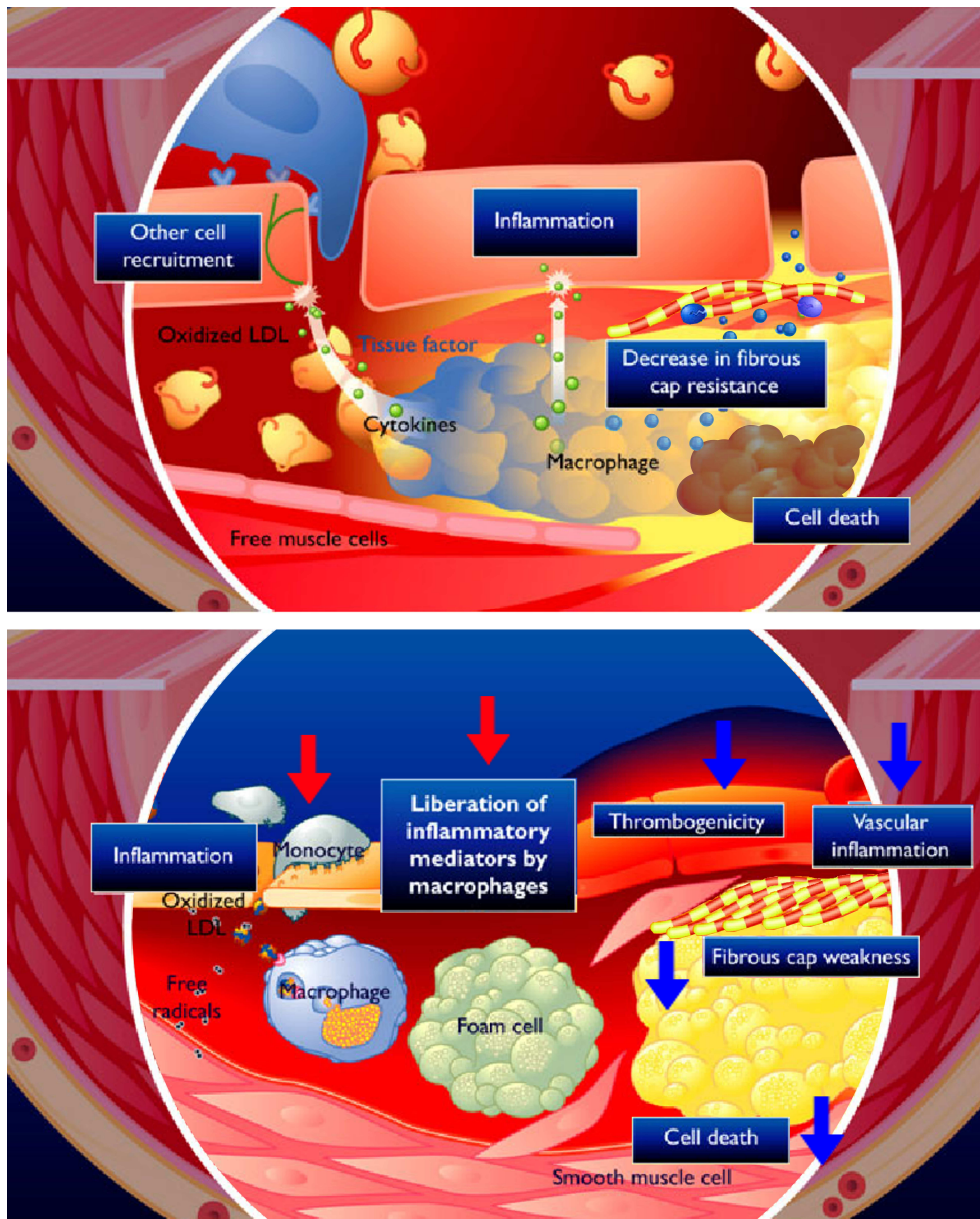
Analogous to the observations in mice, the level of CX<sub>3</sub>CR1 expression defines two major human monocyte subsets, referred to as CD14<sup>hi</sup>CD16<sup>-</sup> and CD14<sup>lo</sup>CD16<sup>+</sup>, that share phenotype and homing potential with their mouse counterparts [62]. Interestingly, increased levels of human CD14<sup>hi</sup>CD16<sup>-</sup> monocytes, deemed to be the human counterpart of mice Ly6c<sup>hi</sup> cells, correlate with high risk and predisposition to coronary heart disease [63].

Taken together, these data suggest that subset specific blood monocytosis might be determinant of the phenotype taken on by atherosclerotic lesions, both in humans and mice. In addition, hyperlipidemia, as observed in atherosclerosis, dysregulates medullar homeostasis by promoting bone marrow hematopoietic stem cell mobilization and intravasation from the bone marrow niche to the peripheral circulation [64]. This is of enormous importance considering that once in the circulation these cells can extravasate and differentiate into tissue macrophages, without monocyte intermediates, [65]. Furthermore, intravasated hematopoietic stem cell progenitors migrate to the spleen, where they differentiate, becoming a major source of proinflammatory Ly6c<sup>hi</sup> monocytes that infiltrate atherosclerotic lesions [66], which suggest that besides causing atherosclerotic lesion onset, hyperlipidemia might drive a preferential differentiation of monocytes into pro-inflammatory macrophages.

## 1.7 Thesis Outline

Considering the above, the onset of atherosclerosis seems to have a predetermined fate towards lesion vulnerability to rupture and clinical manifestations such as sudden infarct and death. However, the processes responsible for the development of atherosclerosis are dynamic and can be modulated towards lesion regression and stabilization. In fact, anastomosis of atherosclerotic vessel segments from ApoE<sup>-/-</sup> donors into wild-type recipients causes lesion shrinkage and reduced contents of macrophage derived foam cells by induction of CD68<sup>+</sup> macrophage migration out of lesions [67, 68]; suggesting that lesion regression could be achieved by increasing myelocyte cell efflux out of lesions. Similarly, viral mediated transfer of ApoE into atherosclerotic ApoE<sup>-/-</sup> mice, triggers lesion regression by inducing reduced monocyte diapedesis and intra-lesion macrophage turnover by apoptosis [69].

The modulation of monocyte and macrophage homeostasis towards resolution of inflammation and wound healing could therefore represent a successful strategy to develop improved treatments to reduce the mortality and cost associated with this disease.



**Figure. 1.5.** The ratio of proinflammatory and tissue repairing macrophages is a key determinant of atherosclerotic lesion stability. Macrophages have the potential to activate proinflammatory (top panel) or tissue repairing and wound healing (bottom panel) differentiation programs. Proinflammatory macrophages release cytokines that induce inflammation and proteases that decrease fibrous cap resistance (top panel). Tissue repairing macrophages in contrast induce wound healing and resolution of inflammation. Cell death, thrombogenicity and endothelial dysfunction are consequently reduced. Adapted with permission from [40]

This thesis presents the results of a series of experiments performed to modulate leukocyte homeostasis in atherosclerosis using two strategies:

1. Deletion and upregulated expression of target genes known to regulate processes that control leukocyte homeostasis including hematopoiesis, intravasation, leukocyte differentiation, lipid homeostasis, extravasation and cell survival.
2. Time controlled systemic and local induction of macrophage apoptosis in advanced atherosclerosis to assess the impact of this process in intravascular and extravascular leukocyte homeostasis at the end of apoptosis induction, and after a period allowed for recovery and normalization of leukocyte levels.

The role of target genes in monocyte and macrophage homeostasis in atherosclerosis was evaluated using knock-out and lentiviral mediated gene knock-in methodologies, combined with Bone Marrow Transplantation (BMT) to generate atherosclerotic mice chimeras bearing genetic alterations in their hematopoietic cells.

Time controlled macrophage apoptosis was induced in atherosclerotic mice by suicide genes engineered to be expressed in macrophages by use of cell specific gene promoters.

Leukocyte homeostasis and atherosclerotic lesion phenotype was evaluated upon each treatment.

The experiments performed are presented in four chapters arranged in two sections. The first section comprises Chapters 2 and 3. This section uses the first strategy previously described. The experiments presented in this section were performed to evaluate the possibility of increasing the amounts of tissue repairing and anti-inflammatory macrophages, while reducing the accumulation of their lytic and proinflammatory counterparts. This section presents novel results and demonstrate the feasibility of targeting different monocyte and macrophage subpopulations to skew atherosclerotic homeostasis towards lesion regression, resolution of inflammation and fibrous cap stability.

Each chapter in the first section includes an evaluation of the expression of the genes studied in a genomic context. Cluster, network and gene ontology analysis of differentially expressed genes from human macrophages stimulated with  $\text{TNF}\alpha$ , IL-4, IL-17 LPS,  $\text{IFN}\gamma$  or LPS+ $\text{IFN}\gamma$  permitted the location of genes used, in genetic networks controlling macrophage differentiation into proinflammatory and tissue repairing phenotypes, which was related to the expression of those genes in human atherosclerotic lesions obtained from patients undergoing carotid endarterectomy or at autopsy. This analysis provided a framework for analysis of results obtained in mice and their relevance for human atherosclerosis.

The second section is dedicated to the induction of macrophage apoptosis in atherosclerosis. Chapter 4 studies atherosclerotic lesion stability and composition upon induction and after recovery from acute  $\text{CD169}^+$  macrophage apoptosis in LDL receptor deficient atherosclerotic mice. Chapter 5 presents a comparison of the impact that systemic and local induction of  $\text{CD115}^+$  macrophage apoptosis exerts on atherosclerosis and vascular and extravascular leukocyte homeostasis in ApoE deficient mice. The results of this section have important implications for the development of therapies to treat atherosclerosis by inducing apoptosis, thereby reducing lesion macrophage contents.

