Role of neutrophils and leukotrienes in atherosclerotic plaque destabilisation : implication of endotoxemia

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-Implication of endotoxemia-

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Abbreviations

12LO: 12-lipoxygenase

15LO: 15-lipoxygenase

5LO: 5-lipoxygenase

A

AA: arachidonic acid

APOE: apolipoprotein E

APOAI: apolipoprotein AI

B

BLT: leukotriene B_4 receptor 1

BMT: bone marrow transplantation

C

CB: endocannabinoid receptor

CCL: chemokine (C-C motif) ligand

CCR: chemokine (C-C motif) receptor

CMV: cytomegalovirus

cPLA2: cytosolic phospholipase A2

CR: complement receptor

CRP: C-reactive proteint

CXCL: Chemokine (C-X-C motif) ligand

CXCR: Chemokine (C-C motif) ligand

cysLT: cysteinyl leukotriene

D

DAMP: damage-associated motif pattern

DC: dendritic cells

DC-SIGN: dendritic cell-specific intercellular adhesion molecule-3 grabbing non-integrin

E

EC: endothelial cell

ECM: extracellular matrix

ERK: extracellular signal–regulated kinases

F

FAAH: fatty-acid amide hydrolase

FLAP: 5LO-activating protein

FPR: formyl-peptide receptor

G

G-CSF: granulocyte-colony stimulating factor

GROα: growth-related oncogene-α

H

HFD: high-fat diet

HSP: heat-shock protein

I

ICAMs: Intercellular adhesion molecule

IL: interleukin

K

KLF: krüppel-like factor

L

LDL: low-density lipoprotein

LDLR: low-density lipoprotein receptor

LPS: lipopolysaccharide

LT: leukotriene

LTA_4: leukotriene A_4

LTA_4H: leukotriene A_4 hydrolase

LTB_4: leukotriene B_4

LTC_4S: leukotriene C_4 synthase

M

MAPK: mitogen-activated protein kinase

MCP: monocyte chemoattractant protein

MIP: macrophage inflammatory protein

MPO: myeloperoxidase

N

NADPH: nicotinamide adenine dinucleotide phosphate

NAMPT: nicotinamide phosphoribosyltransferase

NF-κB: nuclear factor kappa B

NO: nitric oxide

O

oxLDL: oxidised low-density lipoprotein

P

PAD: peptidyl arginine deiminase

PAMP: pathogen-associated motif pattern

PG: prostaglandin

PGP: proline-glycine-proline peptide

PI3K: phosphoinositide 3-kinase

PKC: protein kinase C

PMN: polymorphonuclear neutrophil

PNCA: proliferating cell nuclear antigen

PRR: pattern-recognition receptors

R

ROS: reactive oxygen species

S

SAA: serum amyloid A

SMC: smooth muscle cells

SPM: specialised pro-resolving lipid mediator

T

TGF: transforming growth factor

TNF: tumour necrosis factor

TYK: tyrosine kinase

V

VLDL: very low-density lipoprotein

W

WT: wild-type

Introduction

I. From healthy to atherosclerotic vessels

A/ Structure of arteries

Since William Harvey's description of blood circulation, the cardiovascular system is commonly acknowledged as an organ system that allows for blood to circulate and deliver nutrients, metabolites and oxygen to cells [1]. Arteries are composed of three main layers: the tunica intima, the tunica media and the tunica adventitia (**Figure 1**).

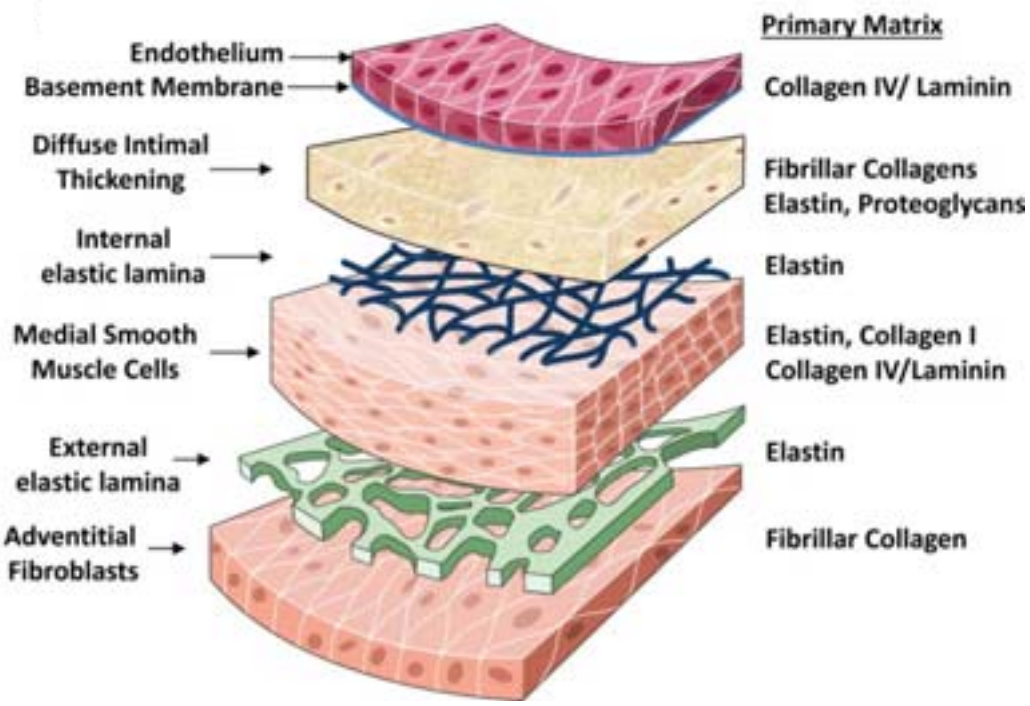

Figure 1. Structure of an artery. *Illustration showing the composition and disposition of the three layers that form arteries (adapted from Yurdagul A Jr, 2016).*

The inner layer, the intima, is formed by a monolayer of endothelial cells (ECs) overlaying a basal lamina rich in type IV collagen. A small number of vascular smooth muscles cells (SMCs) are observed in the intima and are thought to contribute to the production of extracellular matrix (ECM) proteins that constitute the basal membrane.

The middle layer, the media, is made up of interposed elastic laminae and SMC layers that ensure the vessel contractility. The media is rich in type I and III collagen fibres that have important tensile strength. The intima and the media are separated by the internal elastic lamina while the external elastic lamina delimits the interface between the media and the adventitia.

The aforementioned layer of loose connective tissue mainly contains fibroblasts, perivascular nerves, lymphatic vessels, and microvessels named *vasa vasorum*.

B/ Generalities on atherosclerosis

1. Definition

During the XVI and XVII centuries, renaissance anatomists, the most famous of whom Leonardo Da Vinci, described the degeneration of arteries with advancing age, already hinting at the progressive aspect of atherosclerosis [1]. Lobstein coined the term *arteriosclerosis* or *atherosclerosis* in 1829, derived from the Greek '*athere'* (gruel) and '*skleros'* (hardening).

Atherosclerosis develops in the intimal layer of large- and medium-sized arteries, such as carotid arteries, aortas and coronary arteries [2]. The development of atherosclerosis involves complex interactions between blood-derived elements, for instance monocytes or lipoproteins, and vascular wall components, such as SMCs. Lesions start as a lipid deposition in arterial walls. This lipid build-up leads to inflammation within the intima [3] and evolves into atheroma plaques. As such, plaques are mainly asymptomatic. However, they can thicken, leading to a reduction of the arterial lumen, or stenosis. Plaque stenosis alone can sometimes obstruct the blood flow although it is rarely fatal, as downstream tissues are weakly ischaemic [4]. Conversely, acute cardiovascular events are principally caused by the formation of thrombus over the plaque or atherothrombosis. Plaques weaken, rupture and release their content into the bloodstream. Atherosclerotic plaques contain thrombogenic elements capable of activating platelets, leading to atherothrombosis. Thrombi can directly occlude the blood flow or embolise and block smaller vessels. This induces the ischaemia of downstream organs and necrosis of tissues, which can be fatal.

2. Prevalence and risk factors

Atherosclerosis accounts for the majority of cardiovascular diseases, the first cause of death worldwide, with an estimated 15 million deaths in 2015 [5]. Atherosclerosis manifests in multiple forms depending on the affected organ. For instance, ruptured plaques can provoke myocardial infarction, ischemic stroke and acute limb ischaemia.

The main causal factor identified in the initiation of atherosclerosis is low-density lipoprotein (LDL) [6]. These proteins carry cholesterol, are elevated in the blood of hypercholesteraemic patients and can easily accumulate in the vessel walls. Several risk factors for atherosclerosis have been identified and include diet, smoking, genetics, hypertension, hypercholesterolemia, hyperlipidaemia, hyperglycaemia, and diabetes. This multiplicity of risk factors highlights the multifactorial facet of atherosclerosis. Infections are also considered to be risk factor for atherosclerosis [7], [8] and will be discussed in more details in a subsequent part.

However, despite the development of preventive strategies and the use of tension and lipid-lowering drugs such as statins, atherosclerotic plaque rupture is still an important concern and for this reason it is crucial to better clarify the aetiology of plaque destabilisation.

C/ The formation of atheroma or atherogenesis

1. Lipoprotein accumulation and inflammation

Atherosclerosis develops predominantly in areas of low shear stress within arteries, e.g. bifurcations [9]. In these areas, the intima is thickened, probably as an adaptation to resident mechanical constraints [4]. This stage of plaque formation is named 'adaptive intimal thickening' (**Figure 2**).

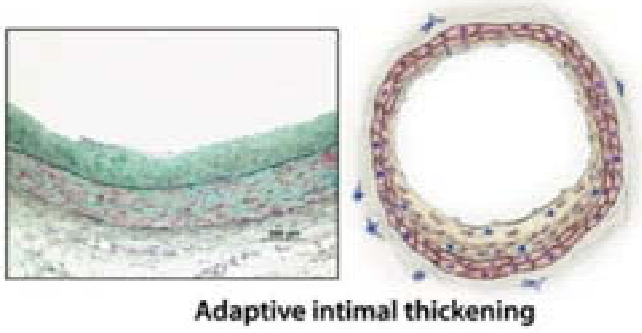

Figure 2. Photograph and representative diagram of a human coronary artery showing adaptive intimal thickening of arteries. *The intima is enriched in smooth muscle cells and matrix fibres in areas of low shear stress (adapted from Bentzon et al., 2014).*

In these sites of predilection, LDLs accumulate in the intimal layer, where they oxidise and aggregate. The oxidation of LDLs is thought to rely on the activity of myeloperoxidase (MPO), lipoxygenases, and reactive oxygen species (ROS). The scavenging of modified LDLs (oxLDLs) by resident macrophages and notably SMCs that reside in the intima results in the accumulation of lipids inside the cells, turning them into 'foam' cells [10]. These processes involve cholesterol efflux and autophagy [11]. OxLDLs stimulate the polarisation of macrophages to a pro-inflammatory phenotype that secretes cytokines and produces reactive oxygen species, which subsequently promote the further retention and oxidation of LDLs in the intima [12].

This type of lesion is classified as intimal xanthoma or Type I-II lesions (**Figure 3**).

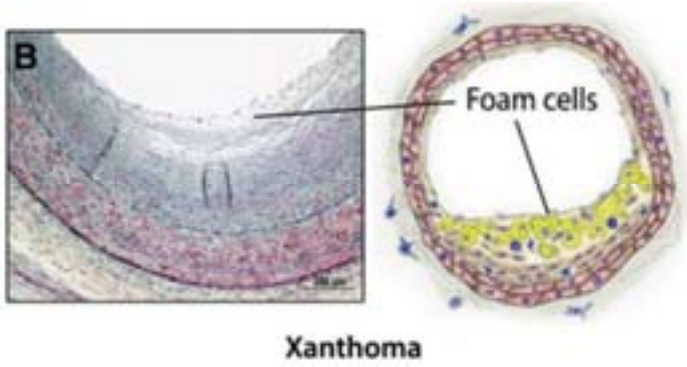

Figure 3. Photograph and representative diagram of a human intimal xanthoma. *This lesion displays an accumulation of foam cells derived from recruited macrophages and SMCs (adapted from Bentzon, 2014).*

Modified LDLs are recognised as danger signals and induce a response to injury in ECs, SMCs, resident macrophages and dendritic cells, stimulating the secretion of chemoattractants and the expression of adhesion molecules. This will lead to the attraction innate immune cells, in particular monocytes, through the interaction of classical monocytic chemokines, such as, CCL2 (monocyte chemotactic protein, MCP-1), CCL5 (RANTES) and CX3CL1 (fractalkine), with their receptors CCR2, CCR5 and CX3CR1 [13]. Recruited monocytes differentiate into specialised phagocytes, i.e. macrophages and dendritic cells, which can further ingest oxLDLs. T cells are also recruited during the initial stages of atherosclerosis in response to cytokines or chemokines secreted by macrophages and ECs.

2. Necrotic core and fibrous cap formation

The ongoing intimal inflammation stimulates the mobilisation of medial SMCs, which migrate to the intima, proliferate and lose their contractile function to acquire a synthetic and phagocytic phenotype. Interestingly, SMC proliferation has been shown to rely on leukotrienes [14]. SMCs then start to produce ECM proteins, such as collagen or elastin. This phenotypic change also leads to the increased retention and oxidation of LDLs, promoting foam cell formation. This type of lesion (Type III-IV) is characterised by a pathological intimal thickening (**Figure 4**).

Figure 4. Representative diagram and photograph showing the pathological intimal thickening of a human coronary artery. *This type of lesion exhibits increased extracellular lipid accumulation in the absence of a defined necrotic core (adapted from Bentzon et al., 2014).*

Foam cell accumulation induces to the apparition of a necrotic and lipidic core composed of apoptotic phagocytes and extracellular lipids in the form of cholesterol esters and crystals [15]. Many factors present in plaques can induce the apoptosis of foam cells and SMCs. In physiological conditions, apoptotic cells are cleared by phagocytes, however in plaques phagocytes exhibit defective efferocytosis, or clearance of apoptotic remnants, which contributes to the necrotic core growth [16]. Progressively, a fibrous cap composed of a matrix of type I and type III collagens mainly produced by intimal SMCs encapsulates this necrotic area [17]. Besides type I and III collagens, fibrous caps contain elastin and other ECM fibres. These lesions (Type V) are characterised as fibroatheroma or fibrocalcific plaques if they display calcifications (**Figure 5**).

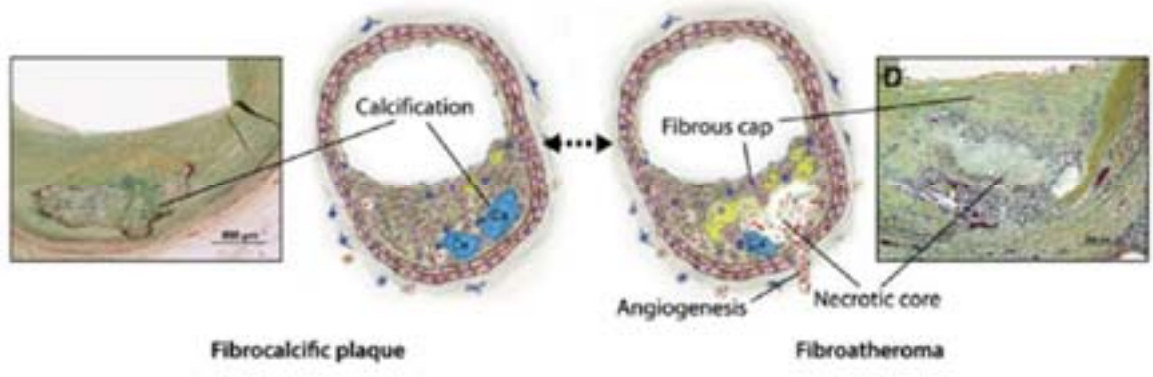

Figure 5. Representative diagrams and photographs of a human fibrocalcific plaque and a fibroatheroma. *Advanced lesions comprise two types: fibrocalcific plaques (left) distinguished by the presence of calcified necrotic cores surrounded with tissue, and fibroatheroma (right) characterised by the presence of a necrotic core, a fibrous cap, and neovessels (adapted from Bentzon et al., 2014).*

II. Atherosclerotic plaques: from stability to vulnerability and rupture

A/ The concept of vulnerable plaques

Stable plaques are generally rich in SMCs that are predominantly localised in a thick fibrous cap. Necrotic cores are rather small or absent in stable lesions. The collagen and elastin present in the fibrous cap maintain the integrity of plaques and limit the extrusion of necrotic core materials into the bloodstream. The balance between SMC proliferation and apoptosis and between the synthesis and enzymatic degradation of ECM fibres determines the thickness of fibrous caps [18].

The presence of a thin cap and large necrotic core is the hallmark of vulnerable plaques [4]. The large necrotic core is associated with a high leukocyte infiltration and the thinned cap with a decrease in SMC and ECM content. These vulnerable plaques can also exhibit haemorrhages.

This type of lesion (Type VI) is named thin-cap fibroatheroma (TCFA) and are considered to be at high-risk of rupture (**Figure 6**).

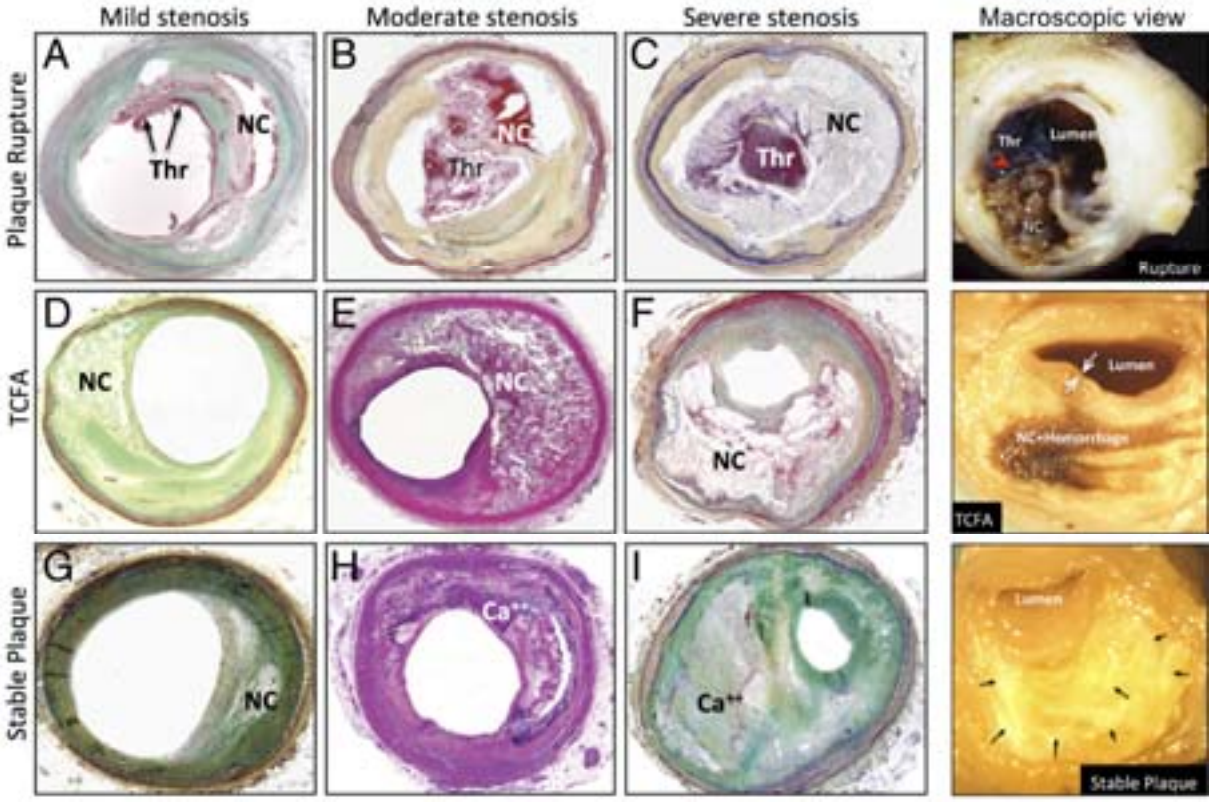

Figure 6. Microphotographs of histological sections and macroscopical views of disrupted, vulnerable (TCFA), and stable plaques with different degrees of stenosis. *(A to C and top right) Ruptured plaques with thrombi (Thr) overlying disrupted fibrous caps (red arrowhead on macroscopic view) and in contact with necrotic cores (NC). (D to F and middle right) Thin cap fibroatheromas (TCFA) showing large necrotic cores (NC) and thinned fibrous caps (white arrows on macroscopic view). (G to I and bottom right) Stable plaques rich in fibrous tissue, with relatively small necrotic cores and frequent calcifications (Ca^{++}, black arrows) (adapted from Narula et al., 2013).*

B/ Mechanisms of plaque destabilisation

1. Cellular death

As aforesaid, SMCs are crucial for plaque stability as they maintaining the fibrous cap thick; therefore SMC apoptosis is an important marker of plaque vulnerability [19]. Indeed, the disappearance of SMCs results in reduced synthesis of matrix fibres and thus thinner caps. Recruited inflammatory leukocytes secrete proteases that induce a loss of SMC-matrix interaction by degrading matrix fibres, resulting in their cellular death by anoikis. Moreover, the ingestion of oxLDLs by SMCs and macrophages engenders endoplasmic reticulum stress and hence promotes apoptosis.

SMC and macrophage apoptosis leads to the necrotic core expansion and the exacerbation of plaque inflammation [20]. Cathepsins released from activated neutrophils are implicated in macrophage death. In addition, activated $CD8^+$ lymphocytes and macrophages release tumour necrosis factor (TNF) which is pro-apoptotic [21]. Mast cells can also promote apoptosis through the activation of Toll-like receptor (TLR) 4 by their enzymes [22].

2. Accumulation of necrotic cells

Clearance of apoptotic bodies by efferocytosis promotes the resolution of inflammatory processes and avoids the accumulation of apoptotic cells. If not cleared, apoptotic cells start to enter secondary necrosis. 'Find-me' and 'eat-me' signals plays a key role in the ingestion of apoptotic cells. However, in plaques, oxLDLs compete with find-me signals as they bind the same scavenger receptors on phagocytes [23]. Moreover, the overabundance of 'eat-me' signals in plaques hampers efferocytosis by overloading scavenger receptors. Finally, several proteases could also cleave these receptors. Altogether, necrotic cells accumulate in plaques, which lead to the expansion of the necrotic core

3. Degradation of the fibrous cap and proteolytic activity

Leukocyte infiltration is known to contribute to inflammatory reactions and proteolysis that destabilise plaque. Indeed, leukocytes, ECs and SMCs release metalloproteases (MMPs) that can digest the fibrous cap. Macrophages and neutrophils can be directly activated by inflammatory components in plaques and are a rich source of MMPs in plaques [24]. In addition, once activated, T cells stimulate macrophages to release matrix proteases. In early stages of atherosclerosis, MMPs are thought to be more protective as they facilitate the migration of SMCs from the media to the intima and thus the formation of a fibrous cap [20], [25]. Conversely in later stages, increased MMP release leads to the digestion of the ECM proteins in the fibrous cap, and therefore weakens plaques. For example, the constitutively-expressed MMP-2, which cleaves type IV collagen, is involved in SMC migration, while MMP-8 is involved in fibrous cap thinning through type I collagen degradation [26].

In addition to MMPs, cathepsins and serine proteases such as the neutrophil elastase digest elastin, and thus thin the fibrous cap. Indeed, the fibrous cap is mainly composed of collagen type I, but also contains elastin and proteoglycans. Expression of collagen type VIII has also been shown in aortic SMC from atherosclerotic mice but not wild-type mice [27].

MMPs have other roles beside proteolytic degradation, and contribute to the ongoing inflammation as they cleave cytokines, pro-MMPs (the secreted inactive form of MMPs), growth factors and receptors. **Table 1** recapitulates the activities and sources of the main MMPs involved in plaque weakening.

Class	Enzymes	Sources	Examples of substrate	Regulators
Collagenases	MMP-1 (interstitial collagenase)	ECs, SMCs, Mφ	Type III collagen (and others), gelatin	oxLDL, TNFα, IL-1β
	MMP-8 (neutrophil collagenase)	Mϕ and neutrophils	Type I collagen (and others), gelatin, laminin, angiotensine I	TNFα, IL-1, IL-6
	MMP-13	ECs, SMCs, Mφ	Type II collagen (and others), gelatin, fibronectin	
Gelatinases	MMP-2 (gelatinase A)	ECs, SMCs	Type IV collagen, gelatin, pro-MMPs-1, -2 and -13, cytokines and growth factors	TIMPs, CRP, LTB_4, statins
	MMP-9 (gelatinase B)	Leukocytes	Type IV collagen, gelatin, pro-MMPs-2, -9 and -13, cytokines and growth factors	TIMPs, NGAL, LTB_4, statins
Stromelysins	MMP-3	SMCs, Mφ, lymphocytes	Type II, IV, IX collagen, pro-MMPs-1, -7 and -8	LPS, IL-1β
	MMP-10	ECs, Mφ	Type II, IV, IX collagen, pro-MMPs-1, -7, -8 and -9	
Matrylisins	MMP-7	Mφ	Type IV collagen, pro-MMPs-1, -2 and -9	statins, LPS, PGE_2
	MMP-12	Mφ	Type IV collagen, fibrinogen, plasminogen	LPS, statins

Mφ = macrophages; TIMPs = Tissue Inhibitors of MMPs; CRP = C-Reative Protein

Table 1. Main MMPs involved in atherosclerotic plaque destabilisation.

4. Intraplaque haemorrhages

As plaques grow, their supply of crucial oxygen and metabolites is lessened. To palliate this nutrient deprivation and ischaemia, neovessels grow into the intima. These neovessels, i.e. *vasa vasorum*, originate mainly from the adventitia, but can also emerge from the luminal side. They provide an entrance for monocytes and immune cells in plaques. Moreover, this neo-angiogenesis gives rise to a fragile and often leaky microvasculature, which promotes the extravasation of red blood cells [15]. Haemoglobin from erythrocytes is highly cytotoxic and thus promotes apoptosis of resident plaque cells

[28]. These intraplaque haemorrhages are frequent in advanced plaques and have been shown to result in the accumulation of protease-rich neutrophils in plaques [29]. Therefore by conveying neutrophils, intraplaque haemorrhages can also contribute to plaque weakening.

5. Calcification and cholesterol crystals

Apoptotic, necrotic and degraded matrix materials trap calcium granules and deposits. When expanding, this trapped calcium results in the calcification of plaques. The genesis of these calcification is however not fully understood. Another type of crystals found in plaques derive from cholesterol, which can be synthesised by SMCs in response to collagen type I [30]. These cholesterol crystals can exhibit several forms, including nodules, plaques and spikes [31]. While the role of calcification, especially the nodular type, in plaque destabilisation is still debated, cholesterol crystals are found perforating the fibrous cap at sites of rupture.

C/ Resolution versus chronicity of inflammation

The evolution of plaques is a slow process that occurs over years in the life of an individual. Lesions are thought to be able to regress or to remain stable. Several processes occurring in plaques display a duality that counterbalance the inflammation to avoid a swift exacerbation. The endoplasmic reticulum stress generated by the ingestion of oxLDLs activates a kinase involved in macrophage survival and autophagy, and thus reduces the excessive apoptosis [32]. The pro-inflammatory activation of plaque cells by inflammatory lymphocytes can be countervailed by regulatory lymphocytes [33]. The prostaglandin PGE_2 expressed in plaques promotes macrophage activation, but also increases the production of anti-inflammatory IL-10 [34]. Macrophages themselves differentiate into either a pro- or anti-inflammatory phenotype and can egress from lesions [12]. Moreover, lipoxygenases, such as 12/15-lipoxygenases oxidise LDLs, while they also produce pro-resolutive lipoxins and resolvins [35]. The balance between these inflammatory and resolutive signals determines the progression and ultimately the fate of the lesion.

D/ From vulnerable plaque to cardiovascular events

1. Mechanisms of plaque rupture

For a plaque to rupture, it must be structurally weak to be prone to tearing in the fibrous cap that exposes the thrombotic material to the bloodstream. In ruptured plaque specimens obtained from autopsy, thinned fibrous caps are consistently evidenced and are particularly localised in the shoulders of eccentric plaques, i.e. plaques that form an arc in the blood vessel [4]. As previously mentioned, fibrous cap thinning involves both the loss of SMCs, thus the reduction of ECM fibres synthesis, and

the digestion of matrix fibres by enzymatic activities [19]. Concurrently, the infiltration of leukocytes such as monocytes and neutrophils results in their subsequent activation and MMP, cathepsin, and plasminogen activator releases [36], accelerating both the thinning of the fibrous cap and enhancing the thrombogenicity of plaques.

The underlying causes of plaque rupture remain undefined. Physical or psychological stress, infection, and temperature variation have been evoked as triggers of cardiovascular events. Circadian rhythm may also play a part, as myocardial infarctions are more frequent in the morning [37], [38]. Mechanistically, the activation of the sympathetic nervous system is the main pathway evoked, as it leads to increased heart rate and blood pressure. Vasoconstriction, vasodilatation and wall shear stress could thus participate in plaque rupture [18], [39].

Observational findings have shown that only a minor part of ruptures results in symptoms. Therefore, it is possible that plaques with limited thrombogenicity also rupture, but only induce a minor thrombus formation and heal silently.

2. Plaque erosion

Atherothrombosis also forms on ‘eroded’ plaques, i.e. with an absence of endothelium over an intact fibrous cap [40]. These plaques are more fibrous and rich in SMCs (**Figure 7**). Local shear stress combined with oxidative stress promotes endothelial dysfunction and death. The subsequent endothelial denudation activates platelets causing them to form a thrombus.

3. Healing of plaques

Observations of human specimen have revealed the presence of thrombi incorporated into plaques and layers of dense type I collagen and loose type III collagen [41]. This suggests that plaques rupture silently and heal by rebuilding layers of tissue (**Figure 7**). This fibrotic remodelling could also favour the stenosing of plaques.

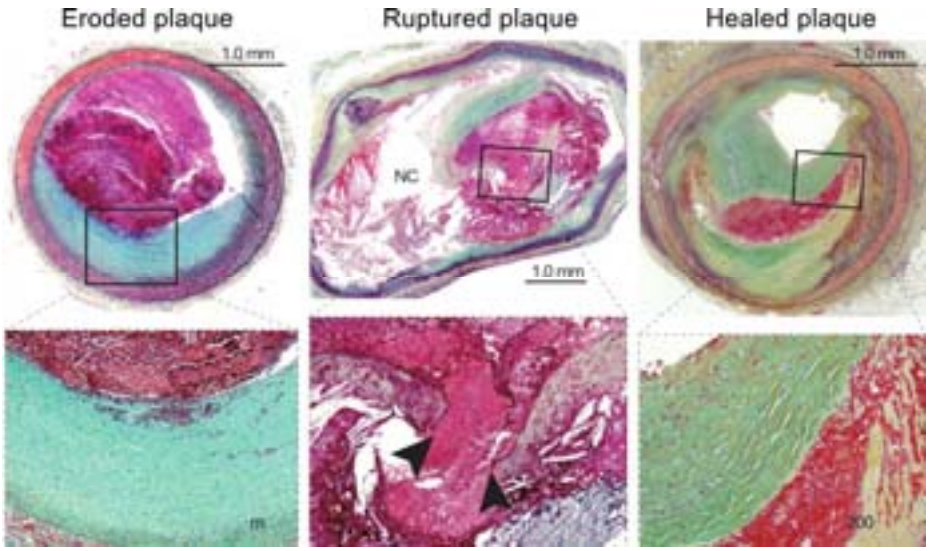

Figure 7. Microphotographs and magnifications of an eroded plaque, a ruptured plaque and a healed plaque. *(Left) Plaque erosion is characterised by a thrombus overlying an intact and thick fibrous cap. (Middle) A ruptured plaque showing an occlusive thrombus in direct contact with the thrombogenic necrotic core (NC); arrowheads indicate the disruption of the thinned cap. (Right) A thrombus has been incorporated into the plaque which displays distinct layers of collagen, representative of a healed plaque (adapted from Patri et al., 2013)*

E/ Murine model of atherosclerosis

Mice do not develop spontaneous atherosclerosis diseases, unlike pigs, non-human primates and surprisingly, birds [42]. Mice are atheroresistant, but some backgrounds are more susceptible to atherogenesis than others when fed a high fat diet (HFD), for example the C57BL/6 background. The most frequent murine models of atherosclerosis are genetically knock-out *Apoe*$^{-/-}$ and *Ldlr*$^{-/-}$ mice [43]. In both models, hyperlipidaemia results from a defective cholesterol trafficking. Apolipoprotein E (APOE) is the ligand of the low-density lipoprotein receptor (LDLR) and other receptors on hepatocytes necessary for lipid uptake of chylomicrons and very low-density lipoprotein remnants. *Apoe*$^{-/-}$ mice are hyperlipidaemic under a chow diet and develop plaques early. Plaque progression can be accelerated by a HFD, leading to plaques rich in macrophages and lipids. *Ldlr*$^{-/-}$ mice do not have such an increased lipidemia, as other receptors than LDLR can uptake lipoproteins. These mice need to be fed a HFD in order to observe the early apparition of lesions. To palliate this disadvantage, *Ldlr*$^{-/-}$ mice have been crossed with other mouse lines such as *Apoe*$^{-/-}$ mice or mice knock-in for human ApoB100 transgene. Interestingly, after HFD feeding, *Apoe*$^{-/-}$*Ldlr*$^{-/-}$ mice show significant coronary lesions and suffer from myocardial infarction, in particular in stress conditions [44]

In terms of atherosclerosis modelling, aged chow-fed *Apoe*$^{-/-}$ mice show lesions that are morphologically closer to human lesions. After 20 weeks, lesions are fibrous, rich in SMCs and ECM. In older mice, intraplaque haemorrhages have been evidenced, suggesting that these plaques could

spontaneously become vulnerable. However, APOE is expressed on bone marrow-derived cells and therefore bone transplantation from mice expressing APOE is not possible, as it will interfere with atherogenesis. Moreover, *Apoe*$^{-/-}$ mice exhibit an increased leucocytosis and cognitive dysfunction, underlining the multiple roles of APOE.

III. The role of infectious diseases in atherosclerosis

The notion of the implication of bacterial infections in atherosclerotic disease stems from clinical observations. As early as in the 19th century, physicians were noticing a connection between the incidence of stroke and infections [45]. Thereafter, a large body of evidence has emerged from both clinical and experimental studies, linking atherosclerosis with different pathogens [46]–[49]. These include bacteria such as *Chlamydia pneumoniae* (*C.pneumoniae*), *Porphyromonas gingivalis* (*P.gingivalis*), *Helicobacter pylori* (*H.pylori*), but also viruses, such as influenza or cytomegalovirus (CMV). Nevertheless, the role of infection in the pathogenesis of atherosclerosis and associated disorders remains elusive, undoubtedly because of the complexity of this possible contribution.

A/ Potential implications of bacterial and viral infections

1. Clinical arguments

Identification of pathogens in plaques: *C. pneumoniae*, a bacteria responsible for low-grade respiratory infections, is one of the first pathogens identified in human atherosclerotic vessels. Microscopic observations by immunohistochemistry or electronic microscopy and PCR identification of this pathogen were later confirmed by the isolation of viable organisms from human plaques [50]. *C. pneumonia* was found in cells from human atherosclerotic vessels, but not from healthy tissues, but its presence was not correlated to plaque instability. Genomic material from several commensal bacteria and viruses have been further identified in human plaques. For example, a large variety of dental germs have been detected by PCR or immunocytochemistry in human plaques [51].

Association with cardiovascular events: In the early 2000s, a large cohort study involving more than 40 000 patients confirmed previous smaller-scale studies on the association between acute infection and myocardial infarction or stroke [52]. Numerous seroepidemiological studies demonstrated associations between bacterial or viral seropositivity and cardiovascular outcomes [49], [53]–[56]. However these associations are weakened by conflicting results, showing how heterogeneities in studied parameters or in study designs could lead to opposing results. Moreover, the lack of suitable and reliable techniques for the identification of pathogens could also underlie this variability.

Antibiotherapy trials: Following these studies, several clinical trials were implemented using antibiotherapy targeting *C. pneumoniae* but showed no benefits on cardiovascular mortality. These studies are however partially inconclusive, as *C.pneumoniae* is an intracellular pathogen and hence result in a persistent infection, in which the pathogen resides in cells for long periods without proliferating and escapes antibiotic treatment. Furthermore, the timing and duration of antibiotic treatments may have been either too late or too short in the course of the infectious process. Moreover, prolonged antibiotherapy leads to an alteration of the lipid metabolism and atherogenesis. Finally, other studies have suggested that cumulative exposures to a large number of pathogens is responsible for the increased risk of cardiovascular events.

The concept of infectious burden: Early studies already showed that the higher the number of pathogens identified, the higher the prevalence of coronary artery disease [57]. In the Northern Manhattan prospective study, the risk of stroke [58] and the thickness of carotid plaques [59] were found to increase in patients seropositive for at least five pathogens, and correlated positively although non-significantly with seropositivity for only one pathogen. Therefore, as different pathogens are identified in plaques, it is possible that the cumulative actions of different bacteria and viruses renders plaques vulnerable, as opposed to the single action of one specific pathogen.

2. Animal studies

In the 70s, an American group showed that following infection with the avian herpes virus, chickens develop atherosclerotic-like lesions in arteries [60]. Since then, numerous models in mice, rabbits and rats have clarified the impact of infection on atherosclerosis.

Repeated injections of *C.pneumoniae* increases lesion size in *Apoe*$^{-/-}$mice, *Ldlr*$^{-/-}$ mice, and cholesterol-fed rabbits, but the antibiotic treatments of these animals does not provide consistent data. In advanced atherosclerosis, infections results in increased MMP activity and reduction of fibrous cap thickness but do not increase lesion size.

Another well-studied group of bacteria in atherosclerosis are dental germs. Oral or systemic deliverance of *P.gingivalis* increases systemic inflammation [61], [62] and accelerates atherosclerosis in *Apoe*$^{-/-}$ mice and rabbits [63]. These increases are also associated with an augmentation of inflammatory cells, lipids, and cytokines within plaques. In several studies, genomic material from *P.gingivalis* has been detected in aortic plaques in murine models of periodontitis. Other dental pathogens accelerate atherosclerosis, in particular during polymicrobial dental infection, which implies a role for the infectious burden in atherogenesis [64].

The impact of *H.pylori* and *Mycoplasma pneumoniae* on atherogenesis is still controversial because of conflicting data.

Viral infections are potential accelerators of atherosclerosis, as infections of mice with CMV or influenza virus enhance T cell and macrophage infiltration and lesion size.

B/ Possible mechanisms

1. Direct action of pathogens on plaques

Bacteria and viruses could directly act on plaques or infect macrophages, SMCs or ECs, resulting in pro-inflammatory responses and expression of adhesion molecules [65] (**Figure 8**). For example, CMV infection triggers the expression of enzymes from the 5LO pathway in SMCs [66] and could hence favour pro-inflammatory responses. Bacterial infections also promote LDL oxidation and foam cells formation [67].

2. Indirect impact of pathogens on plaques

Molecular mimicry: PAMPs or Pathogen-Associated Molecular Patterns expressed at the surface of bacteria present structures that resemble host proteins (**Figure 8**). For example, heat shock proteins (HSPs) found at the surface of *C.pneumoniae, H.pylori* or *P.gingivalis*, are similar to human HSPs [68]. The cross-reactivity between these HSPs can trigger a humoral response directed against both pathogenic and human HSPs, initiating an auto-immune response against plaque cells.

Systemic inflammatory responses: The presence of infectious agents at distant non-vascular sites such as the teeth or the lungs induces an augmentation of circulating cytokines and acute phase proteins which may reach the vascular site of plaques. Circulating levels of IL-6 and C-reactive protein (CRP) are for example elevated in patients with periodontal disease [68], [69] and in murine models of periodontal diseases [61]. Moreover, circulating bacterial or viral products increase the activation of blood leukocytes, which directly affect plaques, as discussed in more detail in the following section.

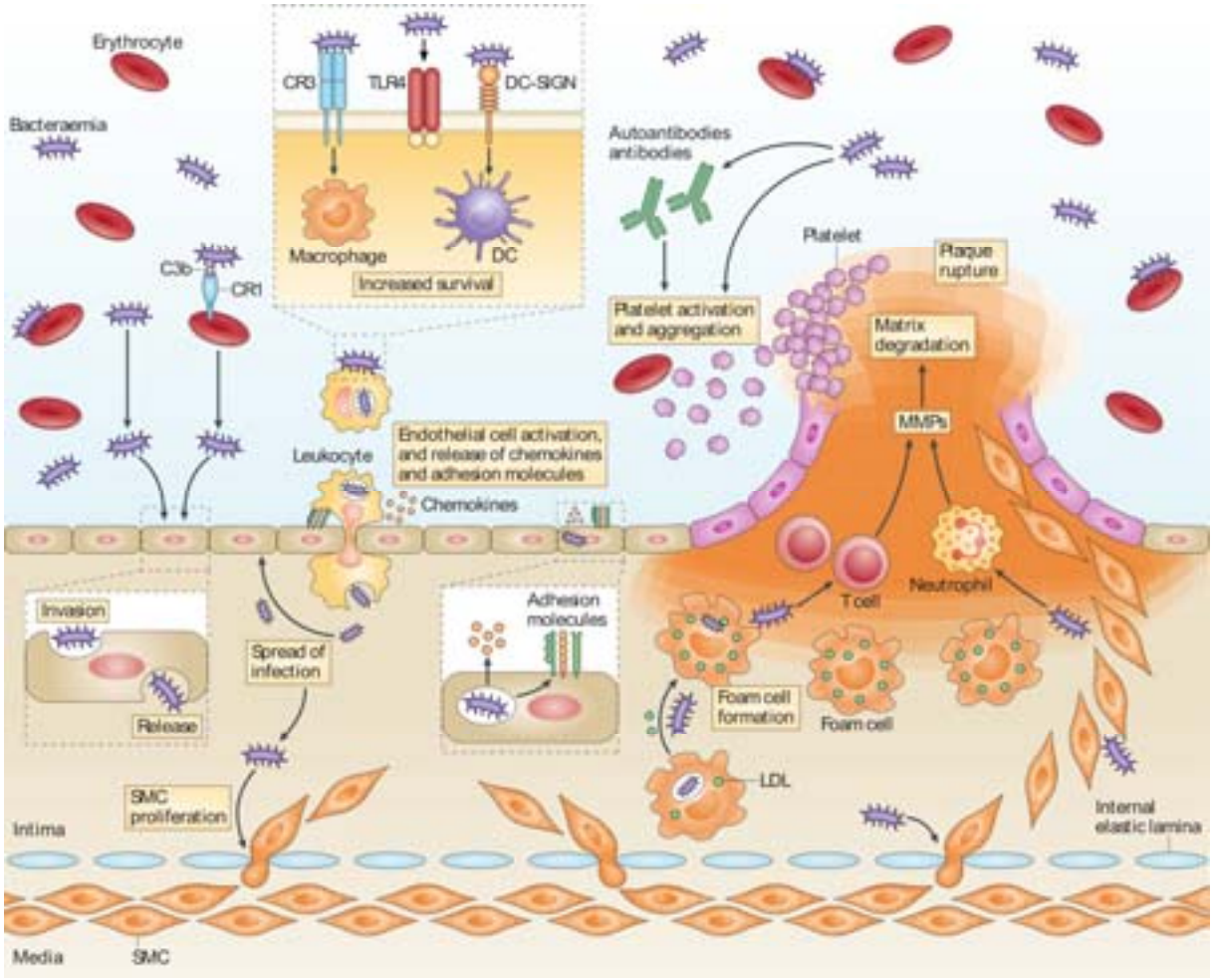

Figure 8 Potential mechanisms of plaque growth and destabilisation mediated by bacteria. *Bacteria directly invade leukocytes and ECs and increase systemic inflammation through TLR4 signalling, leading to the upregulation of adhesion molecules, the secretion of chemokines and the subsequent transmigration of leukocytes. Pathogens enter safely within leukocytes by the activation of complement receptor-3 (CR3) and dendritic cell-specific ICAM-3 grabbing non-integrin (DC-SIGN). The presence of bacteria in the intima induces the proliferation and migration of smooth muscles cells and promotes the uptake of oxLDLs by macrophages. Bacteria, along with inflammatory signals, induce the secretion of MMPs and consequently degrade the fibrous cap. Bacterial signals also enhance platelet aggregation by a direct action and by the production of pro-thrombotic autoantibodies (adapted from. Hajishengallis, 2014).*

C/ Endotoxemia and atherosclerosis

When gram-negative bacteria are dividing or dying, their cellular wall breaks down, leading to the release of their surface components, such as endotoxins or lipopolysaccharides (LPS). LPS is composed of polysaccharides bound to a hydrophobic moiety called lipid A, which has different immuno-potencies depending on the species of bacteria. When hydrophobic LPS enters the blood flow, it is

carried by proteins such as LPS-binding proteins (LBP) or APOE. These proteins either activate target cells or lead to hepatic clearance [70]. LPS is known to induce pro-inflammatory responses and cell death through pathways involving TLR, CD14, MYD88 and NF-κB. Various conditions can lead to the translocation of LPS into the circulation, for example intestinal leakage [71].

1. TLRs signalling in atherosclerosis

LPS binds preferentially to TLR4, however LPS originating from *P.*gingivalis has been shown to exert its effect through both TLR4 [72] and TLR2 [73]. Genetic deletions of TLR2, TLR4 or the adaptor protein *Myd88* in *Apoe*$^{-/-}$ mice decrease macrophage infiltration and diminished atherosclerotic lesion size [74]. Accordingly, human polymorphisms in the TLR4 or TLR2 genes promote or reduce susceptibility to cardiovascular events [75].

Other ligands of TLR4/CD14 complex and TLR2 include saturated fatty acids, which are also known to promote LPS translocation and metabolic diseases [76], [77]. Moreover, macrophagic TLR4 binds oxLDL [78] and its activation by LPS and LDL also upregulates chemokine expression in macrophages [79].

2. Endotoxemic models in atherosclerotic mice

Systemic administration of LPS: Lehr *et al.* first showed that repeated intravenous injections of LPS in hypercholesteraemic rabbits results in accelerated atherogenesis [80]. More recently, repeated intraperitoneal LPS injections for 10 weeks in *Apoe*$^{-/-}$ mice was shown to increase plaque size, oxidation of LDLs and natural killer cell infiltration. *Apoe*$^{-/-}$ mice lacking APOCI are protected from LPS-induced atherosclerosis and their plaques contain less T cells and macrophages, emphasizing the role played by lipoproteins in the regulation of LPS-induced inflammatory responses [81]. Similarly, APOAI protects mice from accelerated atherogenesis and the increased infiltration of CD68-positive macrophages [82]. Moreover, in a model of accelerated atherosclerosis induced by collar placement, chronic LPS exposure in HFD-fed *Apoe*$^{-/-}$ mice promotes macrophage infiltration and vulnerability, in particular in stressful conditions [83]. Finally, in mice injected with subclinical/super-low doses of LPS for two months, monocytes polarise towards an inflammatory phenotype, impairing the resolution of subsequent inflammatory responses in plaques [84].

LPS-induced lung inflammation: A recent study pointed out the role of neutrophils in plaque vulnerability following LPS exposure in a model of acute lung inflammation induced by intratracheal LPS instillation in HFD-fed *Apoe*$^{-/-}$ mice [85]. In this model of plaque destabilisation occurs relatively fast, i.e. 24 hours after LPS administration, which is consistent with the rapid reactivity of neutrophils known to be fast effector in inflammatory processes.

LPS from dental germs: In *Apoe*$^{-/-}$ mice, chronic infusion of LPS from *P.gingivalis* activates the COX-2 pathway in plaque macrophages and also accelerates atherogenesis [67], [86]. This is in agreement with the increased TLR expression in atherosclerotic plaques in murine models of periodontal disease [73] and the induction of inflammatory responses in ECs by LPS from *P.gingivalis* [62], [65].

Metabolic endotoxemia: Mice fed a HFD develop what is called metabolic endotoxemia, which has been implicated in metabolic disorders such as diabetes or obesity [87]. This translocation of LPS due to increased intestinal permeability is dependent on modifications of the gut microbiota [88]. Administration of *Akkermansia muciniphila* in HFD-fed *Apoe*$^{-/-}$ mice restores the integrity of the intestinal barrier, prevents metabolic endotoxemia and reduces the atherosclerotic burden [89].

3. Association between endotoxemia and atherosclerosis in humans

Human blood vessels respond to LPS by expressing and producing inflammatory signals: chemokines (IL-8, CCL2…), cytokines, ROS, PAF, and adhesion molecules [8].

Plasma levels of endotoxin and cardiovascular events: In 1999, results from the Bruneck study provided evidence that plasma levels of LPS correlates to the incidence of atherosclerosis [90]. In patients undergoing peritoneal dialysis, the elevated level of plasmatic endotoxin is associated with increased carotid intimal thickness [91]. Increased plasmatic LPS levels correlates to the risk of metabolic syndromes and the 10-year cardiovascular mortality [92]. Endotoxemia hence is related to cardiovascular events in humans.

Periodontal diseases: During human periodontal diseases, LPS released from dental germs enters the bloodstream [93]. Periodontitis-induced endotoxemia is associated with cardiovascular diseases, as shown in the FINRISK study [94].

Metabolic endotoxemia: High-fat feeding in humans potentially results in the translocation of LPS in the blood, as suggested by studies showing that high-fat meals induce endotoxemia [95]. The levels of plasmatic endotoxin are increased in patients with chronic heart failure presenting a bowel wall oedema that induces gut leakage [96] and LPS translocation [97]. The increased risk of cardiovascular events and mortality observed in endotoxemic patients with chronic kidney disease can also be linked to increased gut permeability [98]. In addition, LPS translocation occurs during long term western-type diets, which are rich in fats and is known to contribute to the onset of atherosclerosis and metabolic syndromes [99]. Metabolic endotoxemia and the gut microbiome are thus likely contributing to atherosclerosis in humans [100].

I. The life of neutrophils

A/ The granulopoiesis

1. Production

Neutrophils belong to the myeloid cell line, which emerges from the differentiation of hematopoietic stem progenitor cells (HSPCs) and includes granulocytes, monocytes, dendritic cells, platelets and erythrocytes [101]. Granulocytes comprise basophils, eosinophils and neutrophils, and are identified by polylobed nuclei and the presence of cytoplasmic granules.

Granulopoiesis takes place in bone marrow niches, where HSPCs are committed under the instruction of chemokines and transcriptions factors to differentiate into mature neutrophils (**Figure 9**), by going sequentially through different stages: myeloblasts, promyelocytes, myelocytes, metamyelocytes and band cells.

2. Release and retention

Once mature, neutrophils can exit the bone marrow to enter the bloodstream (**Figure 9**). This release is antagonistically regulated by the two receptors CXCR4 and CXCR2 [102], [103]. CXCR4, highly expressed on newly formed and senescent neutrophils, binds CXCL12 produced by bone marrow ECs and osteoblasts [104]. CXCR4 retains mature neutrophils in the bone marrow and targets senescent neutrophils back to the bone marrow. Inversely, CXCR2 and its ligands, CXCL1 (GROα, growth-related oncogene-α/KC) and CXCL2 (macrophage inflammatory protein, MIP-2), both expressed by ECs, facilitate the egress of neutrophils into the circulation.

3. Regulation of neutrophil production

Granulocyte-colony stimulating factor (GCS-F) stimulates bone marrow neutrophil production and egress, by shifting the balance between CXCR4 and CXCR2 (**Figure 9**). Bacterial products, IL-1 and TNF can also promote neutrophil release into the blood circulation during ‘emergency’ granulopoiesis [105]. Conversely, the ingestion of apoptotic neutrophils by macrophages reduces IL-23 secretion through a LXR-dependent transcription [106]. IL-23 stimulates the production of IL-17 by specialised lymphocytes which will in turn promote the production of G-CSF and hence the release of neutrophils.

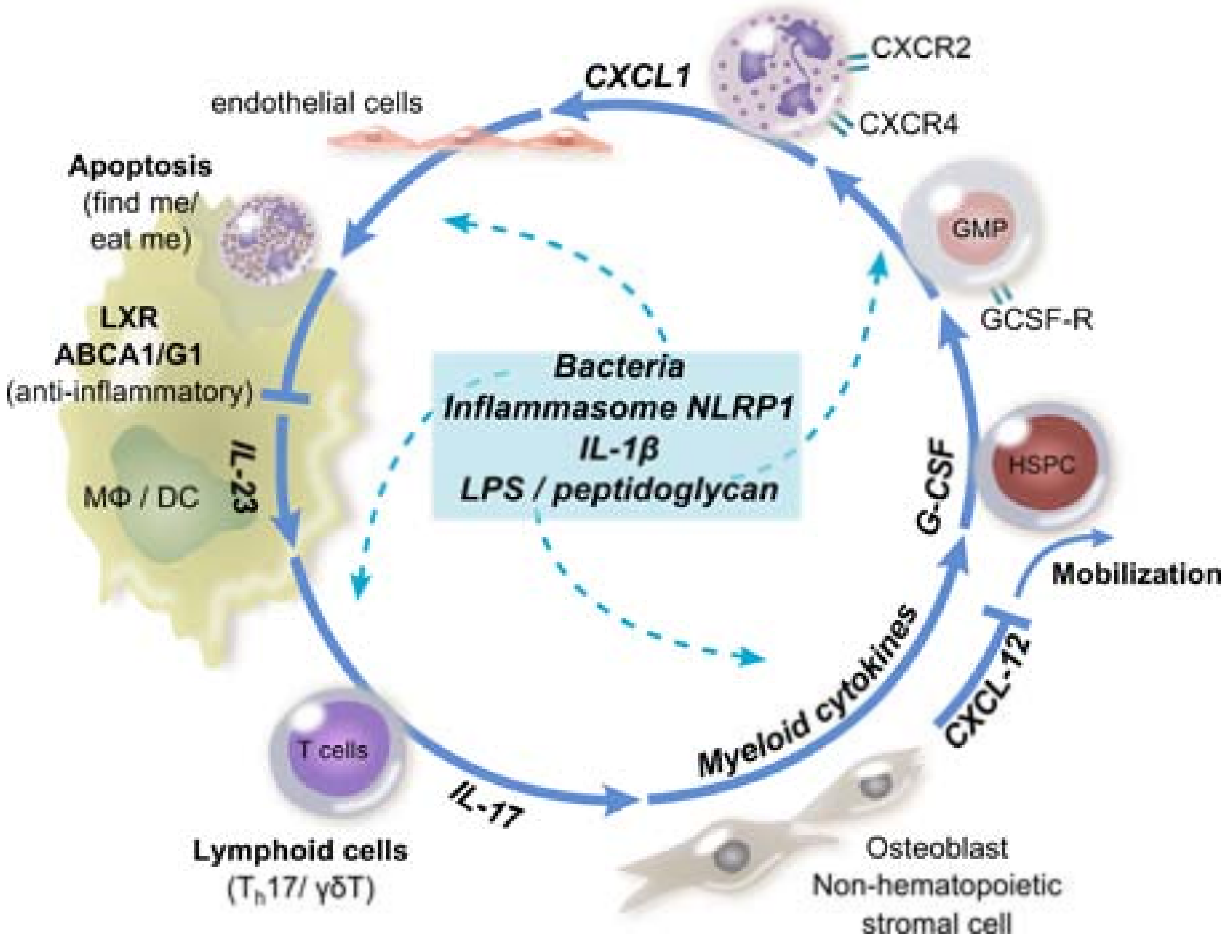

Figure 9. Regulation of granulopoiesis. *Hematopoietic stem progenitor cells (HSPCs) differentiate into granulocyte/macrophage progenitors (GMP), which following stimulation by G-CSF differentiate into mature neutrophils. Osteoblasts and stromal cells in the bone marrow inhibit the mobilisation of neutrophils by secreting CXCL12, the ligand of CXCR4. The egress and mobilisation of mature neutrophils is promoted by the axis CXCR2/CXCL1. The ingestion of apoptotic neutrophils by tissue macrophages (Mϕ) or dendritic cells (DC) inhibits the secretion of IL-23 via LXR-dependent transcription. IL-23 conversely induces the release of IL-17 by specialised lymphocytes (T_h17/γδT), resulting in increased G-CSF production. Bacteria and their products or inflammatory signals, such as NLRP1 (NLR family, pyrin domain containing 1) inflammasome, tune the production of neutrophils (adapted from Wirths et al., 2014).*

4. Granule formation

Belonging to the granulocyte family, neutrophils are rich in granules and secretory vesicles, filled with proteins necessary for their functions from recruitment to microbial defence [107]. Neutrophil granules are divided into four types and are sequentially formed and released in a reverse fashion, meaning that the first formed is the last released (**Figure 10**).

- Primary/azurophil granules are firstly formed and contain high amounts of MPO and serine proteases.
- Secondary/specific granules contain high levels of lactoferrin and collagenases.
- Tertiary/gelatinase granules, as stated in their name, are rich in gelatinase, i.e. MMP-9.
- Secretory granules or vesicles are formed by endocytosis and are rapidly released upon chemotactic stimulation. They are an important reservoir of membrane surface receptors involved neutrophil priming and adhesion, such as CD11b/CD18, complement receptors or the co-receptor of TLR, CD14.

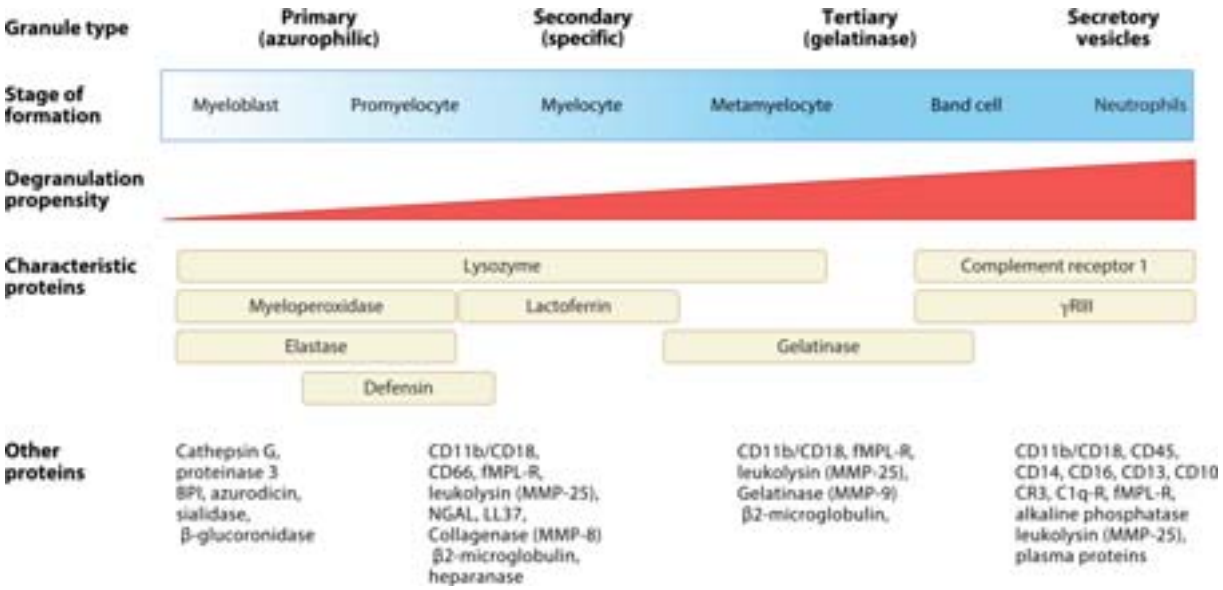

Figure 10. Neutrophil granules types, stage of formation and contents. *Four types of granules are formed during granulopoiesis and contain various factors necessary for neutrophil functions, such as antimicrobial proteins, receptors, adhesion molecules, and proteases. The first granules formed are the last released following neutrophil activation (adapted from Amulic et al., 2012).*

B/ Trafficking of neutrophils

1. Circulation, margination and lifespan

In humans, neutrophils represent about 50 to 70% of the circulating leukocytes, but only about 10 to 25% in mice. However, under inflammatory stimulation, mice show a significant neutrophil mobilisation. In physiological conditions, the number of circulating neutrophils fluctuates throughout the day due to the circadian control of HSPCs [108].

Beside the bone marrow, neutrophils are found in steady state in the lungs, the spleen and the liver [109], [110]. This pool of neutrophils marginated along the organ blood vessels is thought to constitute reservoirs for rapid deployment during acute processes.

Neutrophils are considered as short-lived cells with an estimated lifespan of 3 to 16 hours in the circulation [111]. In the absence of inflammatory signals, neutrophils enter spontaneous apoptosis, however during inflammatory processes their lifespan is prolonged, mainly through the inhibition of apoptosis pathways [34], [112], [113].

2. Recruitment of neutrophils into tissues

Following an injury or invasion of microorganisms, tissue-resident sentinel leukocytes and ECs, expressing pattern-recognition receptors (PRRs), recognise damage-associated molecular patterns (DAMPs) or pathogen-associated molecular patterns (PAMPs). Consequently, activated sentinel cells secrete cytokines and lipid mediators. In response to these signals, ECs start to express leukocyte adhesion molecules [114] that promotes the recruitment of neutrophils. Sentinel cells also attract neutrophils to affected tissues by releasing chemokines and lipids mediators. The cascade of recruitment and transmigration of neutrophils into tissues is well-described [115] and comprises several steps (**Figure 11**):

1. Tethering and rolling: A weak binding between P-selectin and E-selectin, expressed on the surface of ECs, and PSGL-1 (P-selectin glycoprotein ligand 1), expressed on neutrophils, leads to the rolling of neutrophils on surface of the endothelium.

2. Firm adhesion: Rolling neutrophils are primed by cytokines, chemokines and/or DAMPs/PAMPs present at the endothelial surface. This priming leads to conformational changes that increase the affinity of surface integrins, CD11a/CD18 (LFA-1) and CD11b/CD18 (Mac-1), and the release of intracellular integrin stores. When integrins bind intracellular adhesion molecules (ICAMs) on ECs, neutrophils arrest firmly on the endothelium.

3. Diapedesis: Once adherent, neutrophils start to crawl onto the endothelium to search for an appropriate site of entry into tissues. To extravasate, neutrophils need to cross the endothelium and the basement membrane. This transmigration is mainly paracellular but can also occur transcellularly.

As an alternative to rolling, neutrophils can form ‘slings’ and ‘tethers’ enriched in CD11a/CD18 to ensure their arrest in the high shear forces occurring in large arteries [115] (**Figure 11**). Moreover, platelets could contribute to the endothelial adhesion of neutrophils, notably through the secretion of chemokines, for example CCL5 [116].

Interestingly, neutrophils undergo reverse transmigration in certain pathological conditions, such as ischemia-reperfusion [117]. In such condition, the reverse transmigration of neutrophils is dependent on the degradation of junctional adhesion molecules by neutrophil elastase released upon LTB_4

stimulation [118]. This process could prevent neutrophils from residing in tissue when not needed to fight infections.

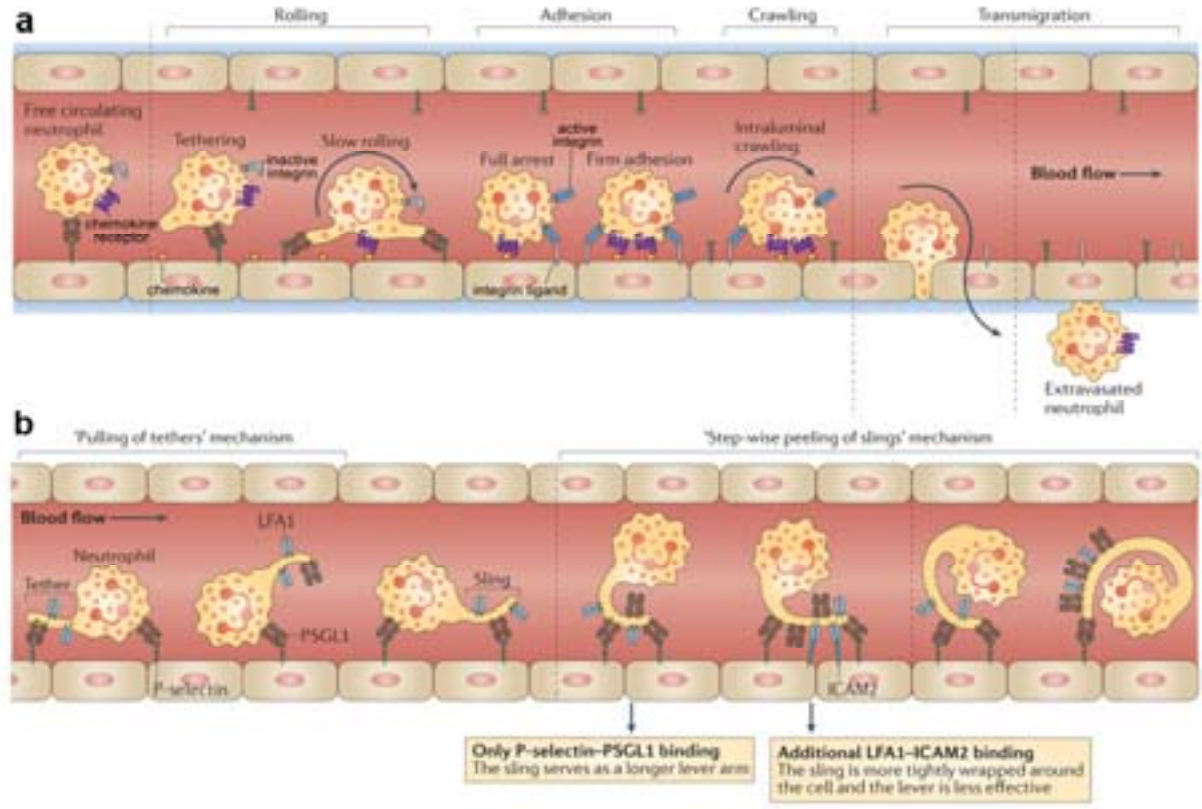

Figure 11. The neutrophil recruitment cascade. *(a) In response to an inflammatory stimuli, ECs express adhesion molecules which allow the tethering of rolling neutrophils. Neutrophils then crawl and adhere to the endothelium. After firm arrest, neutrophils start diapedesis. (b) Alternatively, neutrophils can form tethers and slings to anchor themselves to the endothelium and overcome high shear stress (adapted from Kolaczkowska et al., 2013).*

II. Immune functions of neutrophils

Neutrophils are the first line of defence in bacterial infection. Once in tissues, they navigate towards the inflammed site following a hierarchy of chemotactic molecules which includes LTB_4, IL-8 (CXCL8), CXCL1, CXCL2, CCL3, CCL5, C5a, and fMLP [119], [120]. On the inflammatory site, neutrophils activate to perform their defensive functions.

A/ Defence systems

Neutrophils present various mechanisms involved in microbial killing and immune functions, from phagocytosis to granule release [105], [115], [121]–[123], which will be briefly described herein.

1. Phagocytosis

Similarly to macrophages, neutrophils have a high phagocytic capacity for pathogens and cellular debris after recognition by PRRs, Fc receptors or complement receptors. Once in the cell, pathogens are destroyed in a specialised compartment, the phagosome that becomes functional upon its fusion with granules and the assembly of NADPH oxidase.

2. Reactive oxygen species

The respiratory burst occurs following neutrophil activation and results in the generation of Reactive Oxygen Species (ROS). On the plasma or phagosomal membrane, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase is assembled into a complex that reduces molecular oxygen to superoxide ions ($^{\bullet}O_2^-$). Those ions can further be converted into various species including hydrogen peroxide (H_2O_2) by dismutation or peroxynitrite ($ONOO^-$) after reaction with NO. ROS can contribute to the reversible regulation of several enzymes, such as metalloproteases.

3. Primary granule proteins

Myeloperoxidase (MPO) has an important oxidant activity. During the respiratory burst, MPO reacts with hydrogen peroxide to form hypochlorous acid (HOCl). It can also chlorinate tyrosine residues in proteins, such as the HDL, ApoAI, and form reactive nitrogen intermediates [124]. MPO can either be released into the phagosome or into the extracellular milieu.

Defensins are cationic antimicrobial peptides that can form pores in bacterial membranes and promote monocyte and T cell chemotaxis.

Another antimicrobial protein localised in primary granules is the bactericidal/permeability-increasing protein (BPI) of which the primary function is to bind LPS.

Finally, primary granules contain the three main serine proteases of neutrophils: proteinase-3, cathepsin G, and elastase. These enzymes digest various ECM components, including type IV collagen and elastin. They can also promote the activation of ECs, macrophages and lymphocytes and the attraction of monocytes and T cells.

4. Secondary and tertiary granule proteins

Secondary granules contain several antimicrobial peptides, such as the neutrophil gelatinase-associated lipocalin (NGAL) which plays an important role in iron-depleting immune strategies. Other peptides include LL37 (CRAMP), involved in the chemotaxis of neutrophils, T cells and monocytes, and metal-chelating proteins: lactoferrin which sequestrates iron and calprotectin (S100A8/S100A9) which chelates zinc and manganese.

Neutrophils have an important matrix-degrading capacity, especially since their granules contain high amounts of zinc-dependent MMPs [125]. Secondary granules contain neutrophil collagenase (MMP-8) which preferentially degrades type I and III collagens. Tertiary granules are rich in gelatinase (MMP-9) which degrades type IV collagen, elastin and type I gelatin (collagen hydrolysate). The membrane bound MMP-25 or leukolysin is present in neutrophils, however its functions remain poorly defined, despite its potential role in chemotaxis. MMPs are stored as inactive preforms and are activated upon release by cleavage. Moreover, tissue inhibitors of metalloproteases (TIMPs) can balance the activity of these proteases to limit host damage.

5. NETosis

Activated neutrophils can cast structure named Neutrophil Extracellular Traps or NETs [126]. NETs are composed of decondensed chromatin decorated with histones (H2, H3, and H4) and cationic granular proteins, such as MPO or elastase. The formation of NETs requires reactive oxygen species, generated by NADPH oxidase and MPO, the activity of elastase and the citrullination of histones by a peptidylarginine deiminase (PAD4).

As stated in their name, NETs are released out of the cells by rupture of nuclear and cytoplasmic membranes [127]. First identified following activation with high doses of phorbol 12-myristate 13-acetate (PMA) [128], NETosis is regarded as a neutrophil-specific form of cell death that occurs during inflammatory processes. However, this form of cell death appears mainly incidental rather than regulated [129] owing to the lack of clear signals triggering NETosis *in vivo* and distinct signalling pathways [130], [131]. Nevertheless, it is possible that during phagocytosis neutrophils maintain their integrity in a process named “vital” NETosis. This process ensures the release of NETs while only partially diminishing phagocytic and migratory capacities [132]. This type of NETosis has been demonstrated *in vivo* during phagocytosis of bacteria, where traps are released by vesicles or membrane blebbing (**Figure 12**).

NET formation and functions are still debated. Nevertheless, NETs have been implicated in various functions from pathogen trapping to thrombus formation, autoimmune diseases and macrophage priming in atherosclerosis [133].

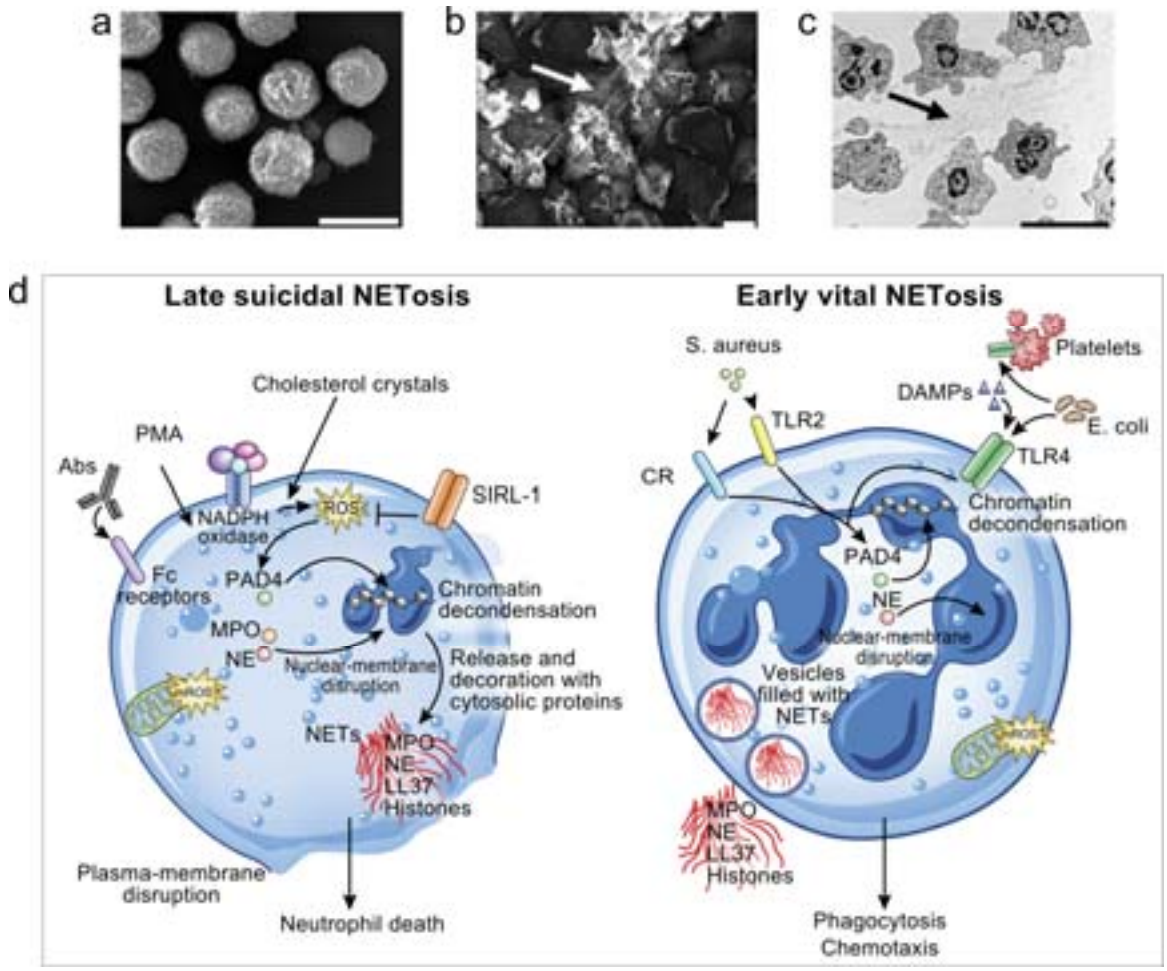

Figure 12. Electronic photographs and mechanisms of NETosis. *(a) Resting neutrophils present a round morphology .Upon stimulation with phorbol 12-myristate 13-acetate (PMA; b) or IL-8 (c), neutrophils release NETs (arrows), as shown by scanning (b) and transmission (c) electron microscopy. Scale bars = 10 µm. Adapted from Brinkmann et al., 2004. (d) Different mechanisms are involved in suicidal and vital NETosis. Suicidal NETosis induces the disruption of the plasma membrane and neutrophil death, while NETs are secreted in vesicles during vital NETosis allowing the neutrophil to still perform its functions, such as phagocytosis and chemotaxis. SIRL-1, signal inhibitory receptor on leukocytes-1; CR, complement receptors (adapted from Jorch et al., 2017).*

B/ Immune cell crosstalk

As neutrophils are present early on inflammatory sites, they secrete the cytokines and chemokines necessary for the build-up of immune responses [134]–[136]. Neutrophils can indeed synthesize *de novo* CXC and CC chemokines, cytokines, lipid mediators, growth and angiogenic factors [137].

Neutrophils contribute to the activation and regulation of various actors of the adaptive immunity (**Figure 13**): NK cells, dendritic cells, B and T cells [136], [138]–[140]. Moreover, neutrophils and monocytes/macrophages exhibit a considerable interplay. Macrophages recruit neutrophils and

monocytes at the beginning of immune responses, resulting in a cluster of monocytes and neutrophils at inflammatory sites. Macrophages and neutrophils cooperate to recruit other inflammatory cells and to mutually enhance their respective antimicrobial and phagocytic activities [141]–[143]. In addition, macrophages enhance the survival of neutrophils, while the ingestion of apoptotic neutrophils by macrophages and dendritic cells promotes the resolution of inflammation.

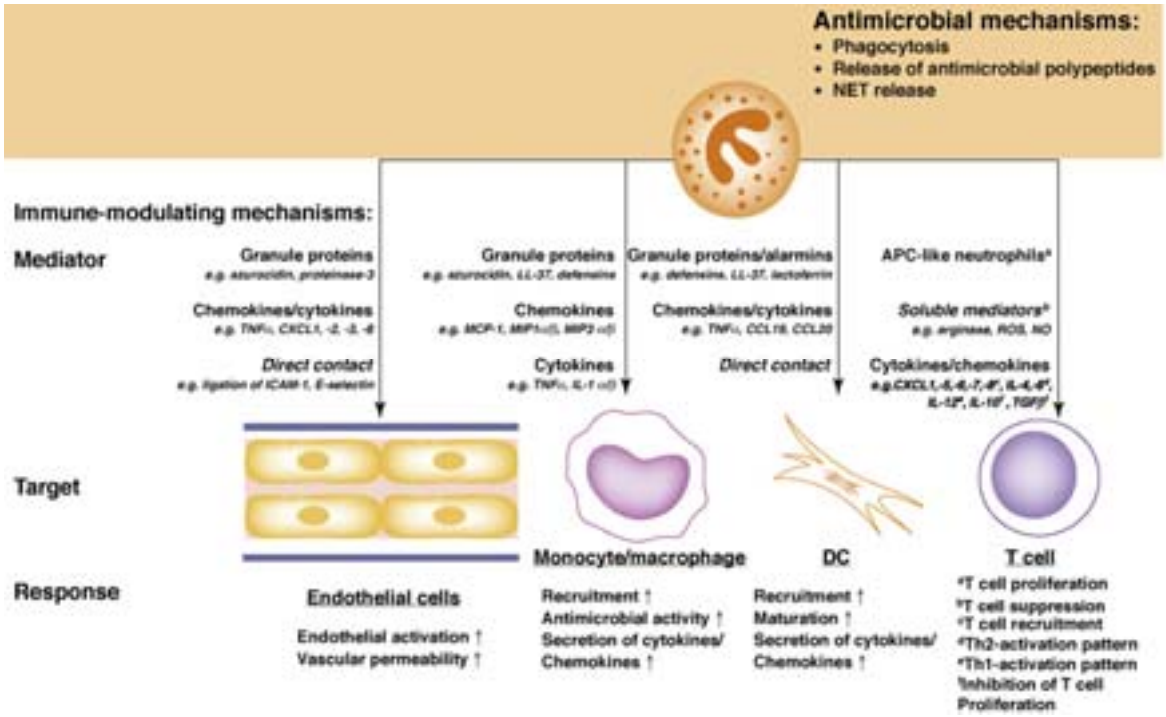

Figure 13. Interactions of between neutrophils and the immune system. *Neutrophils participate in the activation of various actors of the immune system through the release of their granule proteins and the synthesis of cytokines and chemokines. APC, antigen-presenting cell (Soehnlein, 2009).*

C/ Resolution and apoptosis

The clearance of apoptotic neutrophils by macrophages through eat-me and find-me signals is crucial in the resolution of inflammation. Their efferocytosis promotes the production of the anti-inflammatory cytokines TGF-β, IL-10 and PGE_2 by macrophages [34], [144]. Moreover, the engulfment of neutrophils by macrophages inhibits the TLR-mediated production of cytokines.

In addition, neutrophils are themselves involved in the lipid mediator class switch by producing pro-resolving lipid mediators, i.e. lipoxins, protectins and resolvins, through their interactions with various cell types in their vicinity (ECs, fibroblasts and platelets) [35], [145], [146]. These mediators can enhance macrophage phagocytic capacities, reduce the recruitment of inflammatory cells by inducing chemokine scavenging, favour the egress of phagocytes, and promote tissue repair and regeneration.

III. Emerging roles of neutrophils in atherosclerosis

Neutrophils have long been ignored in atherosclerosis, potentially because of the difficulties to specifically identify them and their short lifespan. However, since their identification in both murine and human plaques, they have been regarded as important contributors in the development, growth and destabilisation of atherosclerotic lesions.

A/ Perturbation of neutrophil homeostasis in atherosclerosis

1. Hyperlipidaemia and neutrophilia

In patients with hyperlipidaemia, neutrophils release more MPO and are primed, as shown by increased CD11b expression [147]. Following post-prandial hypertriglyceridemia, neutrophils from healthy volunteers have increased surface expression of CD11b and CD66b [148]. In line with this, hypercholesteraemic mouse lines as *Ldlr*$^{-/-}$ and *Apoe*$^{-/-}$ display increased numbers of circulating neutrophils, especially after high-fat feeding [149]. In this context, APOE regulates the proliferation of HSPCs and thus the count of circulating leukocytes. Moreover, hypercholesterolemia promotes the production of G-CSF, which positively controls bone marrow neutrophil production [116]. This increased neutrophil production is also linked to IL-17 [150]. In addition to a control of bone marrow neutrophil production, hypercholesterolemia increases the expression of CXCR2, the receptor of CXCL1 (KC the murine analogue of IL-8), on murine neutrophils and thus favours their release [116]. Similarly, hyperlipidaemia elicits neutrophil egress from the bone marrow by reducing the concentration of bone marrow CXCL12 and neutrophil expression of CXCR4, therefore impairing neutrophil homing to the bone marrow. Interestingly, in patients with cardiovascular disease, CXCR4 expression on circulating neutrophils is also diminished [151].

Collectively, these data indicate that hyperlipidaemia can disturb neutrophil homeostasis and increase peripheral neutrophil count.

2. Systemic increase and activation of neutrophils in human atherosclerosis

Leukocyte count, in particular peripheral neutrophil count, has long been considered as a predictor of cardiovascular events [152]. Indeed, numerous epidemiological studies have associated blood neutrophilia with an increased risk of myocardial infarction and stroke, independently of serum cholesterol levels [153]–[155]. Moreover, the ratio of neutrophils to lymphocytes in the blood is associated with the risk and the severity of stroke, coronary artery disease and limb ischemia [156]–[159].

Various neutrophil activation markers are known to be predictive of cardiovascular events, such as NGAL, α-defensin, or NETosis markers [160]–[162]. Circulating levels of enzymes abundant in neutrophil granules, for example MPO, NGAL, or MMP-9, are associated with increased risks of

cardiovascular events [163], [164]. Moreover, circulating neutrophils from patients with unstable angina present decreased MPO content, reflecting their activation in the blood [165]. These associations strongly suggest that not only neutrophil mobilisation, but also their activation, contribute to atherosclerotic plaque vulnerability.

3. Other factors linked to atherosclerosis can affect neutrophil homeostasis

Hyperglycaemia: Diabetic mice present monocytosis and neutrophilia due to an increased production of S100A8/S100A9 by blood neutrophils that is associated with the exacerbation of atherosclerosis in *Ldlr*$^{-/-}$ mice [166].

Chronic stress: Neutrophil content in plaques of *Apoe*$^{-/-}$ mice increases after stress and correlates with levels of noradrenaline, a stress hormone that enhances the synthesis of CXCL12 [167]. This increased neutrophil presence is thus due to an enhanced myelopoiesis.

B/ The identification of neutrophils in plaques

1. In human vulnerable plaques

Since *Trillo et al.* identified neutrophils in aortic fatty streak lesions from African green monkeys [168], the presence of neutrophils in human plaque specimens has been clearly evidenced. A first study in 2002 revealed an infiltration of neutrophils, identified by their specific marker CD66b, in ruptured and eroded coronary plaques while they are mainly absent from earlier stage atherosclerotic lesions [169]. Their presence in plaques is also elevated in patients with unstable angina pectoris. More recently, neutrophils have been found at the shoulders of human carotid plaques, next to intraplaque haemorrhages (**Figure 14**) [29] and underneath the luminal endothelium [170]. Moreover, azurocidin, NGAL, defensins, and LL-37 are all neutrophil granule proteins and have all been identified in atherosclerotic lesions [171]–[174].

2. Identification of neutrophils in murine plaques

In early atherosclerotic lesions of *Apoe*$^{-/-}$ or *Ldlr*$^{-/-}$ mice fed a HFD for 4 to 6 weeks, neutrophils are localised, by the detection of their specific membrane protein Ly6G, in the sub-endothelial and intimal areas of plaques (**Figure 14**) [116], [175]. In more advanced lesions of *Apoe*$^{-/-}$ or *Ldlr*$^{-/-}$ mice fed a HFD for more than 10 weeks, neutrophils locate in the plaque shoulders and fibrous caps as well as in the adventitia [175], [176]. Interestingly, neutrophils localise next to monocytes in advanced lesions, suggesting a cooperation between the two cell types.

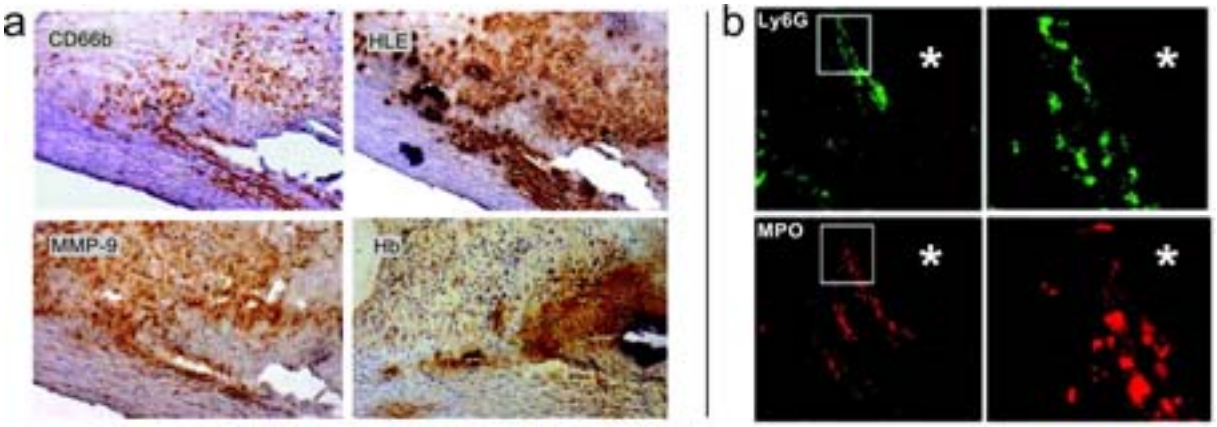

Figure 14. Immunostaining showing the colocalisation of neutrophils with their enzymes in human culprit plaques and murine aortic root lesions. *(a) Serial human carotid plaque sections immunostained for the human neutrophil marker CD66b, human neutrophil elastase (HLE), MMP-9 and haemorrhages (Hb, haemoglobin). Objective original magnification x 10 (adapted from Leclercq et al., 2007). (b) Serial sections of plaques from Apoe$^{-/-}$ mice showing the colocalisation of neutrophils (Ly6G) and MPO (adapted from Zernecke et al., 2008).*

C/ Role of neutrophils in atherogenesis

In humans, in various conditions associated with increased atherogenesis, such as hypertension, sleep apnea, hyperglycaemia or periodontitis, circulating neutrophils are activated [177], suggesting that neutrophils could contribute to plaque formation.

1. Implication of neutrophils in murine atherogenesis

Several studies have implicated neutrophils in atherogenesis. Van Leeuwen *et al.* showed that neutrophils are more abundant in early plaques of *Ldlr*$^{-/-}$ mice compared to advanced stage lesions, suggesting that neutrophils are primarily involved in early atherosclerosis [175]. This implication was further confirmed in *Apoe*$^{-/-}$ mice in which neutrophil aortic counts are reduced after 16 weeks of HFD [116]. Moreover, the depletion of neutrophils reduces lesion size in *Apoe*$^{-/-}$ mice, while conversely the number of circulating neutrophils correlates to lesion size.

The link between plaque neutrophils and atherogenesis has been shown in several other mice models. Indeed, the disruption of the CXCR4-CXCL12 axis [151], [178], the IL-17 pathway [150], the CCL3 pathway [179] and the endocannabinoid pathway [180], [181] also linked increased plaque size to neutrophil content.

Finally, plaque size is decreased in mice in which the neutrophil enzymes, MPO and NADPH oxidase, are inhibited [182]. Similarly, blocking of NET formation limits the extent of atherosclerosis in *Apoe*$^{-/-}$*Ldlr*$^{-/-}$ mice.

2. Potential mechanisms

The main mechanisms by which neutrophils impact atherogenesis are recapitulated in **Figure 15**.

Promotion of endothelial permeability: Neutrophil products are known to affect ECs, as shown in studies on microvasculature. For example, azurocidin induces EC activation, contraction and permeability [183].

Attraction of monocytes: In neutropenic mice fed a HFD, aortas contain less monocytes and macrophages, suggesting that neutrophils enhance early monocytic infiltration [184]. In mice lacking CRAMP (LL-37 murine homologue), lesion size and macrophage content diminishes because of the defective adhesion of monocytes to plaques [185], mediated potentially through formyl-peptide receptor 2 [186]. In addition to increasing monocyte adhesion, neutrophil-derived proteins such as LL-37, azurocidin, cathepsin G and α-defensin exert direct chemotactic activities on monocytes [187]. Neutrophil serine proteases also enhance the chemotactic activities of monocyte chemokines. Moreover, azurocidin and proteinase 3 elicit ECs to express adhesion molecules and secrete CCL2, leading to monocyte recruitment.

Formation of foam cells: Neutrophils are cells with substantial oxidative capacities mediated through MPO, NADPH oxidase, lipoxygenases and ROS, and thus they contribute to the oxidation of LDL retained in the vascular walls [188]. Moreover, MPO promotes the oxidation of HDL, which further impairs ABCA-1-dependent cholesterol efflux, and thus contribute to the formation of foam cells [189]. Neutrophil α-defensin also induces the release of ROS by ECs and macrophages. Moreover, azurocidin, LL-37 and α-defensin promote scavenger receptor expression in macrophages, therefore contributing to oxLDL internalisation and foam cell formation.

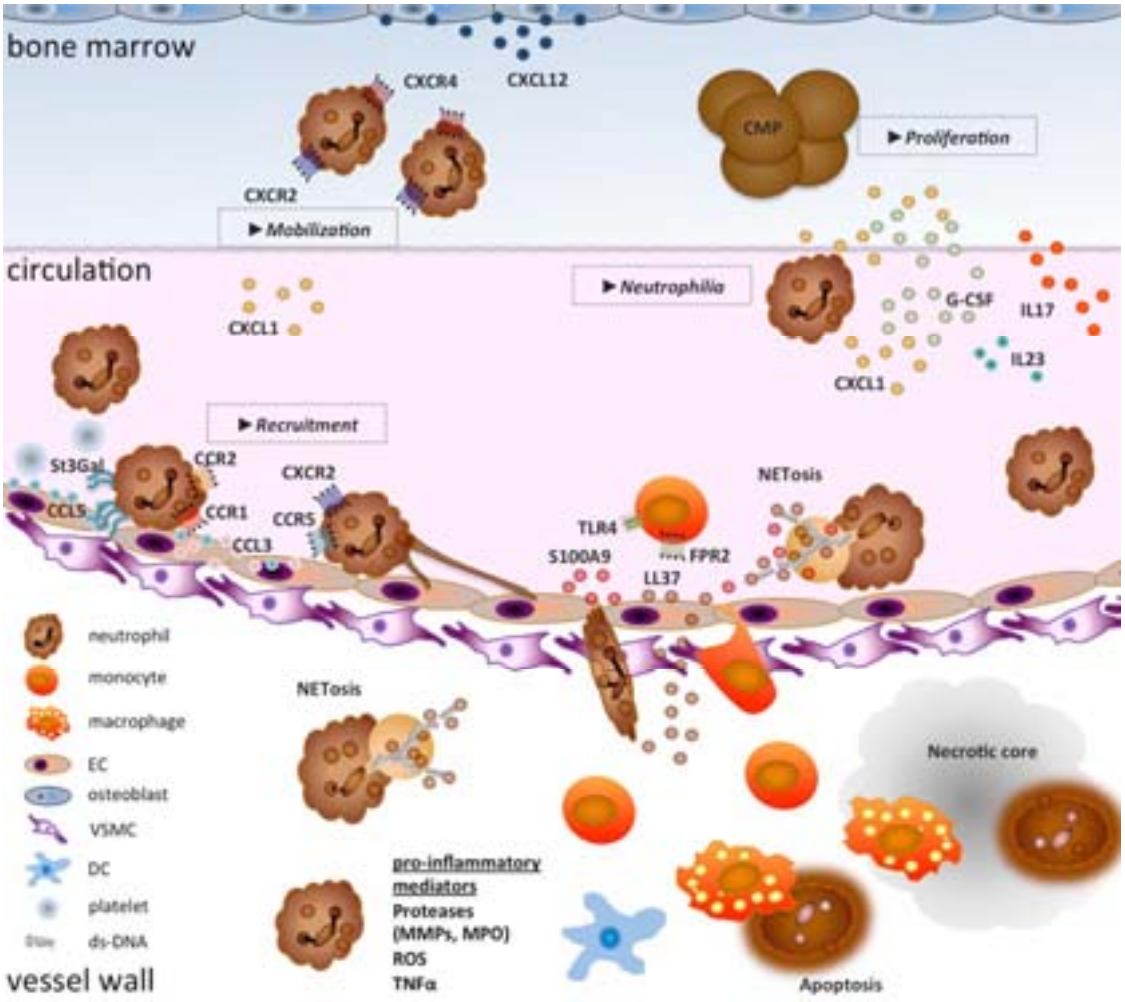

Figure 15. Roles of neutrophils in atherogenesis. *Neutrophil mobilisation and subsequent neutrophilia is promoted by G-CSF, IL-23, the disruption of the CXCL12-CXCR4 axis and increased plasma levels of CXCL1. The recruitment of neutrophils to plaques involves the endothelial deposition of platelet-borne CCL5 and macrophage-derived CCL3, acting through CCR1, CCR3 and CCR5. Once in plaques, neutrophils contribute to the attraction of monocytes by releasing NETs and proteins such as LL37 or S100A8/A9. Moreover, neutrophil mediators promote the formation of foam cells, the activation of dendritic cells (DC) and the formation of a necrotic core. St3Gal, ST3 beta-galactoside alpha-2,3-Sialyltransferase; FPR, Formyl peptide receptor; ds-DNA, double-strand DNA.*

D/ Neutrophils play a part in plaque destabilisation

1. Murine plaque vulnerability and neutrophils

Neutrophils could also impact plaque features of vulnerability. Indeed, besides its role in atherogenesis, neutrophilia contributes to plaque vulnerability, as it increases neutrophil, macrophage, and apoptotic cell content while reducing SMC proportion [178]. A number of studies found decreases

in collagen content, increases in MMP-9 and decreases in SMCs to be concomitant to neutrophil, but not macrophage, plaque infiltration [180], [181], [190]–[192]. However, the deletion of the neutrophil chemokine CCL3 do not impact plaque vulnerability, despite limiting neutrophil numbers in lesions, which could be attributed to an increased turnover rather than a reduced recruitment. It is worth noting that statins also reduce neutrophil and macrophage infiltration, and collagenolysis in plaques of HFD-fed *Apoe*$^{-/-}$ mice in a model of accelerated atherosclerosis induced by collar placement [193].

Finally, a recent study showed that LPS-induced lung injury results in plaque destabilisation in a neutrophil-dependent manner [85]. In this study, neutrophil depletion prevented plaque rupture, as defined by the manifestation of intraplaque haemorrhages or superimposed thrombi (**Figure 16**). This study therefore suggests that neutrophils could link infection to atherosclerotic plaque rupture.

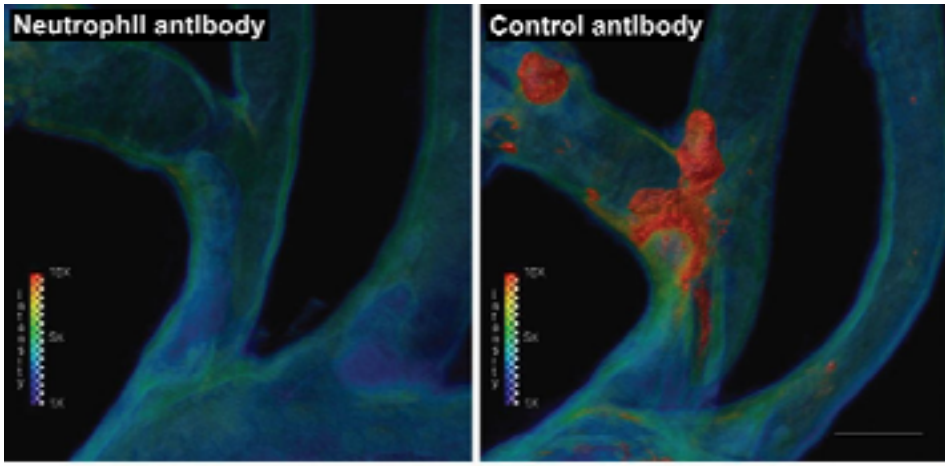

Figure 16. Optical projection tomography fluorescence intensity maps showing murine plaque disruption during LPS-induced lung injury. *Intensity maps of the brachiocephalic trunk from LPS-exposed mice injected with neutrophil-specific antibodies (left) or control antibodies (right). The red colour reflects the autofluorescent signal of blood and thrombi. Scale bar = 500 µm (adapted from Jaw et al., 2016).*

2. Neutrophils are linked to human plaque destabilisation

As previously mentioned, in patients with unstable angina pectoris or in patients who died of myocardial infarction, advanced type IV lesions contain neutrophils [169]. *Leclercq et al.* demonstrated that neutrophil products are released in higher quantity in culprit human plaques compared to non-culprit plaques (**Figure 14**) [29]. Moreover, they showed that neutrophils colocalise with intraplaque haemorrhages, a feature of plaque vulnerability, and with proteolytic enzymes, such as MMP-8, MMP-9, proteinase 3 and elastase. The number of neutrophils in plaques also correlates to features of carotid plaque vulnerability: enlarged lipid core, increased macrophage presence, and decreased SMC and collagen contents but also to plaque levels of IL-8, MMP-8 and MMP-9 [170], [190], [194], [195].

Moreover, neutrophils and macrophages could be important sources of oxidant enzymes, as they are localised in human plaques in oxidant-producing areas [196].

These data are indicative of the role of neutrophils and neutrophil-derived proteases in human plaque vulnerability.

3. Potential mechanisms of neutrophil-mediated plaque vulnerability

Induction of endothelial erosion: Neutrophils release MPO when activated by oxLDL or C-reactive protein. MPO generates HOCl which stimulates EC apoptosis [188] and reduces the bioavailability of NO. Moreover, MMP-2 and MMP-9 degrade type IV collagen, the constituent of the subendothelial basement membrane, and could thus induce to the detachment of ECs. Finally, MMP-8 and proteinase 3 have been shown to induce directly EC apoptosis.

Fibrous cap thinning: The colocalisation of neutrophils with MMP-8, MMP-9, elastase and proteinase 3 suggests they contribute to the degradation of matrix fibres in plaques [29], [170]. Indeed, MMP-9 and elastase degrade type IV collagen and elastin, and MMP-8 degrades the type I and III interstitial collagen of the fibrous cap. In several mouse models and human studies, reduced plaque collagen content correlates to the presence of neutrophils and MMP-9 [190], [195]. Moreover MPO-derived products favour the activation of MMPs from their preforms and the inactivation of TIMPs [197], [198].

Activation of macrophages: Secretory products of neutrophils stimulate the differentiation of monocytes to a pro-inflammatory phenotype and increase macrophage phagocytic capacities. In patients with acute coronary syndrome, neutrophil elastase has been shown to impair clearance of haemoglobin in plaques by macrophages through CD163 cleavage. This could favour a more vulnerable plaque phenotype [199].

NET-mediated priming: NETs are found in murine [200] and human plaques [201], [202]. Cholesterol crystals induce NET formation by neutrophils, which stimulates the production of IL-1β by macrophages (**Figure 17**) [133]. Therefore NETs can induce proinflammatory responses in plaques.

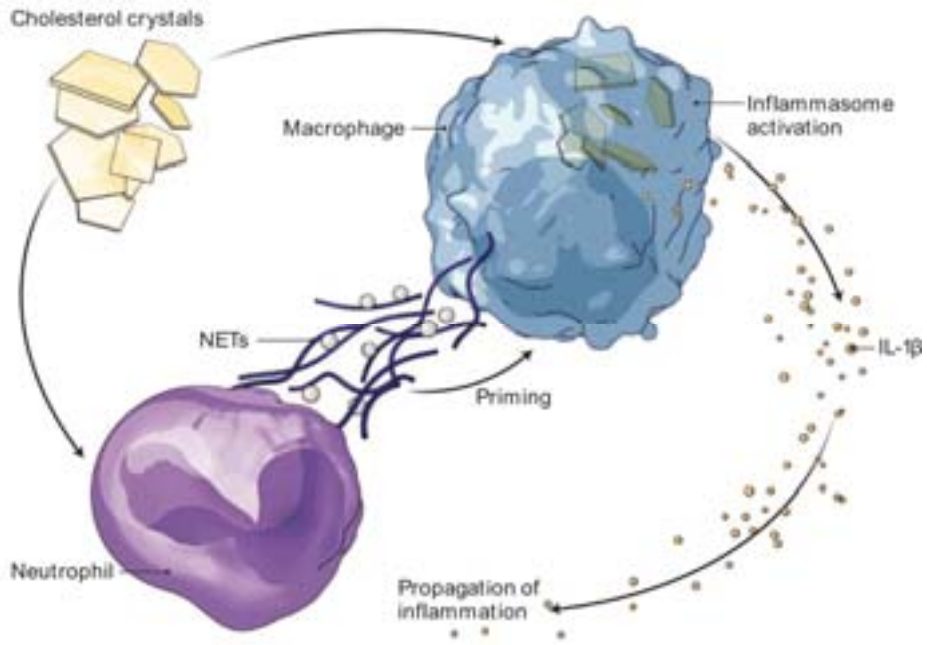

Figure 17. Cholesterol crystals induce the release of NETs which prime macrophages.*Neutrophils are activated by cholesterol crystals in atherosclerotic plaques and release NETs which induce the activation of inflammasome and prime macrophages to secrete IL-1β.*

E/ Recruitment of neutrophils to plaques

1. Lesion entry

In human plaques, neutrophils localise next to neovessels or areas of intraplaque haemorrhages [29], [203], suggesting they could enter through the plaque microvasculature. Such a recruitment has been shown in *Apoe*$^{-/-}$ mice with advanced lesions [204], in particular through the venules that are present in plaques. Microvessels are not present in early atherosclerotic plaques, and therefore this mechanism of entrance could potentially occur only in late-stage atherosclerosis.

In less advanced lesions, neutrophils and other leukocytes are thought to enter the lesion by transmigration from the arterial lumen. Despite the shear stress in arteries being relatively high, neutrophils can form tethers and slings when adherent to the endothelium which slow down rolling under high shear forces. Moreover, during atherosclerosis and hyperlipidaemia, ECs express more adhesion molecules, which favour the adhesion of leukocytes. In murine carotid arteries, neutrophils were found to be rolling on the endothelium of the carotid artery [116], [205] and the abdominal aorta [206] (**Figure 18**). In support with a luminal entry, several studies showed neutrophils adherent to the plaque endothelium [151] and NETs to be localised at the luminal surface of both murine and human plaques [201].

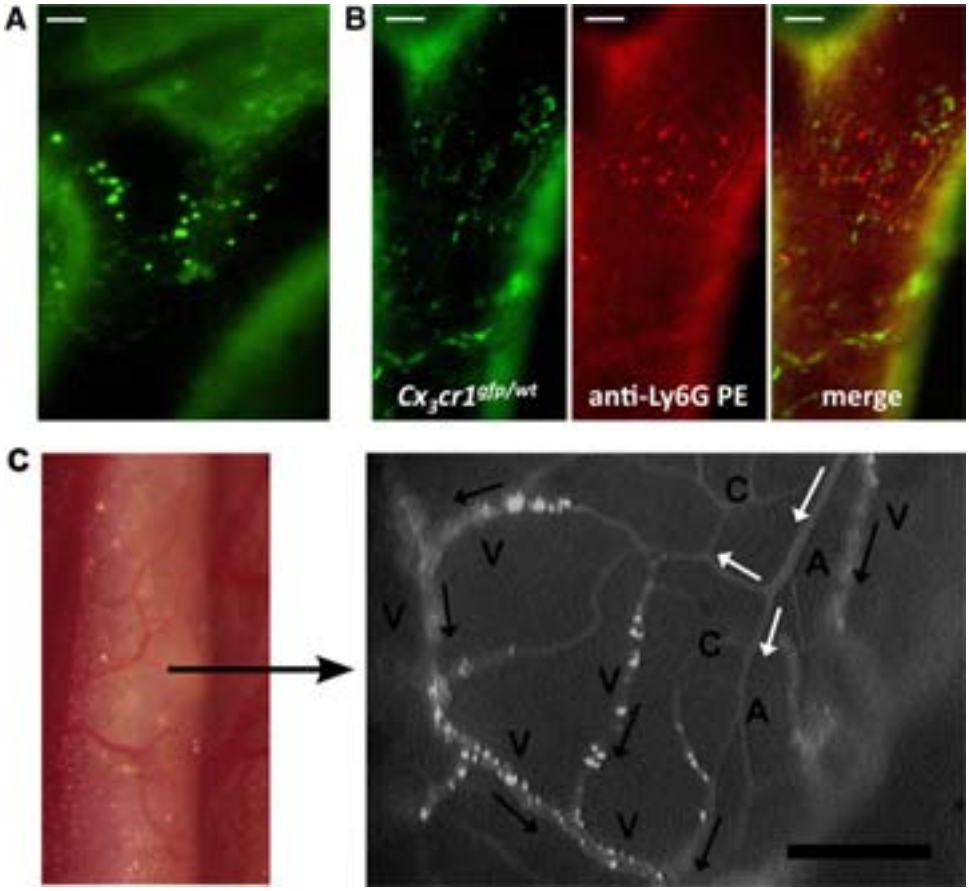

Figure 18. Possible mechanisms of arterial neutrophil recruitment. *(A-B) Intravital microscopic observations of neutrophils adherent to the external carotid artery of Lysm$^{egfp/egfp}$Apoe$^{-/-}$ mice depleted in monocytes (A) and Cx3cr1$^{egfp/wt}$Apoe$^{-/-}$ mice injected with anti-Ly6G PE (red) antibody (B). Scale bar = 100 µm (Soehnlein, 2012). (C) Intravital microscopic observations of microvessels in an advanced abdominal aortic plaque of an Apoe$^{-/-}$ mouse. (Right) image showing the presence of visible microvessels (red) on the plaque (perlaceous). (Left) Staining of P-selectin in plaque microvessels by injection of fluorescent beads coated with a fluorescent anti-mouse P-selectin antibody. Arrows show the direction of the arterial (white) and venous (black) blood flow. A, arterioles; C, capillaries; V, venules. Scale bar = 200 µm (adapted from Eriksson, 2011).*

2. Pathways involved in neutrophil recruitment to plaques

Various chemokines and pathways are directly or indirectly implicated in the recruitment of neutrophils to atherosclerotic plaques (**Table 2**).

CC chemokines: In hyperlipidaemic *Apoe*$^{-/-}$ mice, neutrophil infiltration is dependent on the deposition of CCL5 by platelets on the surface of the plaque endothelium [116]. Interestingly, neutrophil numbers are also increased in the blood, which could be attributable to higher levels of CXCL1 in serum and the increased expression of CXCR2 on bone marrow neutrophils, facilitating their mobilisation from the

bone marrow. Another chemokine involved in neutrophil recruitment during atherogenesis is CCL3 [179], as shown in *Ldlr*$^{-/-}$ mice transplanted with the bone marrow of *Ccl3*$^{-/-}$ mice. These *Ccl3*$^{-/-}$ neutrophils are less responsive to CXCL1 which could explain their reduced number in the blood. Therefore both CCL3 and CCL5 seem to favour neutrophil bone marrow mobilisation and infiltration in plaques during lesion development.

CXCL1: CXCL1 itself is a chemoattractant for neutrophils. In mice, treatment blocking nicotinamide phosphoribosyltransferase (NAMPT) reduces intraplaque levels of CXCL1 and neutrophil recruitment to plaques [192]. Similarly, Evasin-3, an inhibitor of CXCL1 and CXCL2, decreases neutrophil plaque infiltration [191]. Accordingly, in *Apoe*$^{-/-}$ mice lacking the IL-17 pathway, neutrophils are less recruited to plaques because of the reduced CXCL1 expression in aortic lesions [150]. Moreover, the involvement of the CXCL1-CXCR2 axis has also been evoked as a potential means by which mast cells recruit neutrophils to atherosclerotic plaques [207].

The endocannabinoid pathway: The endocannabinoid pathway involves two cannabinoid receptors CB1 and CB2 that bind neuromodulatory lipids which can be degraded by the fatty acid amide hydrolase (FAAH). In human and murine plaques, CB2 expression associates to neutrophil and MM9 contents. Antagonising CB2 prevents neutrophil infiltration in plaques of *Apoe*$^{-/-}$ mice, while plaques from *Apoe*$^{-/-}$ mice knockout for FAAH conversely display increased neutrophil and MMP-9 contents [181], [190]. This was further confirmed with an inhibitor of FAAH [208]. It is thus possible that endocannabinoids play a part in neutrophil plaque infiltration.

Endocannabinoids could mediate their effects through CXCL1, as blocking of a potential endocannabinoid receptor increases CXCL1 and CXCL2 aortic expressions [180] while FAAH deficiency reduces CXCL1 content in plaques.

ApoA1: Treatment of *Apoe*$^{-/-}$ mice with anti-ApoA1 augments neutrophil plaque content probably as a result of increased chemokine production and enhanced migratory capacities [195]. Interestingly, these associations are also found in plaques from patients seropositive for antibodies anti-ApoA1.

		Mouse genotype	Strategy	Diet (weeks)	Parameters assessed			Cellular population				Mecanisms
					Lesion	collagen	NC	SMC	Mϕ	L_T	PMNs	
	Atherogenesis	*Apoe*$^{-/-}$	Neutrophil depletion	HFD (4)	↓				↓	↑	↓	CXCL1, CXCL2 and platelet deposition of CCL5
		Apoe$^{-/-}$	IL-17 double KO	HFD (15)	↓				↓	↓	↓	IL-17 control of CXCL2/1 chemokine expression
		Ldlr-/-	myeloid KO for KLF2	HFD (8)	↑						↑	Increased neutrophil adhesion
Vulnerability		*Ldlr*$^{-/-}$	CCL3 KO BMT	HFD (12)	↓	↔	↔		↔	↔	↓	CCL3-mediated recruitment
		Apoe$^{-/-}$ *and Ldlr*$^{-/-}$	BMT/KO/ inhibitor CXCR4	HFD (6)	↑		↑	↓	↑	↓	↑	CXCR4-induced neutrophilia
		Apoe$^{-/-}$	Anti-ApoA1	chow	↑	↓			↔	↔	↓	Migration towards chemokine induced by anti-ApoA1
		Apoe-/- and collar	CB2 antagonist	HFD (12)	↔	↔			↔		↑	Related to endocannabinoid receptor CB2
		Apoe$^{-/-}$	FAAH KO	HFD	↓	↔		↓	↔	↓	↑	FAAH deficiency increased CXCL1
		Apoe-/- and collar	NAMPT inhibitor	HFD	↔	↑			↔		↓	Decrease in CXCL1 by NAMPT activation
		Apoe-/- and collar	Inhibition of CXCL1	HFD (12)	↔	↑			↔		↓	Decrease in CXC chemokines (CXCL1)
		Apoe$^{-/-}$	Inhibition of endocannabinoids	chow	↔	↓	↔		↔	↔	↑	Increase in CXCL1/ CXCL2

BMT = bone marrow transplantation; CB = endocannabinoid receptor 2; FAAH = fatty acid mide hydrolase; KLF = Krüppel-like factor; KO = knockout; NAMPT = Nicotinamide phosphoribosyltransferase

Table 2. Involvement of neutrophil recruitment in murine models of atherosclerosis.

I. Biosynthesis of leukotrienes

A/ Reaction pathway

Eicosanoids constitute a group of lipid mediators derived from arachidonic acid (AA), a polyunsaturated ω6 fatty acid. They include prostaglandins and leukotrienes (LTs) and participate in inflammatory responses, but also contribute to vascular tone and endothelial permeability [146]. The synthesis of LTs involves several enzymes (**Figure 19**).

First, AA must be released from the cellular membrane by the cleavage of phospholipids, a reaction catalysed principally by cytosolic phospholipases A_2 α ($cPLA_2$). Then, AA is converted to the unstable epoxide LTA_4 by the 5-lipoxygenase (5LO) in a two-step catalytic process: the conversion of AA to 5S-HETE, and 5S-HETE to LTA_4. AA is presented to 5LO by a helper protein, named FLAP or 5LO-activating protein. FLAP does not have catalytic activities and belongs to the MAPEG (membrane-associated proteins in eicosanoids and glutathione metabolism) family. Finally, LTA_4 serves as a substrate for the biosynthesis of biologically active LTB_4 by the zinc aminopeptidase LTA_4 hydrolase (LTA_4H) or LTC_4 by LTC_4 synthase. Once released in the extracellular milieu, LTC_4 can further be converted into two other cysteinyl leukotrienes (CysLTs), LTD_4 and LTE_4.

B/ Cellular production of LTB_4

The term leukotriene, which derives from *leukocytes* and *trienes* (three conjugated double bonds), is indicative of their origin. Indeed, LTs are mainly produced by leukocytes, as 5LO expression is restricted to bone marrow derived cells [209]. Neutrophils, monocytes/macrophages, mast cells, dendritic cells and B-lymphocytes contain significant amounts of the enzyme. Non-leukocytes generally have insufficient amounts of 5LO and FLAP to be able to synthesise LTs, even though 5LO expression has been shown in ECs in pulmonary arteries [210]. Moreover, the epigenetic regulation of the 5LO transcript can promote its expression in non-myeloid cells [66].

LTA_4H, on the other hand, is ubiquitously expressed, for instance in SMCs or ECs. Leukocytes express different amounts of LTA_4H, as neutrophils, monocytes, mast cells and lymphocytes are rich in LTA_4H in contrast to eosinophils, basophils and platelets.

Notwithstanding leukocyte-restricted LT production, *in vitro* and *in vivo* studies have shown that LTB_4 can be produced in non-leukocytic cells by transcellular biosynthesis [211]. This process relies on the transfer of LTA_4 produced in leukocytes that express 5LO and FLAP to other non-leukocytic

cells which express LTA_4H. Although LTA_4 is highly unstable, chaperone proteins facilitate its transport to other cells.

C/ Control and regulation of leukotriene production

The production of LTs is a tightly regulated process, the control of which starts with the $cPLA_2$ (**Figure 19**). This ubiquitously and constitutively expressed enzyme is activated by phosphorylation and in response to low level of intracellular Ca^{2+}. Its expression can be modulated by inflammatory mediators, such as cytokines or bacterial products [212], [213]. Moreover, the LPS/TLR4 pathway can increase $cPLA_2$ activation in macrophages following MyD88 activation [214]–[216]. When activated, $cPLA_2$ translocates to the nuclear membrane, resulting in its increased activation.

Similarly to $cPLA_2$, 5LO is Ca^{2+}-dependent and its expression is increased by inflammatory signals, through notably a NF-κB-dependent transcription [217]. Furthermore, 5LO expression is elevated during the differentiation of monocytes into tissue macrophages [218]. To carry out LTA_4 biosynthesis, 5LO must translocate from the cytosol to the nuclear membrane, in close proximity with $cPLA_2$. The phosphorylation of the enzyme can prevent this translocation, while inversely the MAPK and ERK pathways promote it. Once located at the nuclear membrane, 5LO interacts with FLAP, which expression correlates with LT synthesis. Both TNFα and LPS upregulate FLAP expression [219].

LTA_4H is activated during pro-inflammatory responses, for instance following the activation of NLRP3 inflammasome [220].To limit LTB_4 biosynthesis, LTA_4H is covalently inactivated by its substrate, a process named substrate inactivation. During the resolutive phase of inflammation, LTA_4H has dual role, having also been shown to cleave the neutrophil chemotactic PGP peptide (Pro-Gly-Pro), therefore inhibiting neutrophil recruitment.

Finally other mechanisms have been proposed to contribute to the regulation of LT biosynthesis. Another enzyme, the LTB4-inactivating enzyme leukotriene B4 dehydrogenase, has recently been shown to inactivate LTB_4 [221]. In addition, LTC_4 synthase is segregated to the outer nuclear membrane, while LTA_4H is found in the cytoplasm and within the nucleus (**Figure 19**). It has been suggested that the targeting of 5LO to the outer or inner membrane of the nucleus balances the biosynthesis of LTC_4 and LTB_4 [222]. Moreover, LTA_4 availability determines the balance between the formation of CysLTs and LTB_4.

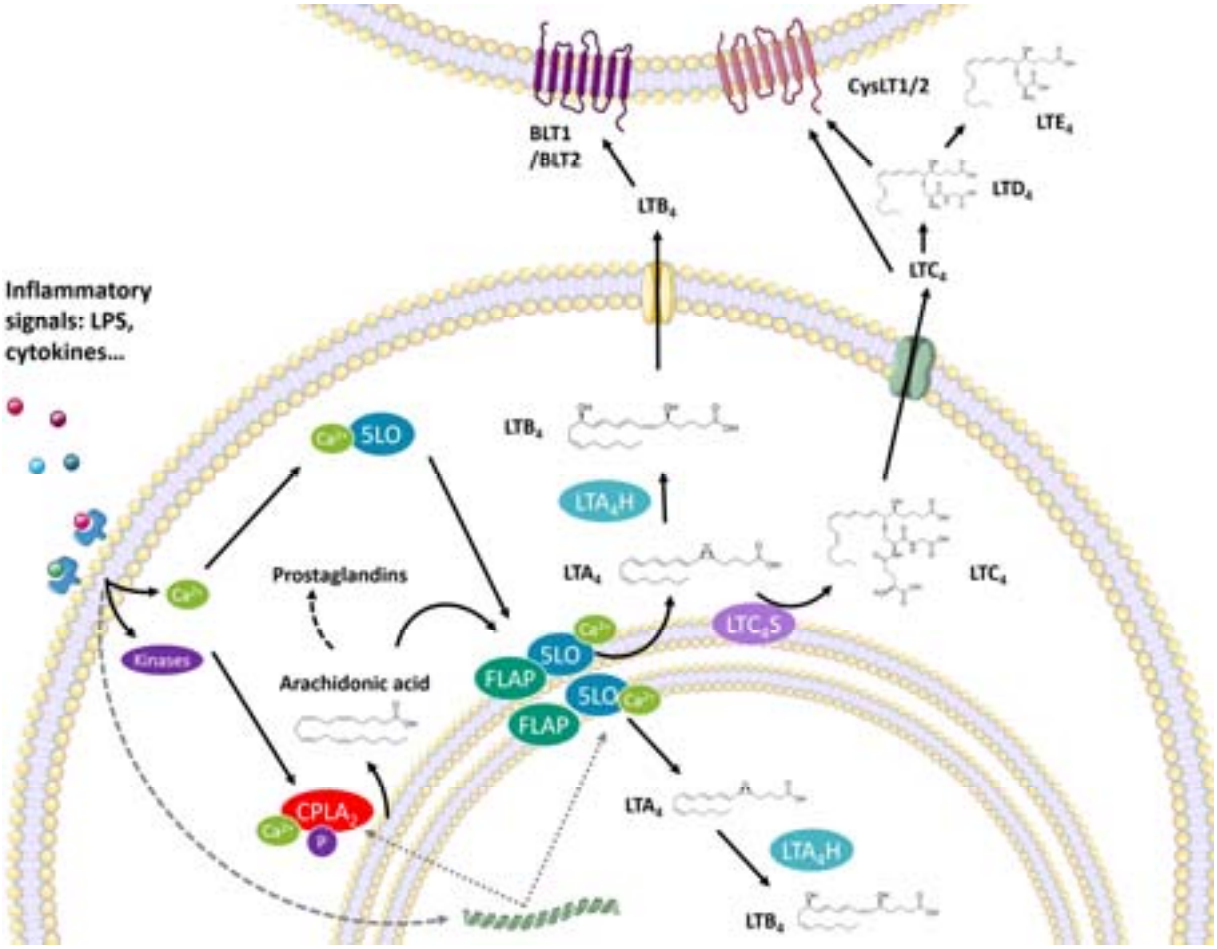

Figure 19. Cellular biosynthesis of leukotrienes. *Upon activation by inflammatory stimuli, $cPLA_2$ and 5LO translocate to the nuclear membrane. $cPLA_2$ releases arachidonic acid from the nuclear membrane, which is then converted into LTA_4 by the enzymatic complex FLAP-5LO. LTA_4 serves as a substrate for the synthesis of LTB_4 and CystLTs. Once secreted, these lipid mediators bind their respective receptors onto target cells.*

D/ Alternative pathways: the SPMs and the 5-oxo-ETE

Two alternative pathways involving enzymes or intermediates of the LT biosynthetic pathway can lead to the formation of lipid mediators from the SPM family or specialised pro-resolving lipid mediator family. First, LTA_4 can be converted into anti-inflammatory lipoxins by two other lipoxygenases, 12LO and 15LO [35]. Second, the action of both 5LO and 12/15LO can result in the transformation of polyunsaturated ω3 fatty acids into resolvins, maresins and protectins, which all have pro-resolutive functions.

These lipids are principally formed during the lipid mediator class switch. This switch is characterised by a temporal change in the type of lipid mediators produced from the same substrate, shifting from pro-inflammatory to pro-resolving mediators. This change could rely on the localisation and phosphorylation of 5LO [145] (**Figure 20**).

In addition, prior to LTA_4 conversion, the intermediary metabolite 5S-HETE can be oxidised into 5-oxo-HETE, which is a potent chemoattractant and activator for eosinophils and, to a lesser extent, for neutrophils [223]. It is worth noting that mice lack the receptor for 5-oxo-HETE [224].

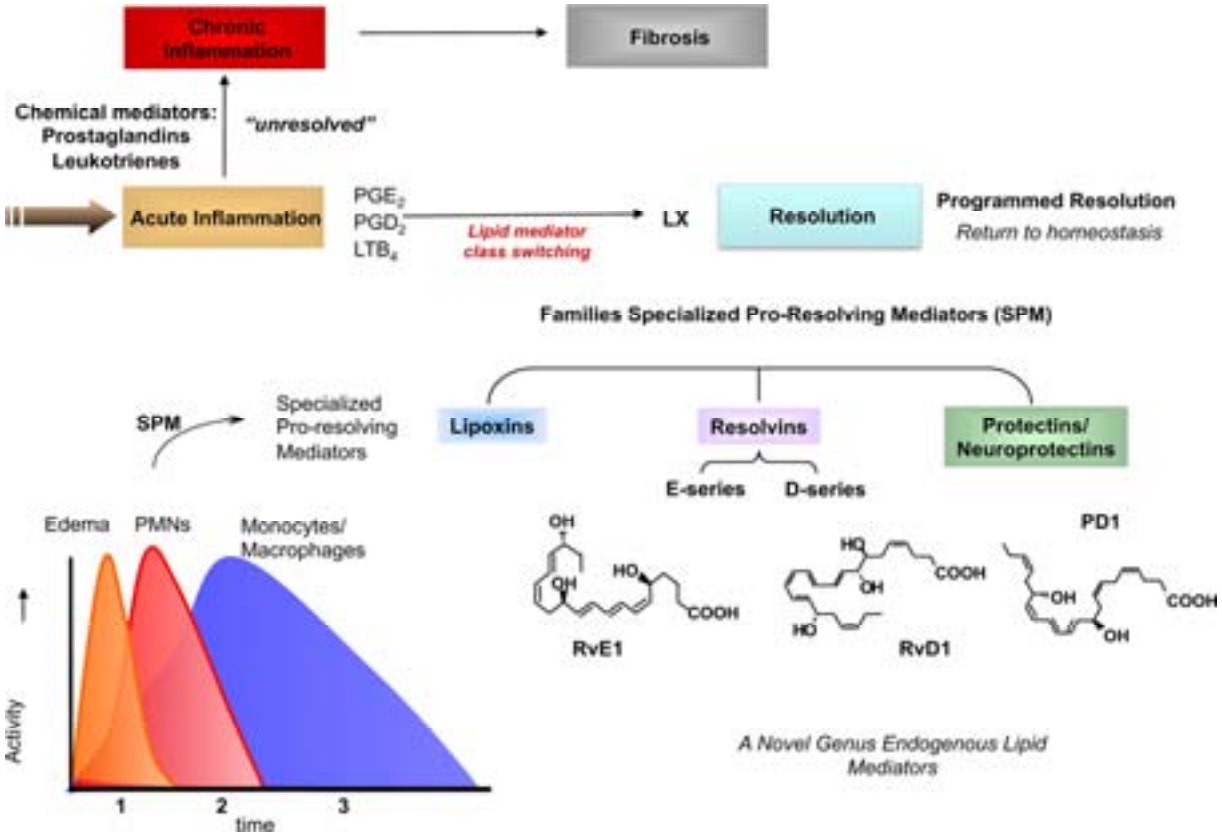

Figure 20. The lipid mediator class switch. *During the transition from the initiation of inflammatory responses to their resolution, the lipid mediators produced by leukocytes temporally switch from pro-inflammatory to pro-resolutive. Tissue oedema is caused by the increased permeability mediated by prostaglandin (PG) and CysLTs. Then, polymorphonuclear neutrophils (PMNs) enter tissue in response to leukotriene B_4 and contribute to inflammatory responses by recruiting monocytes. Subsequently, the apoptosis of neutrophils and their clearance by macrophages switch the production of inflammatory lipid mediators to SPM production, promoting tissue repair (Serhan, 2010).*

II. Functions of leukotrienes

The main actions of leukotrienes are summarised in **Table 3**.

LTs	Producing cells	Target cells	Target Receptors	Responses	Potential pathways
LTB_4	Neutrophils, monocytes, macrophages, dendritic cells. *Transcellular metabolism:* SMCs, endothelial cells	Neutrophils	BLT1	Chemotaxis, recruitment to inflamed tissues	Ca2+, MAPKs, PI3-K
				↑ endothelial adhesion (CD11b, Mac-1)	TYK
				Respiratory burst	PI3-K
				Delayed apoptosis	PI3-K/ERKs
				↑ expression of TLR 7/8/9	NF-κB, MAPKs
			BLT1/BLT2	Degranulation: MMPs, MPO elastase, defensins, azurocidin, LL37	Ca2+ , TYK/MAPKs
		Monocytes/ macrophages	BLT1/BLT2	Chemotaxis	MAPKs
			BLT1	↑ endothelial adhesion (integrin)	
				Production of IL-6 MCP-1, TNFα, IL-1β	NF-κB, MAPKs
				↑ FcR-dependent phagocytosis	PKC, Syk
		Mast cells	BLT1/BLT2	Recruitment of immature mast cells	ERK
		DCs	BLT1/BLT2	↑ chemotaxis of immature and mature DCs in lymph nodes	
				↑ expression CCR7 and production of IL-12 and IFN-γ	
		Eosinophils	BLT1	Chemotaxis after IL-5 priming	
		Lymphocytes	BLT1	↑ T cell recruitment to peripheral tissues	
				↑differentiation to Th17 and ↓differentiation to Treg	
				↑ expression CD23 in resting B cells	
		vSMCs	BLT1	↑ Proliferation and migration	MAPKs, ERKs
				MMP2 release	
		ECs	BLT1	Release of vasoactive factors after LPS and IL-1β stimulation	
LTC_4/LTD_4*	Mast cells, eosinophils, basophils, monocytes/macrophages, dendritic cells. *Transcellular metabolism:* platelets, endothelial cells	Eosinophils	CysLT1	Activation, adhesion and migration	ERK, PKC
		Monocytes/ macrophages	CysLT1	↑ Production of MCP-1, TNFα, MMP9	NF-κB, MAPKs
		Neutrophil	CysLT1	-	
		Mast cells	CysLT1	Proliferation and activation	ERK
				↑ Synthesis of IL-5, TNFα, MIP-1β	MAPK/ERK
			CysLT2	IL-8 release	p38
		SMCs	CystLT1/2	Vaso- and broncho-spasms	ERK
		ECs	CystLT2	↑ permeability and plasma leakage	PKC
				PAF accumulation, leukocyte adhesion (P-selectin)	
				NO production, IL-8 and MIP-2 expression	
			CysLT1	Release of contractile factors after IL-1β stimulation	

LTC4/LTD4 also act on DCs, lymphocytes, basophils, and fibroblasts

ERK = extracellular signal–regulated kinase; MAPK = mitogen-activated protein kinase; PAF = platelet-activating factor; PI3-K = phosphatidylinositol-4,5-bisphosphate 3-kinase; PKC= protein kinase C; TYK = tyrosine kinase

Table 3. Principal sources, targets, and functions of leukotrienes.

A/ Leukotriene receptors

Once synthesised, LTs are transported out of the cell via multidrug resistance protein (MRP) pumps (**Figure 19**). They act on target cells by activating G protein-coupled receptors (GPCRs), leading to increased intracellular Ca^{2+}, reduced cAMP and activation of downstream kinases.

BLT1 and BLT2 are the two known receptors for LTB_4. The high-affinity receptor BLT1 is expressed mainly on inflammatory cells such as neutrophils, monocytes and lymphocytes. However, it was recently shown that BLT1 expression can be induced in non-leukocytic cells. For example, LPS and cytokines up-regulate the expression of BLT1 in SMCs [14], [225] and ECs [226]. On the contrary in monocytes and neutrophils, TNFα and LPS are known to decrease BLT1 mRNA and protein expression, supposedly to limit the spreading of inflammation.

BLT2 has a 20-fold lower affinity for LTB_4 than BLT1 but is on the contrary ubiquitously expressed. The physiological role of BLT2 is not yet known although it has been implicated in LPS responses, neutrophil degranulation, and macrophage and mast cell chemotaxis [214].

CysLTs act on two major receptors CysLT1 and CysLT2. Moreover, the orphan receptor Grp17 has been proposed as a receptor for CysLTs, and the more stable LTE_4 with low biological activities potentially acts through the "CysLTE" receptor. As for BLTs, cytokines and inflammatory signals (such as LPS) can also regulate the expression of CysLT receptors.

SPMs act mainly through GPCRs such as FPR2/ALX, Gpr32, ChemR23 or Gpr18, and can inhibit BLT1. Not all these receptors are specific of SPMs, as for instance FRP2/ALX can bind the acute phase protein serum amyloid A, which is produced in response to bacterial infection [35].

B/ Responses of immune cells to leukotrienes

1. Neutrophils

LTB_4 is a known neutrophil chemoattractant, which is as potent as IL-8 or C5a in early inflammatory responses [227]. In response to injury signals, neutrophils produce LTB_4 to recruit other neutrophils from distant sites and induce swarming in sterile injured sites [228] (**Figure 21**). Therefore, LTB_4 initiates neutrophil recruitment in response to primary signals, which will in turn amplify the recruitment of other leukocytes by cytokines and chemokines [119].

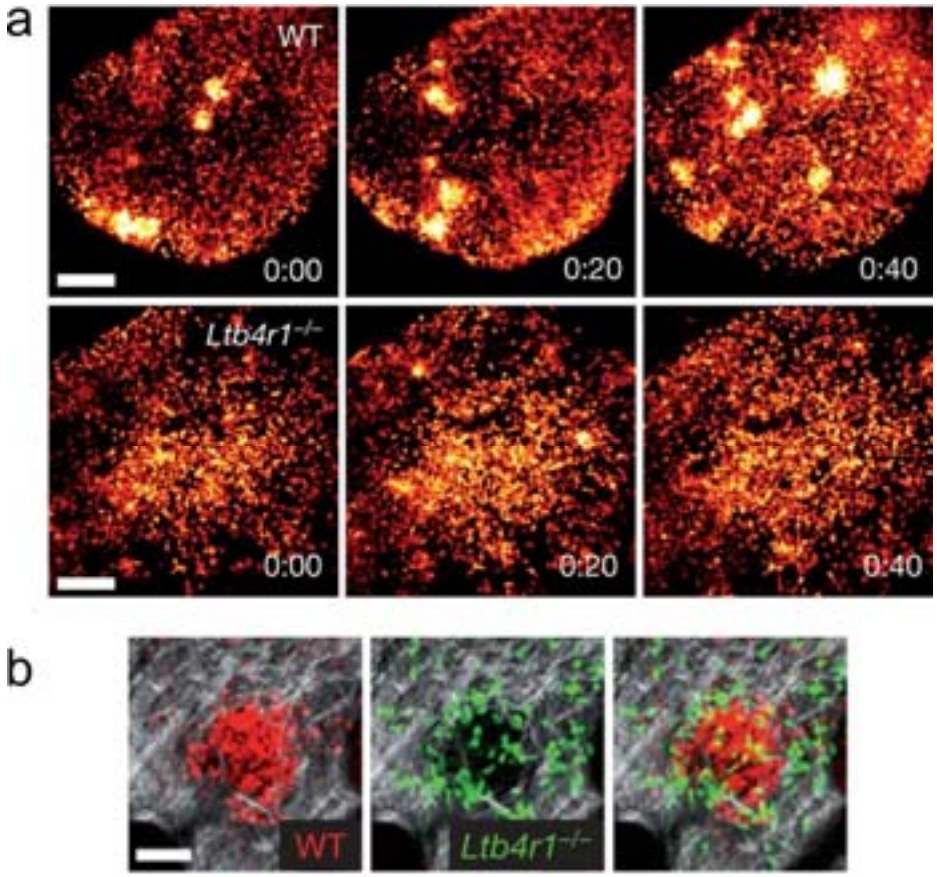

Figure 21. LTB_4 is required for the clustering of neutrophils at sites of infection and injury. *(a) Intravital time-lapse observation of subcapsular sinuses from $Lyz2^{gfp/+}$ (top) and $Blt1^{-/-}Lyz2^{gfp/+}$ ($Ltb4r1^{-/-}$ bottom) mice infected with Pseudomonas aeruginosa showing the LTB_4-dependent neutrophil (GFP-positive view in pseudo-colour) swarming in the infected lymph nodes. Scale bars = 100 µm. (b) Intravital observation of neutrophil clustering following a focal injury (black area) and after co-injection of $Blt1^{-/-}$ ($Ltb4r1^{-/-}$) and wild-type (WT) neutrophils. Collagen fibres were visualised by collagen second harmonic generation. Scale bar = 50 µm (adapted from Lämmermann, 2013).*

In complement to its chemotactic function, LTB_4 primes and activates neutrophils. Indeed, it induces the expression of adhesion molecules, such as CD11b and CD11b/CD18, onto neutrophils [229]. The stimulation of neutrophils with high concentrations of LTB_4 promotes the release of their granule proteins: MMPs, elastase, defensins, MPO and azurodicin [230]. This stimulation also engenders ROS production and enhances the respiratory burst induced by immune-complexes in an autocrine/paracrine-manner [231]. Moreover, *in vivo*, the administration of LTB_4 elevates plasmatic α-defensin levels in humans [232]. Cathelicidin LL37 is another important antimicrobial peptide secreted by neutrophils in response to the activation of the BLT1/LTB_4 pathway. This peptide in turn favours LTB_4 production in neutrophils in a positive-feedback loop [233]. Another antimicrobial neutrophil response induced by LTB_4 resides in the increased TLR expression on the cell surface [137].

Moreover, LTB_4 has been shown to delay neutrophil apoptosis dependently on PI3-K and ERK pathways [234].

In contrast to the powerful effects exhibited by LTB_4 on neutrophils, CysLTs have only a minor priming function [235]. SPMs on the other hand reduce neutrophil infiltration in models of bacterial infections and peritonitis, principally by reducing the surface expression of adhesion receptors, and by counteracting LTB_4 activation [35].

2. Monocytes/macrophages

Monocytes and macrophages express both BLT1 and BLT2 [218], and thus LTB_4 exerts several actions on these cells: chemoattraction, release of pro-inflammatory cytokines such as IL-6 or IL-1β, and the monocyte chemokine CCL2 [236], [237]. LTB_4 also enhances the phagocytic capacities of macrophages for pathogens and FcR-mediated phagocytosis [238], [239]. This is indicative of the antimicrobial functions of LTs on the monocytic system.

CysLTs impact monocyte and macrophage functions, in particular by promoting the production and release of inflammatory cytokines, such as CCL2, IL-8 and TNFα, and proteases, such as MMP-9 [240]. Most action of CysLTs on monocytes are thought to result from CysLT1 activation and downstream transcription by NF-κB [241].

Conversely, SMPs are known to enhance phagocytosis of apoptotic cells and bacterial clearance by macrophages [35].

3. Other immune cells

Mast cells are responsive to both CysLTs and LTB_4. Indeed, LTB_4 has been implicated in mast cell chemotaxis both *in vitro* and *in vivo* [242], [243]. CysLTs have a more potent action on mast cells, as they promote their proliferation, activation and synthesis of cytokines, such as TNFα or IL-8 [230], [244].

LTB_4 also promotes the recruitment of immature and mature dendritic cells to inflammatory sites while CysLTs are involved in their trafficking in lymphatic vessels [245].

The action of LTB_4 on T lymphocytes is rather pro-inflammatory as it promotes their infiltration in peripheral tissue and their differentiation into Th17, while also inhibiting their transformation to a regulatory phenotype. On B cells, LTB_4 has been shown to promote adhesion [246].

CysLTs exert important effects on eosinophils: recruitment, adhesion and activation, whereas LTB_4 only weakly primes eosinophils [213].

C/ Actions of leukotrienes on other cells

1. Smooth muscle cells

Since the discovery of LT receptors on vascular SMCs [14], [225], LTs are acknowledged as actors in vasoconstriction and vasodilatation. For instance, LTB_4 induces the contraction of both human coronary and pulmonary arteries.

CysLTs also induce the contraction of SMCs and promote the proliferation of airway SMCs [230].

2. Endothelial cells (ECs)

ECs do not constitutively express BLT1 or BLT2, but their expression can be differentially regulated depending on the immune stimulus [226]. The resulting responses in ECS are elusive, as LTB_4 could possibly promote the adhesion of leukocytes to ECs. However, this effect might not reflect the direct activation of ECs [247]. LTB_4 could also stimulate the production of vasoactive substances, such as NO [226].

On the other hand, CysLTs are well-known to increase endothelial permeability and to promote the expression of adhesion molecules and the production of vasorelaxant factors [248].

III. Leukotrienes in atherosclerosis

A/ Leukotriene biosynthesis in atherosclerosis

In 1988, De Caterina *et al.* demonstrated that the content of white blood cells in human carotid plaques correlates with LTB_4 production, pointing out the role of leukotrienes in atherosclerosis [249]. In the same decade, CysLTs were shown to be produced in coronary arteries [250].

Human atherosclerotic arteries are enriched in the enzymes of the LT biosynthetic pathway compared to healthy arterial tissues [251]. These enzymes colocalise mainly with macrophages, foam cells, mast cells, neutrophils and dendritic cells, suggesting a myeloid production of LTs in human plaques (**Figure 22**). Furthermore, in arterial tissues, 5LO-positive cells accumulate at the vicinity of T lymphocytes, suggesting the potential involvement of T cells in LT production [218], [252]. Finally, hypercholesterolemia can stimulate LTB_4 production by enhancing the nuclear localisation of 5LO in neutrophils [253], but also in adipocytes [254].

5LO is expressed in the intima, media and adventitia and its expression correlates with atherosclerosis severity [218], [255]. Moreover, in plaques from patients undergoing carotid endarterectomies, Qiu *et al.* demonstrated that human plaques could produce LTB_4 following *ex vivo* stimulation and express 5LO, FLAP and LTA4H [256].

The CysLT receptors and biosynthetic enzymes are also expressed in plaques [218], [256], however CysLT production in atherosclerosis is still controversial, as the level of LTC_4S mRNA is not elevated in human plaques in comparison to healthy tissues [218], [256].

12/15LO and the SMP receptor FRP2/ALX are expressed in murine and human plaques [257], [258].

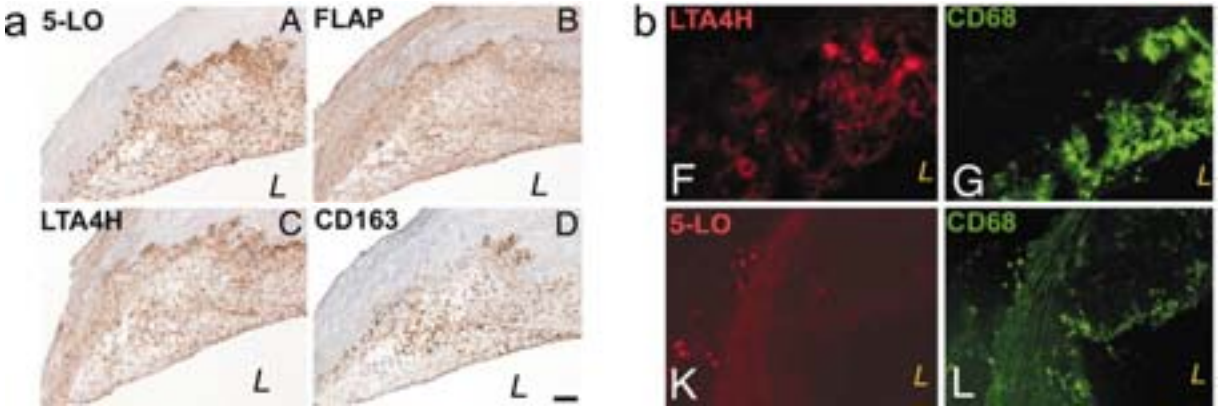

Figure 22. Colocalisation of 5LO, FLAP and LTA_4H with macrophages in human and murine atherosclerotic plaques. *(a) Immunostaining of serial human carotid plaque sections showing the intimal localisation of 5LO, FLAP and LTA_4H in the proximity of CD163-positive macrophages. Scale bars = 200 µm. (b) Co-staining of murine carotid plaque sections for LTA_4H or 5LO with the murine macrophage marker CD68, showing segregated localisation in the intima and the adventitia (adapted from Qiu, 2006).*

B/ Involvement of leukotrienes in cellular responses in atherosclerosis

1. Immune cell recruitment and activation

<u>*Atherogenesis and leukocyte recruitment*</u> (**Figure 23**): BLT1 and BLT2 are localised in areas rich in monocytes/macrophages in human carotid plaques, suggesting that LTB_4 could contribute to monocyte recruitment in atherosclerosis [14]. In line with these findings, plaques from *Apoe*$^{-/-}$ or *Ldlr*$^{-/-}$ mice treated with a BLT1 inhibitor exhibit less macrophages [259]. Monocyte chemoattraction is also amplified by LTB_4-induced expression of CCL2, as shown in *Apoe*$^{-/-}$*Blt1*$^{-/-}$ mice [260]. Consistently, CCL2 expression and leukocyte accumulation is decreased in abdominal aortic aneurysms in *Blt1*$^{-/-}$ mice [261]. Along with BLT1, BLT2 is implicated in monocyte recruitment to plaques [260].

In addition, other cells could be recruited to plaques by LTB_4, and in particular neutrophils, as evidenced in abdominal aortic aneurysms [261]. T cells, which accumulate in close proximity to macrophages in plaques, could also be recruited by LTB_4 [252]. Similarly, in *Apoe*$^{-/-}$ mice knock-out for TFGβ signalling (dnTGFbetaRII) where lymphocytes T are over-activated, T cells have been shown to promote FLAP expression in macrophages. This crosstalk between macrophages and T cells may thus promote the

recruitment of the latter to atherosclerotic plaques in a vicious circle involving LTB_4 [262]. Consistently, MMP-dependent T cell migration is enhanced by LTB_4 [210].

Plaque vulnerability and leukocyte activation: BLT1 and BLT2 colocalise with 5LO and LTA_4H in plaque shoulders, suggesting that they have a role in plaque vulnerability [237]. Accordingly, 5LO, LTA_4H and FLAP expressions correlate with plaque vulnerability in humans [218], [255], [256], [263]. However, this association between intraplaque LTB_4 production and plaque phenotype is still controversial following a recent study [264].

A potential action of LTB_4 on plaque monocytes is the upregulation of NF-κB-dependent inflammatory genes, such TNF-α, IL-6 or CCL2 [237], which could increase the recruitment and activation of circulating monocytes. Moreover, 5LO colocalises with MMP-2 and -9 in plaques of diabetic patients [263] and is associated with increased MMP-2 and CCL3 secretion in murine abdominal aortic aneurysms [252]. MMP-2 and 9 could be either released from T cells, monocytes and neutrophils following LTB_4 activation, or from monocytes through a combined effect of TNF-α and CysLTs [210].

Moreover, LTB_4 upregulates CD36 expression on monocytes and thus favours the differentiation of monocytes and macrophages into foam cells [259], [260], [265]. It is worth noting that this transition is only observed in early but not advanced atherosclerosis.

Finally, in human and murine advanced plaques show an imbalance between LTB_4 and SPMs, which could be explained by the localisation of 5LO in the nucleus rather than in the cytosol [266].

2. Endothelial responses

The role of LTB_4 on ECs is less characterised than the role of CysLTs, which have been shown to promote the production of CXCL2, CCL3 and CCL2, and the expression of P-selectin [248], [252] [267]. In human arteries, LTB_4 receptors are not expressed in ECs, however BLT1 expression can be induced on human ECs *in vitro* by inflammatory stimuli (**Figure 23**), and contributes to the release of vasoactive factors in animals [226]. BLT1 expression is also linked to intimal hyperplasia in human plaques [14].

3. Smooth muscle cells

CysLTs are well known to promote SMC contraction, proliferation and migration. Contrariwise, LTB_4 was shown only recently to induce BLT1-dependent responses in SMCs [14], [225] (**Figure 23**). Indeed, BLT1 immunostaining in both atherosclerotic and healthy arteries revealed that α-actin-positive SMCs express BLT1, demonstrating their propensity to respond to the LTB_4/BLT1 axis. In *Apoe*$^{-/-}$ mice genetically or pharmacologically inactivated for BLT1, atherosclerotic plaques contain less SMCs. Moreover, human coronary SMCs migrate towards LTB_4. In summary, the LTB_4/BLT1 axis mediates the proliferation and migration of SMCs in atherosclerosis [14], [225].

This effect of LTB_4 on SMC proliferation has also been shown in intimal hyperplasia after vascular injury in rabbits. In this context, LTB_4 also promotes the secretion of MMP-2 and integrin signalling in SMCs [268]. This increased secretion of MMPs was later confirmed in *Apoe*$^{-/-}$ mice treated with a BLT1 antagonist [269]. In short, LTB_4 also contributes to plaque proteolytic activities through SMC activation.

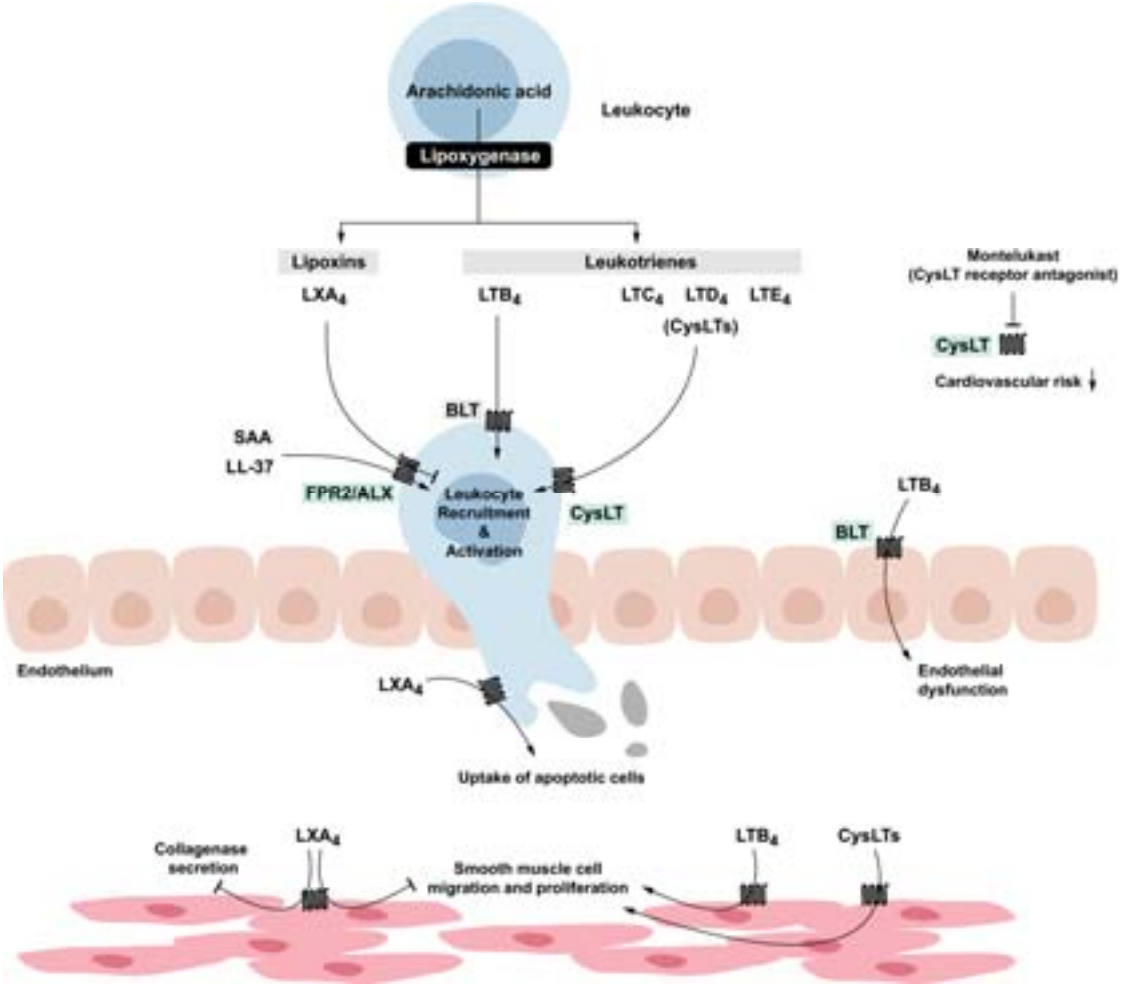

Figure 23. Roles of leukotrienes and other lipid mediators in the regulation of plaque inflammation.
SAA, serum amyloid A; FRP2/ALX, formyl peptide receptor 2/ Lipoxin A_4 receptor (Bäck et al., 2015).

C/ Animal models

The LT pathway in arteries has mainly been studied by genetic or pharmacological targeting in models of atherosclerosis, aneurysms and intimal hyperplasia in mice, rats and rabbits (**Table 4**).

Mouse genotype	Strategy	Age	Diet (weeks)	Gender	Parameters assessed				
					Aortic root lesion	En face	SMC	Macrophages	Necrotic core
C57BL6	*5Lo* $^{-/-}$	26 wk	HFD (18)	F	↓				
Ldlr $^{-/-}$	*5Lo* $^{+/-}$	4-6 mo	HFD (16)	M+F	↓ (-96%)				
	5Lo $^{-/-}$	23 wk	HFD (12)	M+F	↔	↔			
Apoe $^{-/-}$	*5Lo* $^{-/-}$	6-12 mo	Chow	M+F	↔	↔			
	5Lo $^{-/-}$	19 wk	HFD (8)	M+F	↔	↓ (-24%)			
	5Lo $^{-/-}$	8w	HFD (8)	M+F	↔	↔	↔	↔	↔
	5Lo $^{-/-}$ /inhibitor	6 mo	HFD (24)	F	↔	↔	↔	↔	↔
	12 $^{-/-}$ *x 5Lo* $^{-/-}$	6 mo	Chow	F	↓	↓			
	BLT1 antagonist	16-22 wk	Chow	M+F	↓			↓	
	BLT1 antagonist	20 or 32 wk	Chow	M+F	↓	↓	↓	↔	
	BLT2 antagonist	18 wk	HFD (8)	M+F	↔			↔	
	Blt1 $^{-/-}$	<10 wk	HFD (<6)	M+F	↓	↓	↓	↓	
	Blt1 $^{-/-}$	>15 wk	HFD (>8)	M+F	↔	↔	↔	↔	
	Blt1 $^{-/-}$ + hypoxia	>10 wk	HFD (10)	M+F	↓	↓			
	Flap $^{-/-}$	4 mo	HFD (7)	M+F	↔	↔	↔	↔	↔
Apoe $^{-/-}$ *x dnTGFbII R*	FLAP inhibitor	12 wk	Chow	M+F	↓			↔	
Apoe $^{-/-}$ *x Ldlr* $^{-/-}$	FLAP inhibitors	24 wk	HFD (16)	M+F	↓		↑	↓	

Table 4. Study of the leukotriene pathway in murine models of atherosclerosis.

1. 5LO targeting

In the early 2000s, a first study revealed that the 5LO pathway has a deleterious role during atherogenesis, as *Ldlr*$^{-/-}$ mice crossbred to heterozygote *5lo*$^{+/-}$ had reduced aortic root lesion sizes [270]. This reduction of lesion area was also shown later in *5lo*$^{-/-}$ mice on a C57BL6 background, fed a HFD [271]. Therefore, 5LO was thought to exacerbate atherosclerosis. However, subsequent studies have failed to show any protective role for 5LO deletion during atherogenesis [252], [272]. These discrepancies could be explained by differences in the number and age of mice studied in addition to variations in diets. Moreover, the 5LO pathway is thought to be regulated in a sex-specific manner [273]. Consistently, female but not male mice triple knockout for *Apoe*, *5Lo* and *12Lo* show reduced atherosclerotic lesions, suggesting a synergistic effect of 12LO and 5LO in a gender-specific manner during atherogenesis [257]. The sex-specific control of 5LO is however controversial, since another study in *Apoe*$^{-/-}$*5Lo*$^{-/-}$ mice failed to show any difference between males and females [272].

2. FLAP targeting

The targeting of FLAP inhibits only leukotriene formation while 5LO inhibition also prevents the formation of anti-inflammatory lipoxins [210]. In mice models with exacerbated hypercholesterolemia (*Apoe*$^{-/-}$x *Ldlr*$^{-/-}$) or inflammation (*dnTGFBIIR*), the inhibition of FLAP leads to reduce atherosclerotic burden [262], [274], but in more classical models of atherosclerosis, genetic deletion of FLAP do not have any effect on plaque size or inflammatory cell infiltration [275].

3. BLT1 and BLT2 targeting

Contrary to the mixed results obtained following enzymatic inactivation, the targeting of BLT1 has led to convincing data on the implication of LTB_4 in atherosclerosis. Indeed, pharmacological antagonism of BLT1 reduces atherosclerotic lesions and macrophage infiltration [259], confirming the dominant role of the BLT1-LTB_4 axis. Accordingly, double knock-out *Apoe*$^{-/-}$*Blt1*$^{-/-}$ mice exhibit a decreased lesion burden [260].

This reduced atherogenesis is mainly due to the role played by LTB_4 in macrophage and SMC lipid uptake, as *Apoe*$^{-/-}$*Blt1*$^{-/-}$ mice present reduced foam cell content. In these mice, T cell infiltration is also reduced, suggesting a BLT1-mediated chemoattraction of lymphocytes during atherogenesis [218], [252].

Actions mediated by BLT1 seem to occur mainly in early atherosclerosis, as after more than 8 weeks of HFD the protective effects of BLT1 inactivation were no longer observed [225], [260]. Conversely, a recent study demonstrated that pharmacological BLT1 inhibition protected aged *Apoe*$^{-/-}$ mice fed a standard chow diet from atherosclerosis, by decreasing MMP-2/MMP-9 activity, but also showed reduced SMC content in aortic root plaques [269]. This study therefore suggests the implication of LTB_4 signalling in late stage atherosclerosis in mice.

In contrast to BLT1, the targeting of BLT2 by pharmacological inhibition does not modify atherosclerotic plaque phenotype or size [276].

4. Targeting of other leukotriene pathways

Cysteinyl leukotrienes have received little attention in atherosclerosis, despite having been found to have a pro-atherogenic effect as montelukast, a CysLT1 inhibitor reduces lesion size in late stage atherosclerosis in *Apoe*$^{-/-}$*Ldlr*$^{-/-}$ mice [277].

SMPs have begun to be explored in experimental mouse models. In 12/15-LO KO mice where SMPs cannot be produced, atherosclerosis is worsened, while over-expression of 12/15-LO diminishes atherosclerosis [278]. Treatment of *Apoe*$^{-/-}$ mice with SPMs protects against plaque vulnerability induced by HFD [279]. Moreover, in mice deficient for the resolvin receptor FPR2/ALX, plaques exhibit a weakened phenotype with increased SMC-derived MMP-13 and reduced collagen content [258].

5. Limitation of mouse models

In plaques of *Apoe*$^{-/-}$ mice, all enzymes necessary for LT biosynthesis are expressed, but not upregulated [256], [262]. However, when inflammation is exacerbated in advanced atherosclerosis or in hyper-inflamed mice, the expression of these enzymes can be upregulated, suggesting that LTs only

exert an effect in mice when produced abundantly [252], [256], [262]. This potentially explains why the study of the impact of LTs on murine atherogenesis has led to inconsistent results.

Regarding the 5LO pathway, there are considerable differences between mice and humans in the distribution and localisation of LT biosynthetic enzymes. Indeed, while 5LO, FLAP and LTA_4H are associated with plaque macrophages in humans, 5LO is mainly detected in the adventitia of murine plaques, and only LTA_4H colocalises with plaque macrophages (**Figure 22**). Moreover, in double KO mice *Apoe*$^{-/-}$*Ldlr*$^{-/-}$, the expression of CysLT receptors is upregulated in plaque macrophages, while their expression is similar in human plaques and in human healthy arteries [256]. Comparable differences where observed for BLT1 expression on SMCs. Human coronary artery SMCs for example express constitutively BLT1, although this expression is only inducible by pro-inflammatory signals, such as TNF-α, in murine SMCs.

D/ Genetic associations in humans

1. Early studies

Genetic studies provided decisive arguments for the implication of LTs in human atherosclerotic diseases. In the Los Angeles Atherosclerostic Study, Dwyer *et al.* demonstrated an association between allelic variants in the promoter region of the *5LO* gene and the intimal-media thickness in healthy individuals [280]. Later on, Helgadottir *et al.* published two major studies on the association between genetic variants of enzymes from the 5LO pathway and risk of cardiovascular events. In the first, an haplotype of the *FLAP/ALOX5AP* gene was associated with increased risks of myocardial infarction and stroke in Icelandic and British cohorts [281]. In the second study, a haplotype of *LTA4H* was associated with a modestly increased risk of myocardial infarction in Icelandic and American cohorts [282]. In both studies, an increase in LTB_4 production in stimulated but not resting neutrophils was also found in patients who suffered from myocardial infarction.

2. Subsequent studies

The abovementioned genetic associations were confirmed in myocardial infarction [283]–[285], coronary artery disease [286]–[288], stroke [289], and in two meta-analyses [290], [291].

Moreover a study found an association between *BLT1* and the risk of stroke [292] and a new haplotype for *LTA4H* has been associated with protection from increased intimal-media thickness [293].

However, these associations seem to be population-dependent [294]–[297]. Similarly, a recent multi-ethnic study showed no association between *ALOX5* and coronary heart disease [298] and genome wide meta-analyses of coronary artery disease and stroke did not find associations with *ALOX5*, *ALOX5AP* and *LTA4H* [299], [300].

These discrepancies between human genetic studies are mostly due to the heterogeneity of the analysed criteria, the chosen population, and the studied polymorphisms. This indicates that polymorphisms in genes encoding the enzymes of LT biosynthesis could have a population-specific impact on atherosclerosis, but does not however rule out the role of LTs in atherosclerosis.

IV. Role of leukotrienes in other pathologies, with an emphasis on infections

LTs have diverse biological functions, and their implication in various disorders is hence not surprising. As aforementioned, the LT pathways could be involved in cardiovascular diseases, including ischemia, atherosclerosis, aneurysm and pulmonary arterial hypertension.

LTs have also been implicated with allergic diseases, such as asthma, allergic rhinitis, dermatitis, and urticaria. They are well known to act on pulmonary inflammation, for example, in bronchiolitis, chronic obstructive disease, cystic fibrosis, obstructive sleep apnea, or pulmonary fibrosis. Other implications of LTs in inflammatory diseases comprise arthritis, inflammatory bowel syndrome, and vasculitides. In oncology, LT seems also to contribute to solid tumour cancers as well as leukaemia and lymphomas.

Another interesting role played by leukotrienes is in antimicrobial defence during infectious diseases, which will be detailed below with a focus on LTB_4 in regards to its potent effect [213].

The antimicrobial functions of leukotrienes

Bacterial infections: The antibacterial function of LTB_4 was first discovered in murine bacterial peritonitis, where LTB_4 administration promoted bacteria clearance by macrophages, leading to better survival [137]. In 5LO-deficient mice, pulmonary bacterial infection is aggravated [238], [301], because of impaired neutrophil recruitment and bacterial clearance by macrophages [302].

However, in a model of polymicrobial infection, BLT1 deletion protects against organ damage by limiting neutrophil recruitment into the peritoneum [303]. This protective effect was also shown in acute lung injury in response to LPS [304], tuberculosis [221] and polymicrobial sepsis [305], [306].

Viral infections: *In vitro* leukocytic cells are less infected by viruses after LTB_4 treatment [137] and *in vivo*, mortality is reduced following administration of LTB_4 in mice infected with CMV or influenza.

Fungal infections: LTB_4 has be shown to play a role in lung fungal infections through the induction of macrophage phagocytosis rather than neutrophil recruitment [238], [301].

Potential mechanisms: LTB_4 causes neutrophil to release antimicrobial products, such as cathelicidins and defensins from neutrophils [307], enhances phagocytic capacities of neutrophils and monocytes/macrophages [308], stimulates NO and ROS generation [137] and promotes TLR signalling [214], [217], [236].

Aims of the thesis

As highlighted in the introduction, atherosclerotic plaque destabilisation is still an important clinical issue, in spite of the improvement of preventive measures and the identification of its risk factors. Neutrophils are swift effectors of the innate immune system and are rich in oxidant and proteolytic enzymes capable of altering plaque stability. It is hence not surprising that they have emerged as important player in atherosclerotic plaque disruption. One of the main chemoattractants of neutrophils is leukotriene B_4, a lipid mediator that derives from the arachidonic acid pathway. Leukotriene B_4 is a strong pro-inflammatory mediator, which is implicated in chronic and unresolved inflammatory diseases, such as atherosclerosis. These two actors of inflammation are closely linked and are key players in the host defences against pathogens. In line with a concurrent contribution of both neutrophil and leukotriene B_4 to atherosclerosis, bacterial infection has long been evoked as a potential factor of plaque destabilisation. However because of the lack of clear mechanistic evidence, this hypothetical role remains elusive.

Despite their clear involvement in the development and the progression of atherosclerotic plaques, the concomitant actions of neutrophils and leukotrienes in the atherosclerotic disease is still unexplored. Understanding the context in which these two players contribute to the destabilisation of atherosclerotic plaques will provide new insights into the pathogenesis of cardiovascular events and offer new therapeutic approaches in the treatment of atherosclerotic disease.

The objectives of this thesis are:

- To investigate the role of leukotriene B_4 in the recruitment of neutrophils to atherosclerotic plaques.

- To determine whether the infectious context promotes the recruitment of neutrophils to plaques.

- To characterise the deleterious effects of neutrophils on plaque stability.

This work is presented in the form of an article currently in revision.

Results

PUBLICATION

Neutrophils recruited by leukotriene B_4 induce plaque destabilization during endotoxemia

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Article submitted in *Arteriosclerosis, Thrombosis, and Vascular Biology*

Discussion and perspectives

Atherosclerosis is a chronic disease driven by immune responses. Despite the recent advances in the comprehension of its risk factors and the pathways involved in its pathogenesis, atherosclerotic plaque destabilisation and rupture remain a significant clinical issue. Plaque destabilisation results of an imbalance in matrix synthesis and degradation, in cellular survival and death, in enzyme activity and inhibition, and in cell recruitment and egress.

Neutrophils are powerful innate immune actors which cooperate with various cell types, display potent proteolytic and enzymatic activities, and show swift effector functions once recruited to tissues. They have emerged as key players in atherosclerotic plaque growth and evolution [309] and we are only just beginning to understand their recruitment to atherosclerotic lesions. In this context, one of the most powerful chemoattractants of neutrophils, LTB_4, has been proposed as a mediator of human and murine atherosclerotic plaque destabilisation. The chemoattraction of neutrophils by LTB_4 in atherosclerotic plaques has long been evoked [310] but never demonstrated. Interestingly, endotoxemia is a context in which these particular actors are at play, which is itself associated with plaque destabilisation [52], [90].

This work was aimed at determining whether leukotriene B_4 plays a role in the chemoattraction of neutrophils in plaques during endotoxemia and at understanding whether neutrophils tip the balance which maintains the stability of plaques by promoting apoptosis and degrading matrix fibres.

I. Production of leukotriene B_4 in atherosclerotic plaques

Basal production of LTB_4

Murine plaques are able to produce LTB_4 under stimulation with AA [256]. We show herein that unstimulated plaques from *Apoe*$^{-/-}$ mice produce LTB_4 at steady state. This production of LTB_4 at steady state was recently confirmed in early lesions of HFD-fed *Ldlr*$^{-/-}$ mice [266]. However, this production could be result from HFD, which can induce low-grade endotoxemia [88], [89] and promote 5LO nuclear translocation in neutrophils [311]. That is why, in our study, mice were of advanced age and only fed a chow-diet. This model of atherosclerosis also better mimics advanced human atherosclerosis.

The basal LTB_4 production we observed is not unexpected, as both enzymes necessary for its biosynthesis are expressed in murine plaques. Nevertheless, in aortic plaques from *Apoe*$^{-/-}$ mice fed a high-fat diet, intimal LTA_4H was shown to be segregated from 5LO, whose expression was mainly located in the adventitia and only minimally in the intima [256]. This segregation could reflect a protective mechanism by which the production of LTB_4 in plaques is limited to avoid the worsening of inflammation and to maintain plaque stability.

Similarly, we evidenced that non-complicated human plaques, i.e. not at risk of rupture, also produced low basal levels of this lipid mediator, as already reported [249], [256], [263], [264]. Altogether, this indicates that the unstimulated production of LTB4 is not detrimental to plaque stability

Stimulation of LTB_4 production

The production of LTB_4 is enhanced in tissue during inflammatory processes, and therefore, systemic inflammatory conditions could trigger LTB_4 biosynthesis in plaques. We found that systemic inflammation induced by peritoneal LPS injections in *Apoe*$^{-/-}$ mice stimulates the production of LTB_4 in plaques. High levels of intracellular calcium are thought to be required for LT production as both $cPLA_2$ and 5LO are calcium-dependent enzymes. The intracellular calcium level triggered by the LPS-TRL signalling is considered to be insufficient to induce a substantial production of LTs [308]. However, *in vitro* treatment of leukocytes with LPS primes them for LTB_4 biosynthesis through the NF-κB-dependent expression of 5LO [217], and the activation of PLA_2 by MyD88 [214], [216]. Moreover, LPS positively regulates the transcription of FLAP (Serio 2005). Therefore, this LPS-dependent priming of leukocytes could favour a subsequent production of LTs, which is in agreement with the positive correlation between LTB_4 and LPS releases we have evidenced in conditioned media of human culprit plaques.

In addition to leukocyte priming, the LPS/TLR4 signalling could promote LTB_4 production in plaques by inducing cytokine secretion. Indeed, cytokines and leukotrienes are produced in a feed-forward loop (**Figure 24**), as well demonstrated for IL-6 and LTB_4 [236], [237]. It would therefore be interesting to verify that leukotriene B_4 production in plaques during endotoxemia is mediated through TLR4 activation.

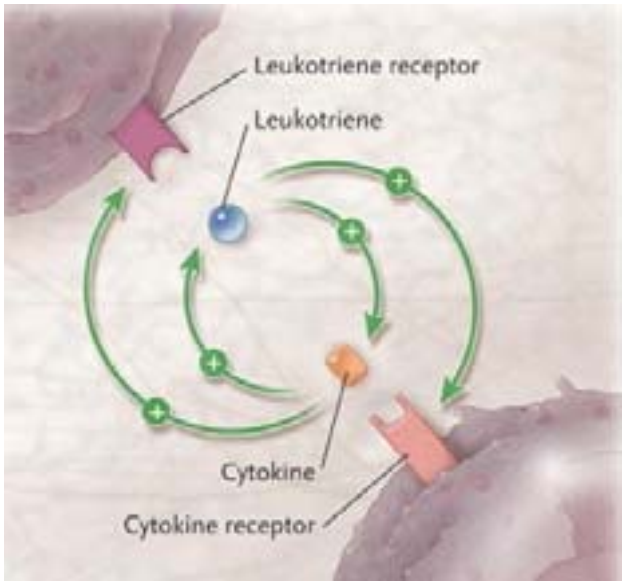

Figure 24. The crosstalk between cytokines and leukotrienes. *Adapted from Peters-Golden, 2007.*

Cellular production in plaques

LTB_4 is mainly produced by leukocytes, and the enzymes involved in its biosynthesis colocalise with macrophages and neutrophils in both human and murine plaques [218], [256]. In our study, neutrophils and monocytes/macrophages are thus likely the main cellular source of LTB_4. Moreover, even though few neutrophils were present in plaques prior to LPS injections, they have a great capacity to produce LTB_4 in a positive-feedback loop [228]. Another plaque leukocyte that could contribute to LTB_4 biosynthesis is the mast cell, especially since mast cells recruit neutrophils by secreting LTB_4 during peritonitis induced by *Escherichia coli* [312].

Besides leukocytes, LTB_4 could also be produced by non-leukocytic plaque cells by transcellular metabolism [211]. Moreover, epigenetic reprogramming induces the transcription of 5LO in SMCs [66] and hypercholesterolemia promotes the nuclear translocation of 5LO in adipocytes [254]. These two cell types could thus contribute to LTB_4 production in aortic tissue. Nevertheless, these potential modes of synthesis may only participate to a limited extent to the biosynthesis of leukotrienes in plaques, as 5LO does locate mostly with plaque leukocytes [218], [270].

The activation of 5LO in leukocytes also promotes the synthesis of CysLTs and SPMs, which could thus be produced during endotoxemia in plaques. However, murine and human plaques only exhibit low levels of LTC_4S [218], [256], and LPS has been shown to inhibit LTC_4S expression in THP-1 monocytic cell lines [313]. CysLTs are hence expected to be only scantily produced. Similarly, SPMs are thought to limit the translocation of 5LO to the nuclear membrane [145], and are thus unlikely to be produced when LTB_4 is synthesised.

II. <u>The specific recruitment of neutrophils by leukotriene B_4 in plaques</u>

The increased neutrophil content is a consequence of a recruitment

LTB_4 exert a powerful chemotactic activity on neutrophils and also primes them enhancing their capacity to adhere to endothelial cells. In plaques of endotoxemic mice, we show that neutrophil infiltration is increased. These endotoxemic mice display a considerable neutrophil mobilisation. As neutrophilia favours neutrophil recruitment to plaques [178], the neutrophil infiltration we observed could be a direct consequence of the significant neutrophil mobilisation. However, the impairment of LTB_4 in endotoxemic mice reduced neutrophil plaque count, despite keeping neutrophil blood count elevated. This shows that the enhanced infiltration was not a mere consequence of neutrophilia, but due to an actual recruitment.

In these experiments, we used a pharmacological inhibitor of LTA_4H to rule out the potential confounding roles of CysLTs and lipoxins which are not produced in double knockout

Apoe$^{-/-}$ *5Lo*$^{-/-}$ mice and mice treated with a pharmacological inhibitor of 5LO. This inhibitor has a high affinity for LTA_4H, but can target other aminopeptidases, and it would be hence interesting to confirm these results using an approach targeting BLT1 or BLT2, as the later has been involved in LPS response and neutrophil chemotaxis [214].

In this work, we identified LTB_4 as a mediator implicated neutrophil recruitment to murine plaques and in human plaques as evidenced by the correlation between LTB_4 and neutrophil markers. The role of LTB_4 in the recruitment of neutrophils to human plaques has been recently challenged [264]. Despite interpretative limitations in this study, it is thus possible that a synergy between the actions of LPS and LTB_4 is necessary to observe significant neutrophil recruitment and plaque destabilisation.

Means by which neutrophils could enter plaques

In addition to being attracted by LTB_4 towards the lesion site, neutrophils must enter plaques located in high shear stress areas. This could occur in several ways. First, both LTB_4 and LPS prime neutrophils by increasing their expression of CD11b and CD11b/CD18 [229]. LPS also triggers integrin expression on ECs and increases endothelial permeability. These activations could enhance the adhesion of neutrophils to the endothelium. Moreover, with the help of slings and tethers, neutrophils have been found to roll at forces equivalent to 10 dyn/cm^{-2} [314]. However, arterial forces are generally comprised between 15 and 30 dyn/cm^{-2}. Platelets could here contribute to neutrophil rolling and arrest, as they adhere to the plaque endothelium and promote the adhesion of neutrophil in murine carotid arteries [116]. In line with a luminal entry, we and others [178] found murine neutrophils to be localised in the luminal part of plaques, suggesting they have entered via the lumen. We also evidenced neutrophils in the adventitia. Therefore, in human and murine plaques the *vasa vasorum* provides an alternative entry route that we cannot exclude.

Potential implications of other chemoattractants

Following LTB_4 impairment, a low number of neutrophils still infiltrated plaques in *Apoe*$^{-/-}$*5Lo*$^{-/-}$ mice. It is possible that a compensatory mechanism is at play in knock-out mice, meaning that another pathway is over-expressed to compensate for the lack of 5LO. As bone marrow transplantation in *Apoe*$^{-/-}$ mice partially restores the level of APOE and thus impact plaques, studying these effects in inducible knockout mice would elucidate this possibility. This is of particular interest as we found that aortic leukocyte count was lower in *Apoe*$^{-/-}$*5Lo*$^{-/-}$ mice, which is consistent with the atherogenic role of 5LO [252].

Nonetheless, we also evidenced a residual neutrophil recruitment in plaques of *Apoe*$^{-/-}$ mice treated with pharmacological antagonists for 5LO and LTA_4H. This suggests that other neutrophil chemokines could

at play. CXCL1 is the principal chemoattractant that have been evidenced in the recruitment of neutrophils to plaques, as CXCL1 inhibition leads to a reduced neutrophil plaque content [191], [192]. Consistently, Butcher *et al.* showed that the disruption of the IL-17/IL-17R axis promotes the expression of CXCL1 in plaques, and the infiltration of neutrophils and macrophages [150]. Interestingly, when neutrophils cast NETs, they promote the secretion of IL-1β by plaque macrophages [133] which upregulates IL-17 secretion and hence neutrophil mobilisation. Therefore, this auto-amplification loop could underlie the residual neutrophil recruitment in plaques of mice with impaired LTB_4 production.

In like manner, CCL3 might also be associated with this residual recruitment. Indeed, macrophages and neutrophils secrete CCL3 during LPS-induced peritonitis [179] which is implicated in the LTB_4-dependent recruitment of neutrophils [315]. Consistently, CCL3-deficient *Ldlr*$^{-/-}$ mice exhibit less neutrophils in their plaques [179]. Notwithstanding the role of CCL3 in neutrophil attraction, the authors concluded that the reduced neutrophil content in plaques in this study was concomitant to an increased neutrophil turnover rather than diminished infiltration. These conclusions however do not exclude the participation of CCL3 to the attraction of neutrophils to plaques during endotoxemia.

During acute lung injury following LPS instillation, platelet-derived CCL5 promotes neutrophil transmigration [316]. Interestingly, the deposition of CCL5 by platelets is responsible for neutrophil recruitment to plaques during hyperlipidaemia [116]. This potential mechanism could hence also contribute to the recruitment of neutrophils during endotoxemia.

It is worth noting that LTB_4 and CysLTs are also activators of mast cells [243]. Mast cells can produce large amounts of IL-8/CXCL1 when activated, and have been implicated in the recruitment of neutrophils to plaques during atherogenesis [207].

Altogether several chemokines and actors may participate in the recruitment of neutrophils to atherosclerotic plaques in endotoxemic contexts, but LTB_4 remains the principal mediator of neutrophil recruitment to plaques during endotoxemia.

Sequential attraction leukocytes in plaques

Prolonged LPS exposure results in accelerated atherosclerosis by promoting macrophage and lymphocyte infiltration [81], [317]. This increased atherogenesis in endotoxemic mice is dependent of LPS binding by high-density lipoproteins [81], [82]. For instance, APOAI protects against LPS-induced atherogenesis. Interestingly, APOAI downregulates neutrophil activation [318], which suggest that the protection is partly mediated by the inhibition of neutrophils. Likewise, anti-APOAI antibodies exacerbate human and murine atherosclerosis and are linked with augmented neutrophil plaque content.

In our work, monocytes, macrophages and lymphocytes were not recruited to plaques after daily injections of LPS for 5 days. This is probably due to the sequential nature of neutrophil and monocyte recruitment (**Figure 26**), which is well characterised both *in vitro* and *in vivo* [319]–[321]. It is hence possible that daily LPS injections reset this sequential recruitment and it would thus be interesting to determine whether such neutrophil-specific recruitment would occur if LPS was infused continuously.

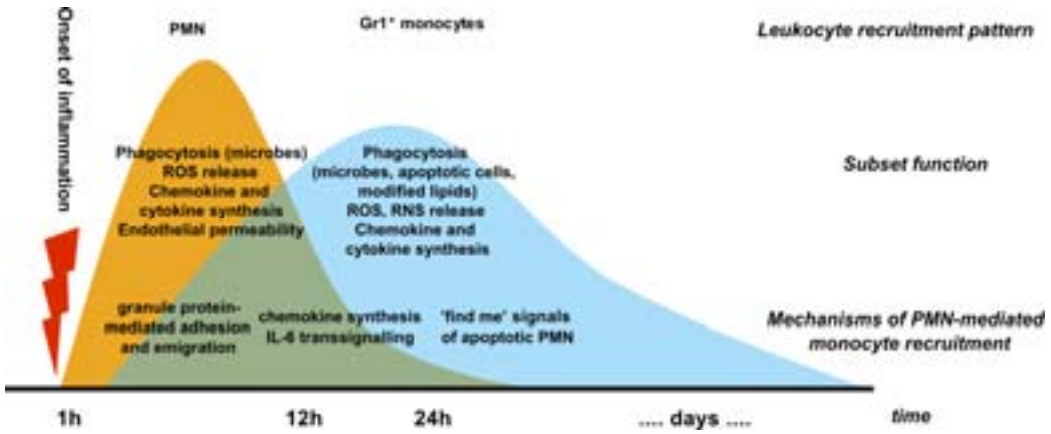

Figure 25. The sequential recruitment and functions of neutrophils and monocytes.

In line with sequential recruitment of neutrophils and monocytes, LTB_4 promotes the release of various microbial peptides from neutrophils, including, LL37 or its murine homologue CRAMP [233]. This cathelicidin enhances monocyte adhesion to plaques [185], [186], suggesting that recruited neutrophils mediate monocyte recruitment to plaques.

Therefore, neutrophils recruited in an earlier phase of endotoxemia may favour the recruitment of other leukocytes in a later phase.

Activation of other plaque cells by LTB_4 and LPS

In addition to the neutrophil-mediated recruitment, LTB_4 itself promotes the recruitment and activation of several cells that constitute the plaque, in particular monocytes and lymphocytes. The attraction of monocytes to murine plaques by leukotrienes may rely on CCL2 (MCP-1), as the protective effect of BLT1 antagonism is lost in CCL2-deficent *Apoe*$^{-/-}$ mice [259]. This could be due to the prominent role of CCL2 in LTB_4-triggered monocyte adhesion [322], [323]. As LTB_4 stimulates the secretion of this cytokine [323], quantifying CCL2 expression in plaques could provide a better insight into the regulation of monocyte recruitment and clarify why monocytes were not recruited in our settings. However, LPS can reduce the expression of BLT1 and CCR2 on monocytes [324], which may explain why we did not observe monocyte recruitment.

LTB_4 is also involved in the migration of SMCs to the fibrous cap [14], [225]. Since *in vitro* LPS induces the expression of BLT1 in SMCs [14], this is consistent with studies that evidence LPS exposure as an enhancer of atherogenesis. As discussed later, in our study, the SMC content in plaques were decreased during endotoxemia, which implies that others mechanisms are at play in advanced atherosclerosis.

To sum up, endotoxemia appears to induce neutrophil-specific recruitment.

III. Effects of endotoxemia, leukotriene and neutrophils on plaque vulnerability and rupture

Neutrophil activation in plaques

Under inflammatory conditions, neutrophils are known to have an increased lifespan. Interestingly LTB_4 has been implicated in the increased survival of neutrophils, dependently on PI3K and ERK pathways [234]. Other anti-apoptotic signals could promote the survival of neutrophils in plaques, such as proliferating cell nuclear antigen (PNCA) [325], hypoxia-inducible factor (HIF) [326], or forkhead box O3A (FOXO3A) [327]. In agreement with the above, when we delivered neutrophils to plaques in a LTB_4-independent assay, we found they only entered apoptosis 6 hours after delivery in plaques, while apoptosis was detected after 1 hour in the adventitia. As apoptosis of neutrophils is pro-resolutive, this prolonged survival can prevent the resolutive phase of inflammation from occurring in plaques.

NETosis is considered as a form a specific cell death but does not necessarily involved the loss of function of neutrophils and could be regarded as an activation process. NETs are known to play a role in infectious diseases and are probable contributors to sterile inflammatory processes. In our study, we evidenced NETosis in murine plaques following endotoxemia and showed that MPO-DNA, a marker of NETosis, was correlated to LTB_4 release in human culprit plaques. Rangé *et al.* previously showed that that these complexes of DNA and MPO are more present in culprit plaques than in non-complicated plaques or healthy arteries [328]. This suggests that signals present in complicated plaques promote the release of NETs by neutrophils following their recruitment by LTB_4. Sterile triggers of NETosis have been identified in atherosclerotic murine plaques. Cholesterol crystals promotes NET formation in neutrophils, resulting in macrophage priming [133]. Moreover the serum of HFD-fed *Apoe*$^{-/-}$ mice enhances the capacity of neutrophils to release NETs [200]. As NETs contribute to the activation of plaque cells and are perceived as danger signals, they enhance the inflammation going on in plaques. Moreover, enzymes bound to NETs are active and thus participate in enzymatic activities and ECM degradatation in plaques [329]. Therefore, the presence of NETs could promote plaque destabilisation.

Other neutrophil functions can be involved in plaque destabilisation, especially functions mediated by their granule proteins. Treatment of human neutrophils with high concentrations of LTB_4 *in vitro* can induce the degranulation of azurocidin at concentrations ranging from 10 nM to 1 μM [330]. The concentration of LTB_4 we found in human culprit plaques ranged from 0.5 to 6 nM, just below the threshold of activation found *in vitro*. Therefore, the correlation we found between LTB_4 and MPO stored in neutrophil primary granules in human culprit plaques might reflect an enhanced neutrophil recruitment rather than an increased degranulation triggered by LTB_4.

Neutrophils have previously been colocalised with proteolytic enzymes in plaques in both murine and human plaques [29], [170], [178], [193], [331]. Similarly, we observed MPO staining in the proximity of neutrophils in murine plaques. Moreover, the increased plaque enzymatic activities for type I and type IV collagens observed after neutrophil invasion in endotoxemia could reflect the degranulation of gelatinase-rich tertiary granules and collagenase-rich primary granules. Accordingly, these increases were mediated in an LTB_4-dependent fashion, strongly suggesting that it is linked to neutrophil recruitment. However, LTB_4 induces the secretion of MMP-2 by SMCs [332] and proteases by macrophages and the inhibition of BLT1 reduces the MMP activity in plaques of aged *Apoe*$^{-/-}$ mice [269]. To evaluate whether neutrophils could rapidly enhance collagenolysis independently of LPS-stimulation and increased LTB_4 production, we designed an *in vivo* invasion assay. Using this assay, we demonstrated that neutrophils alone increase plaque collagenolytic activities and destabilised plaques.

Despite the interpretative limitations of this assay due to the high number of delivered neutrophils, their partial adventitial activation, and the lack of endothelial priming occurring during transmigration [115], its results were decisive in defining the capability of neutrophils to impact plaques and their functional kinetics.

We chose to use naive bone marrow neutrophils which are fully functional [333] to minimise their activation and apoptosis in the adventitial layer [334]. However, endothelial priming is considered crucial to maximise neutrophil effector functions [335] and our observation could hence reflect a cumulative lessened activation of naive neutrophils. An argument against the importance of endothelial priming could be that activated neutrophils in human lesions have been colocalised with areas of intraplaque haemorrhages [29] and therefore are not primed by endothelial adhesion.

Another flaw of the assay is the lack of mechanisms explaining the means of entry of neutrophils. Their presence between the elastic laminae suggests they migrated though the media. In line with this, we observed that elastic laminae were fragmented following neutrophil delivery. However, similar nuclear distortions are observed during the migration of cells from the adventitia to the intima through microvessels [204] (**Figure 26**) and neutrophils can undergo reverse transmigration in the presence of LTB_4 [118], which we found to be produced at steady state in plaques of *Apoe*$^{-/-}$ mice. Therefore, the mechanism of entry in this assay could be more complex than first assumed.

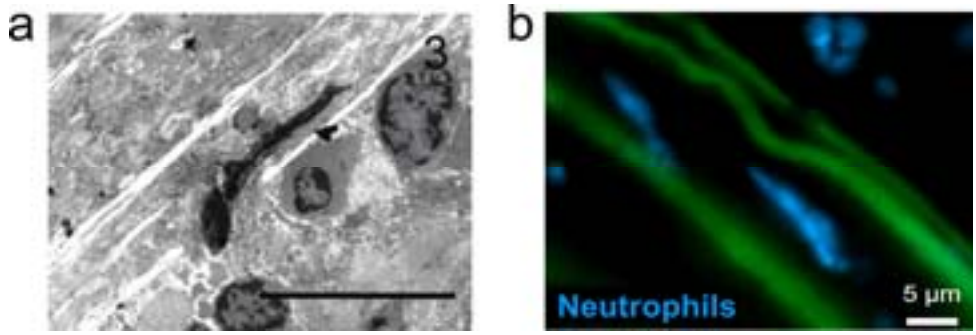

Figure 26. Nuclear distortions observed between the elastic laminae. *(a) Observation by transmission electron microscopy of the cell migrating through the medial elastic laminae. Scale bar = 25 µm, adapted from Eriksson, 2011. (b) Nuclei of neutrophils that have been labelled with a nuclear dye and delivered onto the adventitia in our in vivo invasion assay. Elastic laminae are visible in green.*

MMP activity and collagen degradation

MMP-9 released from neutrophils digests type IV collagen and gelatin. Consistently, we found that neutrophil presence results in increased enzymatic activity for type IV collagen in plaques. A study using an accelerated model of atherosclerosis by collar placement similarly showed that neutrophils were recruited early to these lesions, which exhibited increased gelatinolytic activities and decreased collagen content [193]. Despite the limited interpretation of this model, these results suggests that enzyme from neutrophils could digest plaque collagen. This association between contents in MMP-9, neutrophils and collagen was confirmed in studies involving the endocannabinoid and the CXCL1 pathways [180], [190], [192], [331]. As type IV collagen and gelatin are non-fibrillar collagens unlike the collagen present in the fibrous cap, this collagenolysis is more likely to result in endothelial erosion.

We further observed an increase in type I collagenolytic activity, extracellular MPO, and reduced plaque collagen content following neutrophil infiltration. These results are consistent with the release of primary granules, suggesting that neutrophil MMP-8 could mediate the observed type I collagenolysis and fibrous cap thinning. This is consistent with studies correlating neutrophils, MMP-8 and human plaque vulnerability [26], [170], [336]. Besides the oxidation of lipoproteins [124], MPO-derived products, such as HOCl, could promote the activity of MMPs by promoting the extracellular conversion of proenzymes and inhibiting TIMPs by oxidation of cysteine residues [198], [197]. Furthermore, in preliminary experiments, elastinolysis and breaks in the elastic laminae were increased in our *in vivo* assay, suggesting that neutrophil elastase is at play in neutrophil-mediated plaque vulnerability.

Nevertheless, as neutrophils and monocytes/macrophages display an intricate interplay [337], we cannot rule out the potential contribution of macrophages to these effects, especially since they are rich in MMP-13.

Necrotic core and SMC apoptosis

In mice where neutrophilia is triggered by the disruption of the CXCR4/CXCL12 axis, plaques are enriched in neutrophils, display less SMCs and more apoptotic cells [178]. Accordingly, we and others found similar results following neutrophil infiltration [181], [192]. Interestingly the effect of neutrophil on SMC apoptosis may be mediated by IFN-γ, which has been show to inhibit SMC proliferation, leading to a reduction of collagen content in plaques [338]. Indeed, NETs promote IFN-α and IFN-γ expression in atherosclerotic plaques of *Apoe*$^{-/-}$ mice and could hence induce SMC apoptosis [200].

In contrast to the proliferative effect of LTB_4 on SMCs [14], [225], we found that SMC plaque content is similar in *Apoe*$^{-/-}$*5Lo*$^{-/-}$ and *Apoe*$^{-/-}$ mice, suggesting that 5LO mainly plays a role during atherogenesis. Moreover, we observed that when LTB_4 is intensively produced during endotoxemia SMC apoptosis is induced. It is possible that the apoptosis observed is due to death by anoikis, as we observed a reduction in matrix fibres in plaques.

However, while we did not observe a significant difference in collagenolysis for type IV and type I collagens between 5LO and LTA_4H inhibitions, we found the former to be more protective than the latter. Human coronary SMCs stimulated with LPS express CysLT1 [230], and the activation of CysLT receptors upregulate the expression of pro-atherosclerotic genes [339]. Therefore, even though they may not be produce in large amount, CysLTs might exert a deleterious effect on plaque SMCs, which show phenotypic differentiation. Moreover, the activation of monocytes and macrophages by CysLTs induces the production of TNF-α and MMP-9, which can both contribute to SMC apoptosis [340]. This contrast between proliferation and cell death reflects the duality of the effect of leukotrienes on SMCs in atherosclerosis.

Therefore, cumulative actions of LPS, leukotrienes, and neutrophils precipitate the expansion of the necrotic core by promoting apoptosis of SMCs and plaque cells.

Endotoxemia and plaque rupture

As endotoxemia is associated with cardiovascular events in humans [90], [91], [94], [98], we resolved to explore its role in plaque destabilisation. Although peritoneal infection or sepsis has not been associated with cardiovascular diseases, we choose to employ LPS-induced peritonitis not as a model of severe sepsis or bacterial peritonitis, but as an acknowledged and reproducible model of endotoxemia and systemic inflammation, previously used in the study of atherosclerosis [81], [83], [317]. Interestingly, in HFD-*Apoe*$^{-/-}$ mice chronically exposed to intraperitoneal LPS, plaques formed by the induction of shear stress (collar placement) were shown to rupture, especially when mice were

stressed. As stress has already been involved in plaque rupture in mice [44], testing the impact of stress on plaque disruption in our settings would be of interest.

Jaw *et al.* recently provided strong evidence for the role of the LPS-neutrophil axis in murine plaque rupture. Using a model of LPS-induced acute lung injury, the authors showed that plaque disruption, identified by the presence of superimposed thrombi or intraplaque haemorrhages, was triggered as soon as 24h after LPS instillation. They also observed rupture 8h after lung LPS exposure and show that this rupture relied on circulating neutrophils. In contradiction with our study, they failed to reproduce these results 8h after intraperitoneal LPS injection. A potential reason underlying this discrepancy is the chosen timeframe. Indeed, we found that 8 hours after LPS injection, plaques did not have an increased production of LTB_4, and thus that neutrophils were not recruited. This difference in timing could be explained by the route of administration, as the lung is a highly vascularised organ.

We did not observe superimposed thrombi or intraplaque haemorrhages in our model, even after a 5-day treatment. Moreover, during extensive preliminary experiments in our *in vivo* invasion, we designed a model of acute hypertension induced by repeated injections of angiotensin II to assess by intravital microscopy whether plaque rupture could occur following adventitial delivery of neutrophils. We did not however observe plaque rupture, showing the importance of the endotoxemic context. Several parameters could underlie the differences observed between the model used by Jaw *et al.* and our model. While we used aged (over 50 weeks) chow-fed mice, Jaw *et al.* employed young (8-12 weeks) HFD-fed mice, which could present metabolic endotoxemia and increased neutrophil activation. Moreover, the dose of LPS in this study is elevated and the serotype more potent than in our model. Regardless of the dissimilarities, the study from Jaw *et al.* reinforces our demonstration that neutrophils contribute to plaque destabilisation, and determining whether LTB_4 is involved in this model of plaque rupture would provide novel insight into the progression of atherosclerosis.

IV. Perspectives

Beyond the deleterious role of neutrophils, the resolution

Two days after neutrophil delivery, plaques displayed increased collagen content, reduced collagenolytic activities, and thickened fibrous caps in our *in vivo* invasion assay. A potential explanation underlying these unexpected results could be that once in plaques neutrophils also promote the resolution of inflammatory processes. Indeed, the ingestion of apoptotic neutrophils by macrophages favours the lipid mediator class switch necessary for the cessation and resolution of inflammation. For instance, the efferocytosis of apoptotic neutrophils is known to induce the synthesis of SPMs in macrophages, which increases the phagocytic capacities of macrophages, chemokine scavenging and tissue repair. In line with this concept, both unstable murine and human plaques present an imbalance

between resolving lipid mediators and LTB_4 and chronic administrations of resolvin promotes plaque stability and effective clearance of apoptotic bodies [266]. Therefore, determining whether neutrophils could also contribute to plaque rebuilding following endotoxemic episodes would provide a novel insight into the functions of neutrophils in atherosclerosis.

Targeting LTB_4 in infectious contexts in patients at risk of cardiovascular diseases

The role of infection in cardiovascular events has long been suspected in clinical practice [46], [52], [53], [57], [60], [90], [96]. However, despite significant limitations in negative clinical trials using antibiotherapy targeting *C.pneumonia*, the hypothetical impact of infections on plaques has sidelined [341]. Recently, new light on the role of infectious diseases in atherosclerosis has been shed, following the revelation that other infectious mechanisms could be at play, for instance the involvement of periodontal diseases [63], [94], the potential importance of the pathogenic burden [54] or the contribution of gut microbiota [88], [89], [100].

We have shown here a mechanism by which pathogens could promote plaque destabilisation, and determining whether this LTB_4-neutrophil axis is involved in acute and chronic, mono- and polymicrobial infections would offer wider therapeutic opportunities in the prevention of cardiovascular events.

The therapeutic potential of LT targeting in cardiovascular diseases targeting is already tested in several clinical trials. In a phase II randomised control trial, patients carrier of the *FLAP* and *LTA4H* haplotypes treated with a FLAP antagonist (DG-031) showed a reduction in serum C-reactive protein, an inflammatory marker linked to increased risk of cardiovascular events [342]. In patients with acute coronary disease, a phase II trial for a 5LO inhibitor (*Atreleuton*, VIA 2291) showed a significant reduction in LTB_4 biosynthesis, plasma CRP and in the incidence of 'new' coronary plaques accompanied by a significant reduction in plaque volume [343], [344]. Several LTA_4H inhibitors have been developed such as bestatin, captopril or DG-051. Bestatin (ubenimex) is currently being tested for the treatment of pulmonary arterial hypertension and DG-051 has been envisaged for use in the prevention of myocardial infarction [345]. Therefore, anti-leukotrienes appears as promising therapeutic strategies in the management of atherosclerosis, however using these treatments during infectious might be deleterious owing to the role of LTs in infectious diseases. Combinatory treatments could hence provide a safer option.

As far as the targeting of infections in cardiovascular diseases is concerned, a major drawback of antibiotics is the possible subsequent endotoxemia induced by the death of the targeted bacteria [346]. Therefore, combinatory treatment of antibiotics and anti-leukotrienes would be here too of particular interest for therapeutic interventions. Another approach in targeting the LPS-LTB_4-neutrophil axis

would be to promote the neutrophil-mediated resolution of inflammation through pro-resolutive lipid mediator therapies.

V. Conclusion

In conclusion, this work evidences the recruitment of neutrophils mediated by LTB_4 as a link between endotoxemia and plaque destabilisation. We have herein identified LTB_4 as an important chemoattractant of neutrophils to plaques and showed the deleterious effects of neutrophils on plaque stability. Our results contribute to the comprehension of the mechanism by which systemic inflammation in infectious contexts disturb the fragile equilibrium that maintains plaque stable. This study opens the road to therapeutic approaches aimed at targeting infection, leukotrienes and neutrophils in the prevention of cardiovascular events.

French summary

I. Introduction

A/ Le développement de l'athérosclérose

L'athérosclérose est une maladie qui touche principalement les artères de moyens et gros calibres. Les artères sont composées de trois couches. La couche la plus luminale, l'intima, est formée de cellules endothéliales reposant sur une lame basale de collagène de type IV. La média est la couche intermédiaire, riche en cellules musculaires lisses et collagène de type I et III. L'adventice, la tunique la plus externe, contient du tissu conjonctif lâche, des fibroblastes et des micro-vaisseaux, nommés *vasa vasorum*. L'athérosclérose se développe dans la couche intimale suite à l'accumulation de lipides dans les zones présentant des faibles forces de cisaillement, comme les bifurcations.

Dans ces sites de prédilection, des lipoprotéines de faible densité (LDL) qui transportent le cholestérol s'accumulent et s'oxydent. L'ingestion d'oxLDLs par les macrophages résidents et cellules musculaires lisses intimales les transforment en cellules dites spumeuses. Une strie lipidique est alors formée dans l'intima : c'est le premier stade de la pathologie athérosclérotique. De plus, ces lipoprotéines modifiées (oxLDLs) sont reconnues comme des signaux de dangers par les cellules résidentes du vaisseau et vont ainsi induire une réponse immunitaire. Cette réponse favorisera le recrutement de monocytes qui se différencient en macrophages et ingèrent les oxLDLs.

Suite à l'inflammation ainsi engendrée, les cellules musculaires lisses de la média migrent vers l'intima et se dédifférencient en un phénotype sécrétoire conduisant à la formation d'une chape fibreuse de collagène et d'élastine. Ce type de lésions évolue vers un athérome dit fibro-lipidique suite à l'accumulation des cellules nécrotiques formant un corps nécrotique sous la chape fibreuse.

B/ La déstabilisation et la rupture de la plaque d'athérosclérose

Ces plaques d'athérome peuvent rester stable ou s'épaissir et induire une sténose. Elles peuvent également se fragiliser rompre, et induire la formation d'un thrombus à leur surface ou athérothrombose, menant l'ischémie des organes en aval et qui peut être fatale. Pour qu'une plaque rompe, il faut qu'elle soit vulnérable, c'est-à-dire qu'elle présente une fine chape et un large corps nécrotique. La stabilité de la plaque est maintenue par une balance entre la synthèse et la dégradation des fibres matricielles, la survie et la mort cellulaire, ainsi que l'activation et l'inhibition enzymatique.

L'ingestion d'oxLDLs par les cellules musculaires lisses et les macrophages induit leur entrée en apoptose. A cause d'une efférocytose défectueuse, l'accumulation de débris nécrotiques n'est pas éliminée par les phagocytes des plaques. Ces phénomènes conduisent à l'élargissement du corps nécrotique.

Concurremment, l'augmentation de l'activité protéolytique des métalloprotéases matricielles (MMP) sécrétées par les cellules immunitaires recrutées et activées, comme les monocytes, les lymphocytes et les neutrophiles, conduit à la dégradation des fibres matricielles de la plaque. De plus, cette dégradation

des fibres matricielles induit l'apoptose des cellules musculaires lisses, et donc à un déficit en synthèse de protéines matricielles. La résultante de ces processus conduit à l'affinement de la chape fibreuse.

Ces plaques dites vulnérable à la rupture sont nommées TCFA ou *thin cap fibroatheroma*. Elles peuvent rompre sous l'effet de contraintes mécaniques. En fonction de la thrombogénicité des plaques, la rupture peut induire la formation d'un thrombus bloquant le flux sanguin provoquant la mort des organes en aval ou former un thrombus mineur qui peut être inclus sous une couche de matrice extracellulaire lorsque la plaque se « répare ».

C/ L'infection et l'athérosclérose

Le rôle de l'infection tant bactérienne que virale dans la formation et la fragilisation de la plaque d'athérosclérose a depuis longtemps été suspecté en clinique et lors d'observations expérimentales dans des modèles murins d'athérosclérose, comme les souris *Ldlr*$^{-/-}$ ou *Apoe*$^{-/-}$. Plusieurs arguments suggèrent l'implication des bactéries dans les plaques.

Premièrement, dans les plaques humaines, plusieurs types de bactéries ont été identifiés et de nombreuses études épidémiologiques ont mis en évidence des associations entre infection et risque d'accidents cardiovasculaires. Cependant, plusieurs essais cliniques basés sur une antibiothérapie ciblée n'ont pas montré d'efficacité dans la prévention des risques cardiovasculaires. Devant les limitations de ces études, la question du rôle de l'infection dans la pathogénèse de l'athérosclérose demeure et ce d'autant plus depuis la découverte d'une forte association entre les parodontopathies bactériennes et les accidents cardiovasculaires et l'importante contribution du microbiote intestinal.

Plusieurs mécanismes hypothétiques ont été suggérés pour expliquer l'impact de l'infection dans l'athérosclérose : (i) une action directe des pathogènes sur la plaque, (ii) un mimétisme moléculaire entre bactérie et composant de la plaque, ou (iii) un impact de l'inflammation systémique engendrée par l'infection. Il est aussi possible que les produits bactériens, en particulier le lipopolysaccharide ou LPS, puissent entrer dans la circulation, créer une endotoxémie et contribuer à l'inflammation de la plaque. Plusieurs études ont en effet montré une corrélation entre la présence de LPS dans le sang et l'incidence de l'athérosclérose chez l'humain, notamment lors d'une endotoxémie induite de manière métabolique ou lors de maladies parodontales. De même, dans le modèle murin d'athérosclérose *Apoe*$^{-/-}$, l'induction d'une endotoxémie par l'injection de LPS dans le péritoine ou dans le sang induit une athérogénèse augmentée et favorise le recrutement de cellules inflammatoires dans les plaques, telles que les macrophages et lymphocytes. Une étude récente a également montrée que lors d'une inflammation pulmonaire induite au LPS la plaque était rapidement déstabilisée par un mécanisme dépendant des neutrophiles. Cette étude met en évidence le rôle potentiel des neutrophiles dans la déstabilisation de la plaque au cours de l'endotoxémie.

D/ Les neutrophiles dans l'athérosclérose

Les neutrophiles sont des cellules de l'immunité innée, formé suite à la différentiation de progéniteurs hématopoïétiques dans la moelle osseuse. Les neutrophiles appartiennent à la famille des granulocytes, caractérisée par la présence de vésicules d'exocytose enrichies en protéines dans leur cytoplasme. Les granules des neutrophiles sont divisés en quatre types formés et sécrétés séquentiellement : les granules primaires riches en myéloperoxydase (MPO) et sérine protéases, les granules secondaires qui renferment des collagénases (MMP-8), les granules tertiaires qui contiennent des gélatinases (MMP-9) et les vésicules sécrétoires qui sont un réservoir de récepteurs et de molécules d'adhésion, tel que l'intégrine CD11b/CD18. Suite à leur recrutement par des chimiokines (CXCL-1, CXCL-8, CCL3, CCL5…) ou des médiateurs lipidiques (LTB_4…) dans les tissus selon une cascade de transmigration bien définie, les neutrophiles s'activent et déploient leurs systèmes de défense.

Considéré comme des effecteurs rapides de l'immunité ayant une durée de vie courte (3 à 16 heures dans la circulation), les neutrophiles ont été longtemps ignorés dans l'étude de l'athérosclérose. Cependant, plusieurs études ont contribué à mettre en lumière le rôle des neutrophiles dans l'athérosclérose en révélant leur présence dans les plaques humaines dans des zones dites vulnérables comme les zones d'hémorragies intra-plaques. De plus, les marqueurs d'activation des neutrophiles, tels que la MPO, le NGAL (neutrophil gelatinase-associated lipocalin) ou la MMP-9, dans le plasma ainsi que le nombre de neutrophiles circulants sont à la fois associés et prédictifs du risque d'accidents cardiovasculaires chez l'humain. Les conditions sous-jacentes à l'athérosclérose comme l'hypercholestérolémie favorisent la mobilisation des neutrophiles et leur activation. Dans leur ensemble, ces études indiquent que les neutrophiles pourraient avoir un rôle délétère sur la stabilité des plaques.

Ce potentiel rôle délétère est aussi révélé dans des études expérimentales sur des modèles murins d'athérosclérose. Plusieurs études ont montré que lors des stades précoces de l'athérosclérose les neutrophiles étaient présents dans les plaques de souris *Ldlr*$^{-/-}$ et *Apoe*$^{-/-}$ et pourraient contribuer à l'attraction de monocytes et à la formation de cellules spumeuses. Lors d'une neutrophilie, les plaques murines présentent aussi moins de cellules musculaires lisses et une augmentation du nombre de cellules apoptotiques. De façon similaire, l'infiltration neutrophilaire dans les plaques murines est concomitante à une diminution du contenu en collagène et en cellules musculaires lisses, ainsi qu'à une augmentation en MMP-9. De manière opposée, la déplétion des neutrophiles circulants prévient la fragilisation de la plaque induite par l'inhalation de LPS.

Plusieurs fonctions des neutrophiles pourraient participer à la déstabilisation des plaques. Premièrement, ils sont riches en MPO et MMP-9 qui pourrait favoriser l'apoptose des cellules endothéliales et donc l'érosion endothéliale par une action directe ou par la dégradation du collagène de type IV du sous-endothélium. De plus, via leurs enzymes granulaires telles que la MMP-8 ou l'élastase, les neutrophiles pourraient dégrader le collagène et l'élastine de la chape fibreuse et ainsi

l'affiner. Enfin, les neutrophiles peuvent projetés des réseaux d'ADN décondensé décoré d'histones et de protéines cationiques (MPO, élastase), nommé Neutrophil-Extracellular Traps (NETs). Ces NETs ainsi que les produits sécrétoires des neutrophiles peuvent activer la réponse inflammatoire des macrophages de la plaque. Ainsi les neutrophiles pourraient par plusieurs actions contribuer directement ou indirectement à la fragilisation des plaques.

L'entrée des neutrophiles dans les lésions athérosclérotiques pourraient avoir lieu au travers des micro-vaisseaux ou *vasa vasorum*. Cependant ces vaisseaux sont souvent absents des stades précoces et les neutrophiles pourraient entrer dans la plaque par voie luminale en formant des structures particulières (sling et tether) qui leur permettraient de rouler à des forces de cisaillement élevées. Les plaquettes pourraient également favoriser cette infiltration des neutrophiles. Plusieurs signaux ont été identifiés comme contribuant au recrutement des neutrophiles dans les plaques : les chimiokines CXCL1, CCL3, CCL5, mais aussi des voies impliquant les endocannabinoides ou l'ApoA1. Dans ce contexte, les leucotriènes ont aussi été évoqués comme potentiel chimio-attractant des neutrophiles dans les plaques.

E/ Le leucotriène B_4 dans l'athérosclérose

Le leucotriène B_4 (LTB_4) appartient à la famille des eicosanoïdes, un groupe de médiateurs lipidiques dérivés de l'acide arachidonique (AA). Ils sont synthétisés principalement par les leucocytes par l'action de trois enzymes : la phospholipase cytosolique A_2 ($cPLA_2$) qui libère l'AA des membranes, la 5-lipoxygénase (5LO) qui converti l'AA en leucotriène A_4 et la leucotriène A_4 hydrolase (LTA_4H) qui transforme le LTA_4 en LTB_4. Plusieurs signaux inflammatoires comme par exemple le LPS ou le TNF-α favorisent la synthèse de LTB_4. Une fois synthétisé, le LTB_4 est transporté hors de la cellule et agit au niveau de son récepteur de haute affinité BLT1, exprimé principalement par les cellules inflammatoires, et de son récepteur de faible affinité BLT2, exprimé de manière ubiquitaire. Le LTB_4 agit ainsi sur plusieurs types cellulaires. Son action est majeure sur les neutrophiles car elle induit leur activation, leur chimiotactisme, leur dégranulation, et retarde leur apoptose. Le LTB_4 possède aussi une action chimiotactique sur les monocytes et les lymphocytes et favorise la sécrétion de cytokines et de MMP par les monocytes/macrophages. Il a également été montré que l'axe LTB_4/BLT1 peut avoir une action sur les cellules musculaires lisses des plaques en induisant leur migration et prolifération, ainsi que la libération de leur MMP.

Plusieurs études ont révélé la présence des enzymes impliquées dans la synthèse du LTB_4 dans les plaques humaines et murines. De manière intéressante, une colocalisation entre ces enzymes de synthèse et les macrophages, les mastocytes, les neutrophiles et les cellules dendritiques a été montrée. De plus, la production de LTB_4 par les plaques humaines est corrélée à l'infiltration leucocytaire. Le leucotriène B_4 a été impliqué dans l'athérogénèse dans plusieurs modèles chez des souris *Apoe*$^{-/-}$ ou *Ldlr*$^{-/-}$ déficientes en 5LO, LTA4H ou FLAP (la protéine « helper » de la 5LO). L'action du LTB_4 dans les modèles murins de plaques avancées reste plus controversée. En effet, bien que plusieurs données

indiquent une action délétère du LTB_4 sur la stabilité des plaques, les données issues de souris déficientes en 5LO indiquent un rôle proéminent de ce lipide dans les stades précoces de l'athérosclérose. Ce lien entre la fragilisation de la plaque et LTB_4 chez l'humain a été apporté par de nombreuses études génétiques démontrant une association entre des variants de la 5LO, de FLAP et de la LTA4H et le risque d'accidents cardiovasculaires. Cependant, la force de ces associations varie en fonction de la population étudiée. Ainsi le rôle du LTB_4 dans la rupture de la plaque reste encore aujourd'hui mal compris.

II. Objectifs de la thèse et résultats

A/ Objectifs

L'ensemble de mon travail a pour but de déterminer le rôle du LTB_4 dans le recrutement des neutrophiles dans les plaques d'athérosclérose au cours de l'endotoxémie et de définir l'impact des neutrophiles sur la fragilisation de la plaque.

Notre premier objectif était de déterminer si l'endotoxémie peut induire la production de LTB_4 dans les plaques et si ce dernier peut favoriser le recrutement des neutrophiles, qui fragiliserait ensuite les plaques. Nous avons recherché si les neutrophiles pouvaient induire la vulnérabilité des lésions athérosclérotiques avancées suite à leur activation dans un test d'invasion *in vivo* consistant à délivrer un nombre élevé de neutrophiles à une plaque d'athérosclérose carotidienne par voie adventitielle. Nous avons ensuite déterminé si au cours de l'endotoxémie, les neutrophiles circulants, pré-activés par l'infection, pouvaient être recrutés dans la plaque par l'action du LTB_4 et fragiliser les plaques par leur action protéolytique sur les composants fibreux des plaques. Enfin, nous avons cherché à établir si ce mécanisme pouvait être en jeux dans la déstabilisation des plaques humaines.

B/ Approches expérimentales et résultats

Nous avons pu montrer que l'endotoxémie induite par l'administration intra-péritonéale de LPS (1.5 mg/kg/jour pendant 5 jours) provoque une mobilisation des neutrophiles et une inflammation systémique, confirmée par la présence de sérum amyloïde A (SAA). De plus l'endotoxémie stimule la production de LTB_4 déterminée par un dosage par ELISA dans les plaques aortiques de souris *Apoe*$^{-/-}$ âgées d'au moins 50 semaines, nourries sous régime standard. Nous avons déterminé par cytométrie en flux qu'au cours de l'endotoxémie, le nombre de neutrophiles dans les plaques est augmenté. Nous avons vérifié le rôle du LTB_4 dans cette infiltration neutrophilaire par l'utilisation de souris *Apoe*$^{-/-}$*5Lo*$^{-/-}$ et *Apoe*$^{-/-}$ traitées par des inhibiteurs pharmacologiques de la 5LO (zileuton) et de la LTA_4H (bestatin). Dans ces souris, le nombre de neutrophiles recrutés dans les plaques au cours de l'endotoxémie est diminué, confirmant le rôle majeur du LTB_4 comme chimio-attractant.

Pour tester si l'invasion de la plaque par les neutrophiles peut altérer la plaque à elle seule, indépendamment du LPS et du LTB_4, nous avons mis au point un test d'invasion *in vivo* dans lequel

des neutrophiles quiescents isolés à partir de la moelle osseuse par tri-immunomagnétique sont délivrés sur l'adventice d'une plaque carotidienne préalablement exposée par chirurgie. Nous avons montré par zymographie *in situ* que l'entrée des neutrophiles augmente l'activité protéolytique intraplaque pour le collagène de type I et de type IV. De plus, l'infiltration des neutrophiles fragilise la plaque en diminuant son contenu total en collagène et la taille de la chape fibreuse la recouvrant ainsi qu'en augmentant la taille du corps nécrotique. Ces données montrent que les neutrophiles ont la capacité de fragiliser les plaques d'athérosclérose.

Lors de l'endotoxémie, les neutrophiles recrutés se localisent majoritairement dans les zones sous-luminales des plaques et s'activent, comme déterminé par la présence de MPO couplée aux NETs. L'endotoxémie entraîne une augmentation de la digestion du collagène de type I et IV ainsi que de l'apoptose des cellules musculaires lisses de manière dépendante du LTB_4 ce qui suggèrent un mécanisme dépendant des neutrophiles. L'étude de l'impact de l'endotoxémie sur la stabilité des plaques montre une augmentation de taille du corps nécrotique et une diminution du contenu total en collagène ainsi que de l'épaisseur de la chape. Ainsi au cours de l'endotoxémie, les neutrophiles recrutés par le LTB_4 se localisent dans les plaques, s'activent et contribuent à la déstabilisation des plaques en augmentant l'activité protéolytique des plaques et en favorisant l'apoptose des cellules musculaires lisses.

Chez l'humain, les milieux conditionnés d'endartériectomie contiennent plus de LTB_4 lorsque les plaques sont dites compliquées ou vulnérables par rapport aux plaques non-compliquées. Cette quantité de LTB_4 est corrélée au contenu en LPS, MPO ou MPO-DNA (un marqueur des NETs). Ainsi l'axe LPS-LTB_4-neutrophile serait impliqué dans la déstabilisation des plaques humaines.

III. Conclusions et perspectives

Ce travail soutient l'hypothèse du rôle délétère des neutrophiles dans l'athérosclérose. Nous avons montré que le recrutement des neutrophiles par le LTB_4 comme un lien entre l'endotoxémie et la fragilisation des plaques d'athérosclérose. Ces résultats apportent une meilleure compréhension du recrutement des neutrophiles dans la plaque dans un contexte infectieux. Ils révèlent également un mécanisme par lequel l'inflammation systémique provoquée par l'infection pourrait perturber l'équilibre fragile qui concoure à la stabilité des plaques. Une ouverture à ce travail sera de rechercher l'implication des neutrophiles et du LTB_4 dans l'infection aigue et chronique. De plus, les neutrophiles contribuant aux processus résolutifs de l'inflammation, notamment par leur apoptose, il serait intéressant d'étudier plus en détails la participation des neutrophiles à la stabilisation de la plaque dans des délais plus longs. Cette étude ouvre à la recherche de nouvelles voies thérapeutiques dans la prévention des accidents cardiovasculaires dans des contextes endotoxémiques par le ciblage de l'axe neutrophile-LTB_4.

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Marie-Anne MAWHIN

Role of neutrophils and leukotrienes in atherosclerotic plaque destabilisation
-Implication of endotoxemia-

Abstract

Atherosclerotic plaque destabilisation remains an important clinical issue, in spite of the recent advances in the comprehension of its aetiology. Neutrophils are swift and powerful innate immune actors which display potent enzymatic activities capable of altering plaques. They have emerged as players in atherosclerotic plaque growth and we are only just beginning to understand their role in the evolution of atherosclerotic lesions. In this context, the leukotriene B_4, one of the main chemoattractants of neutrophils, has been proposed as a potential contributor to human and murine plaque destabilisation. A particular context in which these two actors are closely linked is endotoxemia, itself associated with plaque destabilisation. Understanding whether in such a context, these two players cooperate in the destabilisation of plaques will provide new insights into the pathogenesis of cardiovascular events.

This work was aimed at determining whether leukotriene B_4 plays a role in the chemoattraction of neutrophils in plaques during endotoxemia and at assessing whether neutrophils can tip the balance which maintains plaques stable. We have herein evidenced that the recruitment of neutrophils mediated by leukotriene B_4 has a deleterious impact upon plaque stability during endotoxemia by promoting apoptosis and degrading matrix fibres.

In conclusion, this study paves the way to novel therapeutic approaches aimed at targeting the axis leukotriene-neutrophil in atherosclerotic disease.

Keywords: atherosclerosis, neutrophils, leukotrienes

Résumé

La déstabilisation de la plaque d'athérosclérose reste de nos jours un problème clinique majeur, malgré les progrès récents dans la compréhension de son étiologie. Les neutrophiles sont des acteurs rapides et puissants de l'immunité innée possédant de nombreuses activités enzymatiques capables d'altérer les plaques. Ils sont reconnus comme des acteurs de la progression de l'athérosclérose mais leur rôle dans l'évolution des plaques reste à élucider. Un chimio-attractant majeur des neutrophiles, le leucotriène B_4, pourrait être un des contributeurs potentiels de la déstabilisation des plaques murines et humaines en particulier dans l'endotoxémie qui est associée aux accidents cardiovasculaires. Déterminer si dans un tel contexte, ces deux acteurs participent à la déstabilisation des plaques contribuerait à une meilleure compréhension de la physiopathologie de l'athérosclérose.

L'objectif de ce travail a été de définir le rôle du leucotriène B_4 dans l'attraction des neutrophiles dans la plaque au cours de l'endotoxémie et de déterminer si les neutrophiles peuvent basculer le fragile équilibre qui maintient les plaques stables. Nous avons montré que le recrutement des neutrophiles médié par le leucotriène B_4 a un impact délétère sur la stabilité des plaques murines au cours de l'endotoxémie en favorisant l'apoptose et la dégradation de fibres matricielles.

En conclusion, cette étude ouvre la voie vers de nouvelles approches thérapeutiques visant à cibler l'axe leucotriène-neutrophiles dans la maladie athérosclérotique.

Mots-clés : athérosclérose, neutrophiles, leucotriènes