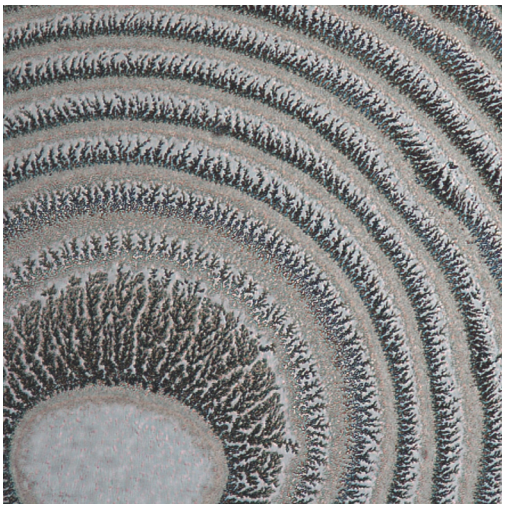
DISSERTATIONES SCHOLAE DOCTORALIS AD SANITATEM INVESTIGANDAM
UNIVERSITATIS HELSINKIENSIS

88/2015

KRISTIINA RAJAMÄKI

# Role of the Inflammasome Pathway in Atherosclerosis



WIHURI RESEARCH INSTITUTE AND
DIVISION OF BIOCHEMISTRY AND BIOTECHNOLOGY
DEPARTMENT OF BIOSCIENCES
FACULTY OF BIOLOGICAL AND ENVIRONMENTAL SCIENCES
DOCTORAL PROGRAMME IN INTEGRATIVE LIFE SCIENCE
UNIVERSITY OF HELSINKI

# ROLE OF THE INFLAMMASOME PATHWAY IN ATHEROSCLEROSIS

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ACADEMIC DISSERTATION

To be presented for public examination with the permission of the Faculty of Biological and Environmental Sciences of the University of Helsinki
at Haartman Institute, lecture hall 1, Haartmaninkatu 3, Helsinki,
on the 27th of November 2015 at 12 o'clock.

Helsinki 2015

*Dissertationes Scholae Doctoralis Ad Sanitatem Investigandam*
*Universitatis Helsinkiensis* 88/2015

ISBN 978-951-51-1644-4 (paperback)
ISBN 978-951-51-1645-1 (PDF)

ISSN 2342-3161 (print)
ISSN 2342-317X (online)

http://ethesis.helsinki.fi

Hansaprint Oy
Helsinki 2015

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*For my parents.*

*'Donner à nos enfants le désir de savoir, éveiller leur curiosité.'*
*'Convey to our children the desire for knowledge, awaken their curiosity.'*

*- Albert Barillé, creator of the Once Upon a Time...Life cartoon -*

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**II**

**Rajamäki K**, Nordström T, Nurmi K, Åkerman KEO, Kovanen PT, Öörni K, Eklund KK. Extracellular acidosis is a novel danger signal alerting innate immunity via the NLRP3 inflammasome. J Biol Chem 2013 May 10;288(19):13410-9.

**III**

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## AUTHOR'S CONTRIBUTION

**I** Author participated in the study design, conducted all the experiments and analyses with the exception of qPCR primer design and the IL-1β Western blot, and participated in writing the manuscript.

**II** Author participated in the study design, conducted all the experiments and analyses with the exception of intracellular pH recordings and cathepsin B activity measurement, and wrote the manuscript.

**III** Author participated in the study design, conducted all the experiments and analyses with the exception of disease status classification of the coronary specimens and some of the statistical analyses, and wrote the manuscript.

The publications are referred to in the text by their Roman numerals. The original articles are reproduced with the permission of the respective copyright holders.

# ABBREVIATIONS

A list of the abbreviations that appear in more than one section are presented here.

| | |
|---|---|
| AHA | American Heart Association |
| AIM2 | absent in melanoma 2 |
| AP-1 | activator protein 1 |
| apo | apolipoprotein |
| ASC | apoptosis-associated speck-like protein containing a CARD domain |
| IFN | interferon |
| IL | interleukin |
| CAPS | cryopyrin-associated periodic syndrome |
| IL-1Ra | interleukin-1 receptor antagonist |
| CARD | caspase activation and recruitment domain |
| CHC | cholesterol crystals |
| CIITA | class II major histocompatibility complex transactivator |
| HDL | high density lipoprotein |
| HFD | high-fat, high-cholesterol diet |
| HLA | human leukocyte antigen |
| KO | knock-out (dKO, double knock-out) |
| LDL | low density lipoprotein |
| LPS | lipopolysaccharide |
| LRR | leucine-rich repeat domain |
| MAPK | mitogen-activated protein kinase |
| MCP-1 | monocyte chemotactic protein 1 |
| M-CSF | macrophage colony-stimulating factor |
| MDM | monocyte-derived macrophage |
| MyD88 | myeloid differentiation primary response 88 |
| NF-κB | nuclear factor kappa-light-chain-enhancer of activated B cells |

| | |
|---|---|
| NLR | nucleotide-binding domain and leucine-rich repeat containing family |
| NLRC | NLR family, CARD domain containing |
| NLRP | NLR family, pyrin domain containing |
| NOD | nucleotide-binding and oligomerization domain |
| oxLDL | oxidized LDL |
| PMA | phorbol-12-myristate-13-acetate |
| PRR | pattern recognition receptor |
| PYHIN | pyrin and HIN domain containing family |
| qPCR | quantitative PCR |
| ROS | reactive oxygen species |
| SR | scavenger receptor |
| TGF | transforming growth factor |
| Th | T helper cell |
| TIR | Toll/interleukin 1 receptor domain |
| TLR | Toll-like receptor |
| TNF | tumor necrosis factor |

# ABSTRACT

Atherosclerosis is the underlying cause of myocardial infarction and stroke, the leading causes of death worldwide. It is a complex multifactorial disease closely linked with obesity, type II diabetes, and metabolic syndrome and, together, these conditions comprise the global epidemic of metabolic disorders that are becoming more and more prevalent, affecting adults and children alike.

Atherosclerosis affects the large arteries that gradully loose their normal structure and function via a degenerative process involving lipid accumulation and chronic inflammation in the arterial wall. The lipid accumulation is driven by high circulating levels of cholesterol-carrying low density lipoproteins that become trapped and modified in the arterial wall. This causes an inflammatory reaction characterized by abundant immune cell infiltrates, mainly monocyte-derived macrophages. The macrophages scavenge large amounts of lipids and become activated to secrete a host of proinflammatory mediators and matrix-degrading enzymes that drive the progression of the disease. These processes result in the focal development of fatty lesions or 'plaques' along the arteries. Over time, more complex lesions develop as a result of inflammatory and fibrotic responses, matrix remodeling, calcification, cholesterol crystallization, neovessel formation, and microhemorrhages. Ultimately, the plaques may rupture, causing thrombosis and acute complications.

Although inflammation is recognized as a major driving force in atherosclerotic lesion development, the mechanisms triggering and maintaining the arterial wall inflammation remain incompletely understood. The aim of this thesis was to study the role of a key innate immune signaling pathway, the inflammasome, in atherosclerosis. The inflammasomes are large cytoplasmic signaling complexes that trigger the proteolytic maturation and secretion of two proinflammatory and proatherogenic cytokines, interleukin(IL)-1β and -18. The inflammasome pathway can be triggered by microbial components or by sterile endogenous danger signals that elicit the activation of cytoplasmic sensor molecules from the NLR (nucleotide-binding domain and leucine-rich repeat containing) or PYHIN (pyrin and HIN domain containing) families. Despite the established roles of IL-1β and -18 in driving atherosclerotic lesion development, the triggers of inflammasome activation in atherosclerotic plaques remained unknown.

Macrophages are the prototypical inflammasome pathway-expressing cells, and thus cultured human macrophages were utilized to identify and characterize atherosclerosis-associated triggers of the inflammasome pathway. Cholesterol crystals and acidic environment were both found to trigger a strong inflammatory response via the activation of NLRP3 inflammasome and secretion of IL-1β and IL-18.

Cholesterol crystals are a hallmark of atherosclerotic lesions, yet they have been considered an inert material that merely acts as a sink for excess free cholesterol in the arterial wall. These new data suggested, however, that cholesterol crystals act as a potent sterile danger signal that may directly link pathological lipid accumulation and inflammation in the lesions. Local extracellular acidosis arises in the growing plaque due to the hindered diffusion of oxygen and the highly active glycolytic metabolism of macrophages. Acidic environment not only triggered the NLRP3 inflammasome, but even a very mild acidification from the physiological pH of 7.4 to 7.0 was sufficient to greatly amplify the IL-1β response to other NLRP3 activators, including cholesterol crystals.

Having showed that the atherosclerotic lesions harbour potent activators of the inflammasome pathway, we further analyzed the expression of this pathway in atherosclerotic human coronary specimens obtained from 10 explanted hearts. For this purpose, we utilized a quantitative PCR array targeting 88 inflammasome pathway-related molecules. Significant upregulation of 12 target genes was found in advanced coronary plaques compared to early lesions from the same coronary trees, including many of the very core components of the inflammasome pathway. Moreover, p38δ mitogen-activated protein kinase (MAPK), a poorly characterized isoform of the stress- and cytokine-activated p38 MAPK family, was consistently upregulated in advanced coronary plaques. Immunohistochemical stainings of human coronary lesions showed strong expression of NLRP3 inflammasome components and p38δ MAPK in macrophages surrounding the cholesterol crystal-rich lipid core. Furthermore, the p38δ MAPK was activated in cultured human macrophages upon NLRP3 inflammasome activation by cholesterol crystals and extracellular ATP, and required for NLRP3-mediated IL-1β secretion.

Taken together, the data presented in this thesis propose novel inflammasome-mediated mechanisms that may trigger sterile inflammation in atherosclerotic lesions and thus drive lesion progression.

# TIIVISTELMÄ

Tässä väitöskirjatyössä tutkittiin tulehdusreaktioiden merkitystä valtimon rasvakovettumataudin eli ateroskleroosin kehittymisessä. Ateroskleroosissa valtimoverisuonien seinämään kertyy sekä veren lipoproteiini-hiukkasten kantamaa kolesterolia että immuunipuolustuksen syöjäsoluja eli makrofageja. Makrofagit yrittävät poistaa liiallista kolesterolia, mutta juuttuvat lopulta rasvarakkuloiden täyttäminä valtimon seinämään ja laukaisevat ateroskleroosin etenemistä edistävän kroonisen tulehdusreaktion. Sen seurauksena rasva-aineiden ja tulehdussolujen muodostamat kertymät kehittyvät vähitellen monimuotoisiksi valtimoa ahtauttaviksi plakeiksi, jotka voivat revetessään aiheuttaa aivo- tai sydäninfarktin.

Inflammasomi on makrofageista löydetty tulehdussignalointireitti, jonka aktivaatio laukaisee voimakkaan tulehdusreaktion käynnistämällä tulehdusvälittäjäaine interleukiini (IL)-1β:n erityksen. Ateroskleroosin hiirimalleissa geneettinen IL-1β-puutos vähentää huomattavasti plakkien kasvua, ja IL-1β:n määrä lisääntyy myös ihmisen valtimon seinämässä plakkien kehittyessä. Plakkien inflammasomi-aktivaatiota ja IL-1β-eritystä laukaisevia tekijöitä ei kuitenkaan aiemmin tunnettu ja väitöskirjatutkimuksen tavoitteena oli tunnistaa tällaisia tekijöitä. Tutkimus osoitti, että plakeissa yleisesti esiintyvät kolesterolikiteet sekä plakin kehittymiseen liittyvä kudosnesteen paikallinen happamoituminen laukaisevat makrofageissa voimakkaan inflammasomi-välitteisen tulehdusvasteen IL-1β-erityksen kautta. Elimistön immuunipuolustus kykenee siis tunnistamaan kyseiset taudinkehitykseen liittyvät muutokset vaarasignaaleiksi ja reagoimaan niihin käynnistämällä tulehdusreaktion.

Kolesterolikiteitä on vuosikymmenien ajan pidetty ateroskleroosin kehityksen kannalta merkityksettöminä sivutuotteina. Tutkimuksen tulokset haastoivat tämän käsityksen osoittamalla, että kolesterolikiteet ovat aktiivinen tekijä ateroskleroosin kehittymisessä ja voivat selittää häiriintyneen rasva-aineenvaihdunnan ja valtimon seinämän tulehduksen välistä yhteyttä. Lisäksi tutkimuksessa havaittiin, että jo hyvin lievä solunulkoinen happamoituminen voimistaa merkittävästi kolesterolikiteiden aiheuttamaa tulehdusvastetta makrofageissa, kun nämä ärsykkeet annetaan soluille samanaikaisesti.

Väitöskirjatyössä inflammasomi-reitin toiminnallisuutta tutkittiin myös sydämen sepelvaltimonäytteissä laajan geeni-ilmentymisanalyysin ja vasta-ainevärjäysten avulla. Tulokset osoittivat, että kaikki inflammasomi-reitin keskeisimmät komponentit ilmentyvät sepelvaltimon seinämän makrofageissa ja useat niistä lisääntyvät merkittävästi ateroskleroottisten plakkien kehittymisen myötä. Geeni-ilmentymisanalyysissa havaittiin myös erään tulehdusta säätelevän molekyylin, p38δ MAP-kinaasin, lisääntyminen sepelvaltimoissa ateroskleroottisten plakkien

kehittymisen myötä. Kyseistä molekyyliä tutkittiin tarkemmin viljellyissä ihmisen makrofageissa, mikä johti uuden tulehdusta säätelevän reitin löytymiseen. Tulokset osoittivat, että p38δ MAP-kinaasin aktivoituminen on keskeinen säätelijä kolesterolikiteiden laukaisemassa inflammasomi-aktivaatiossa.

Ateroskleroottisten sairauksien hoito perustuu tällä hetkellä pääsääntöisesti veren kolesterolipitoisuutta alentavaan lääkitykseen. Valtimon seinämän tulehdusmekanismien tarkka selvittäminen luo perustan uudentyyppisen, kroonista tulehdustilaa hillitsevän lääkityksen kehittämiseen tämän kansanterveydellisesti merkittävän taudin hoitoon.

# 1 INTRODUCTION

*'As expected, it ended in a coronary thrombosis. Obviously, when the fats build up, the passage ways became completely blocked, we couldn't get through anymore.'*

*- A red blood cell in Once upon a time…life, episode 7, The heart –*

This simple view on development of atherosclerosis was presented to me early in childhood by the educational cartoon "*Once upon a time…life*" (original title:"*Il était une fois...la Vie*"; in Finnish "*Olipa kerran elämä*") created in 1986 by the French screenwriter and cartoonist Albert Barillé (1920-2009). The level of accuracy and detail presented in the 26 episodes of the series is astonishing. The very same series also introduced me with the immune system and white blood cells, presented as the patrolling policemen of the circulation (innate immune cells) and the army special task forces releasing swarms of insect-like antibodies to fight the invading microbes (adaptive immune cells). Since then, I have learnt a great deal more about cardiovascular disease and the underlying process of atherosclerosis, involving not only build-up of fats but also chronic inflammation and profound changes in the vessel wall architecture.

Atherosclerosis is a slowly progressing degenerative disease of the large arteries that may ultimately trigger myocardial infarction or stroke, the leading causes of death worldwide. The word 'atherosclerosis' refers to the hardening (from Greek *skleros*, meaning hard) of the arterial walls and deposition of fatty substances, fibrous material, and immune cells to form a plaques with porridge-like consistency (from Greek *athere*, meaning porridge). The disease may develop without symptoms for decades, but sudden complications arise upon plaque rupture and thrombosis that obstructs the blood flow through the artery. The most abundant immune cells in atherosclerotic plaques are the macrophages, innate immune cells that attempt to restore homeostasis by phagocytic clearance of the accumulating lipids. These lipids are derived from plasma lipoproteins, the major carriers of cholesterol and other lipids in circulation. Low density lipoprotein (LDL), or '*bad cholesterol*' in layman's terms, has a pivotal role in plaque development. However, researchers in the 21st century have increasingly acknowledged the key role of inflammatory reactions in disease development (Hansson 2011, Libby 2015).

The hypothesis on defective lipid metabolism as the driver of atherosclerosis began to formulate in the early 20th century. Landmark studies by Ignatowski and Anitschkow showed that feeding of rabbits with a diet rich in animal protein or in pure cholesterol triggered atherosclerosis (Buja 2014). The theory was later fuelled

by epidemiological studies in the 1950's showing a correlation between plasma cholesterol levels and cardiovascular complications in large population studies, including the Framingham study (Dawber 1957). Discovery of the LDL receptor and advances in understanding the regulation of cholesterol metabolism made by Brown and Goldstein in the 1970's and 80's further fortified the lipid hypothesis of atherosclerosis and earned them a Nobel Prize in 1985 (Brown 1974, Anderson 1977, Brown 1980, Brown 1986).

The inflammatory nature of atherosclerotic plaques was acknowledged by several pathologists and physicians already in the 19th century, but these observations were largely neglected amidst the cholesterol frenzy of the 20th century. Most notably, the German pathologist Rudolph Virchow proposed a role for inflammation in the atheromatous process in his highly influential work *Cellular Pathology* (Virchow 1863). Virchow described two distinct early pathological processes in the vessel walls: *"the simple fatty metamorphosis"* and *"a stage of irritation preceding the fatty metamorphosis, comparable to the stage of swelling, cloudiness, and enlargement which we see in other inflamed parts"*. He concludes *"in admitting an inflammation of the inner arterial coat to be the starting point of the so-called atheromatous degeneration"*. In his discussion on more advanced stages of the disease, Virchow describes *"the chronic inflammatory processes going on in the deeper parts* [of aorta]*"* and the rupture of atheromatous depots, causing *"just as destructive results, as we see in the course of other violent inflammatory processes"*.

Thus, Virchow postulated the involvement of inflammatory processes throughout all stages of atherosclerosis, based on purely observational studies of human vessels. It took some 140 years, however, before a major paradigm shift began, marked by a review article titled *Atherosclerosis – an inflammatory disease* by the renowned pathologist Russell Ross published in 1999 (Ross 1999). According to the modern view, atherosclerosis involves a complex interplay between lipid accumulation and the inflammatory responses of arterial wall cells and recruited immune cells to these lipids (Hansson 2011, Libby 2015).

The purpose of this study was to elucidate the role of a pro-inflammatory signaling pathway called *the inflammasome* in atherosclerosis. The inflammasome pathway was discovered in 2002 in macrophages, the key immune cells involved in all stages of atherosclerotic plaque development (Martinon 2002). The inflammasome pathway controls the maturation and secretion of two potent proinflammatory cytokines, interleukin(IL)-1β and -18, that accelerate atherosclerosis in mouse models (Kirii 2003, Elhage 1998, Mallat 2001b, Elhage 2003). The triggers of inflammasome activation and secretion of IL-1β and -18 in atherosclerotic plaques were, however, unknown. We thus set out to identify such triggers in cultured macrophages and to analyse the expression of the inflammasome pathway in human atherosclerotic lesions.

# 2 REVIEW OF THE LITERATURE

## 2.1 MACROPHAGES

Macrophages are integral cells of the innate immune system that perform both immune surveillance and homeostatic functions. Resident macrophages are found in almost all healthy human tissues. This is not a scattered population of few patrolling cells here and there. Instead, resident tissue macrophages form a relatively ordered network comprising ~10 % of cells in many tissues (Sasmono 2003). Macrophages are stellate cells typically occupying a niche in close contact with endothelial or epithelial cells. Tissue macrophages, dendritic cells, and blood monocytes, together with their bone marrow precursors, form the mononuclear phagocyte system (Jenkins 2014). As described by Elie Metchnikoff in the 19th century (Metchnikoff 1892), the key function of macrophages is continuous phagocytosis of material, both host-derived and microbial, from their surroundings (from Greek: *makros*, "large", and *phagein*, "eat"). However, resident tissue macrophages develop highly specialized functions related to their niche. Heterogeneity and plasticity are, indeed, the defining features of macrophages. An excellent summary of the importance of macrophages in host defence was recently presented by Prof. David Hume (Hume 2008): "*A reasonable definition of a pathogen is a microorganism that evades constitutive killing by macrophages.*"

In the following sections, some of the key aspects of macrophage biology will be briefly discussed, pertaining to characteristics common to most macrophage populations.

### 2.1.1 Origin and populations of tissue macrophages

According to the traditional view, resident tissue macrophages differentiate from circulating bone marrow –derived monocytes that extravasate into tissues (Hume 2008, van Furth 1968). However, it is now known that this model is much too simplified (Davies 2013). Thus, some tissue macrophage populations, such as the microglia in the brain, are established already during the prenatal period from the yolk sac (Ginhoux 2010), whereas others, including the Langerhans cells in the skin, are derived from the foetal liver (Hoeffel 2012).

This heterogeneity extends to the maintenance of the various tissue macrophage populations. For example, tissue macrophages in the gut are short-lived and replenished almost exclusively by blood monocytes infiltrating the tissue (Jenkins 2014). Conversely, alveolar macrophages are long-lived and self-renewal occurs via local proliferation. Most often, both self-renewing and monocyte-derived macrophage populations co-exist in the same tissue, as exemplified by macrophages residing in

the peritoneum and in the liver (Jenkins 2014). Furthermore, the tissue macrophage population can be rapidly expanded during infection or injury via enhanced recruitment of blood monocytes, guided by locally produced chemoattractants and adhesion molecules. Current knowledge suggests that the same monocyte subset – CD16-negative in humans corresponding to Ly6C-high in mice (Ingersoll 2010) – is recruited from blood both during steady-state and during inflammation (Jakubzick 2013, Epelman 2014). However, during inflammation, the recruited monocytes more readily differentiate into tissue macrophages, rather than continue migration as monocytes to the draining lymph nodes.

Regardless of their origin, the differentiation, survival, and proliferation of all tissue macrophages is maintained by constant secretion of macrophage colony-stimulating factor (M-CSF) by stromal cells and signaling via its receptor, CSF1R (Jenkins 2014). The balance between M-CSF secretion and consumption defines the size of a tissue macrophage population at any given time. M-CSF-null mice show markedly diminished populations of blood monocytes and tissue macrophages and exhibit marked developmental defects in bones, the pancreas, and the nervous system, as well as infertility (Hume 2008, Wiktor-Jedrzejczak 1982). Intriguingly, comparison of these mice to CSF1R-deficient mice revealed an alternative CSF1R ligand, interleukin-34, that supports macrophage survival in the brain and in the skin (Davies 2013, Dai 2002, Lin 2008). Furthermore, additional cues specific for each niche further define the phenotypes of tissue macrophages, resulting in vast heterogeneity as shown by transcriptomic analysis performed in the Immunological Genome Project (Gautier 2012).

### 2.1.2 Clearance of apoptotic cells – a common homeostatic function

While immune surveillance is a common task for all macrophages, the range of tissue-specific homeostatic functions of macrophages is vast. Bone marrow macrophages support erythropoiesis, whereas splenic macrophages phagocytose senescent red blood cells and regulate iron metabolism and storage; the microglia in the brain remove dead neurons and participate in synaptic remodelling; the lung macrophages regulate the amount of pulmonary surfactant in the alveoli; finally, macrophages in white and brown adipose tissue regulate insulin sensitivity and adaptive thermogenesis, respectively (Davies 2013). Nevertheless, phagocytic removal of senescent, apoptotic, or otherwise dysfunctional or damaged cells and debris is a recurrent theme. This is no small enterprise; for instance, some $10^{10}$ red blood cells are produced every hour in the bone marrow balanced by similar clearance rates (de Back 2014). The clearance of apoptotic cells by professional phagocytes proceeds via a coordinated series of events involving migration, recognition, engulfment, and degradation of the target cell (Poon 2014). Importantly, the process is immunologically silent, a

defining feature differentiating it from the phagocytic clearance of microbes or membrane-permeabilized necrotic cells.

Chemotactic signals produced by apoptotic cells, the so-called 'find-me signals', guide phagocytes to the dying target cell. These include the nucleotides ATP and UTP that are released via the pannexin-1 channel during early apoptosis (Chekeni 2010) and recruit phagocytes by signaling via the $P2Y_2$ receptor (Elliott 2009). Furthermore, apoptotic cells release chemokine-carrying microparticles, as well as lysophosphatidyl choline via caspase-3-mediated activation of calcium-independent phospholipase A2 (Truman 2008, Lauber 2003). Both act as chemoattractants for monocytes and macrophages. Subsequently, 'eat-me signals' on the surface of apoptotic cells are required to direct the physical interaction with phagocytes (Poon 2014). Increased exposure of the inner plasma membrane leaflet lipid phosphatidyl serine (PS) provides the most crucial eat-me signal (Fadok 1992). The phagocytic cells recognize exposed PS moieties both via direct interaction of PS with several receptors - including BAI1, Tim4, and stabilin-2 (Park 2007a, Miyanishi 2007, Park 2008) - and via PS-binding bridging molecules (Poon 2014). In addition to PS receptors, several other receptor classes expressed on macrophages and other innate immune cells contribute to the recognition and engulfment of apoptotic cells, including scavenger receptors, complement receptors, and certain pattern recognition receptors, most notably CD14 (Devitt 2011). Finally, the concurrent loss of 'do-not-eat-me' signals from the target cell is required to trigger engulfment (Poon 2014). Taken together, these redundant mechanisms reflect the essential role of apoptotic cell clearance both in homeostatic functions and in host defence, and highlight the key role of surface-exposed PS in this process.

### 2.1.3 Immune surveillance by macrophages

The macrophages are cells of the innate immunity and, as such, belong to the first line of defence against pathogens. The innate immune cells have evolved several mechanisms to discriminate between self and non-self, between infectious pathogens and harmless commensals, as well as mechanisms to detect 'missing self' and 'modified self' (Medzhitov 2009). These mechanisms rely on germline-encoded receptors, secreted recognition molecules, signaling components, and effector molecules designed to recognize and eliminate a broad spectrum of pathogens.

Many of the key concepts in innate immune recognition evolved through experimentation utilizing insects as a model system. Pioneering work by Hans Boman and co-workers first demonstrated the humoral, non-specific, and inducible nature of the immune response in the fruit fly *Drosophila melanogaster* (Boman 1972), and identified the first secreted antimicrobial peptides using the giant silk moth *Hyalophora cecropia* (Steiner 1981). Based on the rapid bacterial killing mediated by the antimicrobial peptides found accross the animal kingdom, Boman also noted

that the term 'instant immunity' could have been more suited to describe our innate immune system (Boman 2003). Others showed that the induction of antimicrobial peptides in *Drosophila* was controlled by promoter sites resembling the binding sites of an inducible mammalian transcription factor, nuclear factor κB (NF-κB) (Engström 1993, Kappler 1993). NF-κB had been implicated as a transcriptional regulator of immediate early immune response to pathogens (Baeuerle 1994), thus suggesting the existence of a conserved control mechanism between insect immunity and the innate immune response in mammals. Marco Rosetto *et al.* showed in a *Drosophila* blood cell line that the induction of antimicrobial peptide production was controlled by a receptor named Toll (Rosetto 1995). Extending these observations, Bruno Lemaitre *et al.* showed that 'dorsal', the counterpart of NF-κB in *Drosophila*, was activated by binding of 'spätzle', an endogenous ligand induced during fungal infection, to the Toll receptor (Lemaitre 1996). Moreover, this Toll-induced signaling pathway was essential for antifungal immune responses of the fruit fly, as demonstrated by Toll-mutant flies (Lemaitre 1996).

Meanwhile, Charles Janeway had presented in 1989 a very influencial theory on innate immune recognition, yet his theory was not yet backed up by experimental evidence (Medzhitov 2009, Janeway 1989). Janeway proposed that the initial detection of invading micro-organisms in the body is achieved by recognition molecules on innate, rather than adaptive immune cells. These hypothetical germline-encoded receptors would recognize general structural motifs conserved among many pathogens, termed 'pathogen-associated molecular patterns'. An alternative theory by Polly Matzinger proposed that rather than recognizing the pathogen, innate immune system is alerted by 'danger signals' released from host cells upon tissue injury (Matzinger 1994). Experimental evidence for Janeway's pattern recognition theory was provided by the discovery of mammalian Toll homologues, named Toll-like receptors (TLR), that mediated NF-κB activation and antimicrobial responses via direct binding of microbial ligands (Medzhitov 1997, Poltorak 1998, Yang 1998). Similarly, Matzinger's danger theory has received experimental support from several studies identifying modified or 'out-of-place' endogenous molecules that trigger immune responses, many of them even acting upon the same receptors as microbial stimuli (Bryant 2015). Thus, though the original theories by Janeway and Matzinger were to some extent opposing, today both aspects of innate immune recognition are well-appreciated.

#### *2.1.3.1 Pattern recognition receptors*

Innate immune cells have developed a wide selection of germline-encoded pathogen sensor molecules expressed at the cell surface, the cytoplasm, as well as in the intracellular vesicle compartments (Medzhitov 2009). These molecules are called *pattern recognition receptors* (PRRs) according to their ability to recognize conserved

microbial structures. Ligand binding to PRRs triggers various intracellular signaling cascades that culminate in the activation of key transcription factors controlling the mRNA expression of pro-inflammatory cytokines and chemokines, costimulatory molecules, and antiviral interferons (Takeuchi 2010). Some cytoplasmic PRRs assemble into a caspase-1-activating signaling platform called the inflammasome, which mediates the proteolytic maturation and unconventional secretion of the pro-inflammatory cytokines interleukin-1β and -18 (Latz 2013).

PRRs expressed in macrophages include TLRs, C-type lectin receptors (CLRs), RIG-I-like receptors (RLRs), pyrin and HIN domain containing proteins (PYHINs), and nucleotide-binding domain and leucine-rich repeat containing proteins (NLRs) (Takeuchi 2010, Schattgen 2011) (**Fig.1**). The TLRs are transmembrane proteins in charge of immune surveillance at the cell surface and endolysosomal compartments. The TLRs recognize a wide range of microbial structures and endogenous danger signals, as discussed in the following section. The CLRs are expressed at the cell surface and mediate proinflammatory cytokine expression in response to fungal cell wall carbohydrates (Kingeter 2012). The RLRs are cytoplasmic RNA helicases that recognize viral double-stranded RNA, triggering primarily antiviral responses (Yoneyama 2004). Similarly, cytoplasmic/nuclear PYHINs recognize bacterial and viral double-stranded DNA and trigger antiviral interferon responses (Unterholzner 2010) or inflammasome assembly (Hornung 2009, Fernandes-Alnemri 2009). Finally, the cytoplasmic NLRs respond to diverse microbial components, toxins, and endogenous danger signals. Some NLRs trigger pro-inflammatory cytokine expression (Inohara 1999, Ogura 2001), while others mediate inflammasome assembly (Latz 2013).

The activation of macrophages solely via direct PRR-mediated recognition of pathogens, sometimes referred to as "innate activation" (Gordon 2003), occurs in the absence of any contribution by adaptive immune cells. The ensuing release of inflammatory mediators by resident tissue macrophages alerts the local tissue cells and initiates the rapid recruitment of further innate immune cells from circulation into the site of infection or injury. The adaptive immune responses mounted by T and B lymphocytes arriving later at the inflamed site are shaped by the ongoing innate response. Conversely, macrophages are profoundly affected by the mediators released by T lymphocytes, as discussed in section 2.1.6.

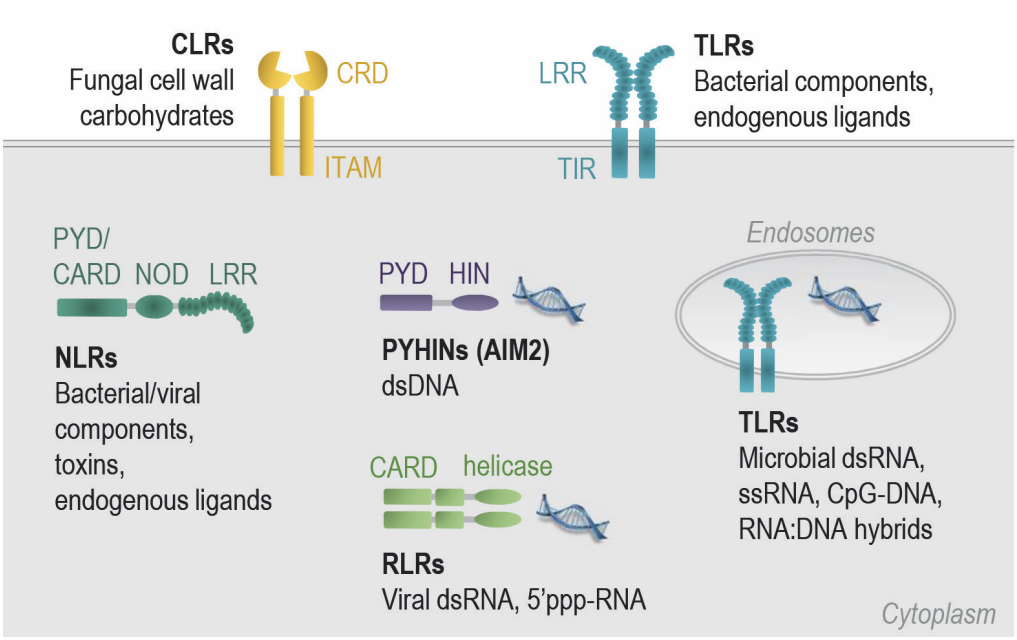


**Figure 1. Macrophage pattern recognition receptors involved in immune surveillance.** The PRRs at the cell surface detect mainly carbohydrates, lipoproteins, and lipids present in bacterial and fungal cell walls, as well as certain endogenous ligands acting as danger signals. The cytoplasmic and endosomal PRRs include a large variety of receptors recognizing microbial nucleic acids, as well as the NLR family recognizing diverse microbial and endogenous molecules. Two PRRs have been identified from the human PYHIN family, the DNA sensors AIM2 (depicted) and IFI16 (not depicted, mainly nuclear). The PRRs from different families share some of the functional domains involved in ligand recognition (LRR) and homotypic domain-domain interactions (CARD, PYD). CRD, carbohydrate recognition domain; ITAM, immunoreceptor tyrosine-based activation motif; LRR, leucine-rich repeat domain; TIR, Toll/IL-1 receptor domain; CARD, caspase activation and recruitment domain; PYD, pyrin domain; NOD, nucleotide-binding and oligomerization domain; HIN, hematopoietic interferon-inducible nuclear protein domain.

### *2.1.3.2 Immune recognition via opsonisation*

Various soluble molecules can mark pathogens for indirect recognition by macrophages. These molecules are collectively referred to as 'opsonins', a name derived from Greek and translating roughly to 'supplying or preparing food'. In the context of host defence, the interaction of opsonins with macrophage receptors triggers efficient phagocytosis and microbial killing by oxidative burst or cytolytic activity towards a damaged or infected host cell, accompanied by the release of inflammatory mediators. Notably, the recognition of opsonins may also result in immunologically silent uptake, as exemplified by clearance of apoptotic cells via bridging molecules that, in fact, function as opsonins in the process (Poon 2014).

Antibodies produced by B lymphocytes are the prototypical opsonins that enhance the uptake of microbes by phagocytic cells. The constant Fc regions of different immunoglobulin classes are recognized by the Fc receptors widely expressed in hematopoietic cells, including the macrophages. Fcγ receptors recognize the most abundant immunoglobulin class, IgG, and trigger phagocytosis via inducing receptor clustering and signal transduction that drives the reorganization of actin cytoskeleton for target engulfment (Goodridge 2012, Nimmerjahn 2008). The Fcγ receptors generally bind IgG with low affinity. Therefore, coordinated receptor interaction with multivalent ligands, such as IgG-opsonized particles or immune complexes, is required to overcome the low affinity barrier (Goodridge 2012, Nimmerjahn 2008).

The humoral arm of the innate immunity also includes many opsonins, such as soluble pattern recognition molecules and components of the complement system. These molecules are synthesized mainly in the liver, but also innate immune cells and endothelial cells can produce a subset of them (Bottazzi 2010). Soluble pattern recognition molecules include pentraxins, collectins, and ficolins that can be considered functional ancestors of antibodies (Bottazzi 2010). Notably, the pentraxins, such as C-reactive protein, bind various microbial structures and act as Fcγ receptor ligands or trigger further opsonisation by complement (Bharadwaj 1999, Lu 2008, Roumenina 2006). The complement system comprises a large group of plasma proteins that trigger a complex hierarchical protease cascade leading to opsonisation of microbes and damaged or infected host cells. Complement activation can also directly trigger membrane permeabilization and lysis of the target microbe or host cell. The classical route and the lectin route of complement activation are both initiated by soluble pattern recognition molecules, the C1q and the collectins or ficolins, respectively (Bottazzi 2010). C1q binds to microbes and dying cells and mainly regulates homeostatic processes, whereas collectins and ficolins promote enhanced phagocytosis of pathogens via their various receptors on immune cells (Bottazzi 2010). Complement activation not only boosts the phagocytotic clearance of microbes and damaged or infected host cells by macrophages, but triggers also macrophage activation and the release of proinflammatory mediators (Hänsch 1984).

### 2.1.4 Toll-like receptors: The prototypes of pattern recognition

Discovery of the mammalian TLR family was based on homology with the *Drosophila* Toll receptor involved in antifungal immune responses (Rosetto 1995, Lemaitre 1996, Medzhitov 1997, Poltorak 1998). Unlike the Drosophila Toll, the vertebrate TLRs directly recognize and bind microbial components and thus act as true PRRs. Ten functional TLRs (TLR 1-10) have been identified in humans, and the TLRs 1-9 conserved between humans and mice have been extensively characterized (Bryant 2015, Kawai 2010). The TLRs are transmembrane proteins characterized by 1) leucine-rich repeat (LRR) domain facing the extracellular space

or lumen of the endolysosomal vesicles and 2) Toll-interleukin-1 receptor (TIR) domain facing the cytoplasm. The LRR domain mediates ligand binding, whereas the TIR domain initiates downstream signaling by the activated receptor.

#### *2.1.4.1 TLR ligands*

The TLRs recognize and bind to a wide array of bacterial, viral, protozoan, and fungal ligands that include proteins, lipids, sugar moieties, and nucleic acids. Traditionally, the TLRs are grouped into two groups according to their subcellular localization (**Fig. 2**). TLRs 1, 2, 4, 5, and 6 are expressed on the cell surface, where they co-operate to detect lipids or proteins found in the surface structures of microbes, including the cell wall and the flagellae involved in bacterial locomotion (Bryant 2015, Kawai 2010). The other group comprises TLRs 3, 7, 8, and 9 trafficking between the endoplasmic reticulum, endosomes, and lysosomes, where they detect microbial nucleic acids (Bryant 2015, Kawai 2010). Notably, TLRs 2 and 4 at the cell surface are activated also by several endogenous ligands, sometimes referred to as danger or damage-associated molecular patterns. For example, the nuclear protein high mobility group box 1 released upon tissue injury activates both TLR2 and TLR4 (Tsung 2005, Park 2006) and the chaperone heat shock protein 60 activates TLR4 (Ohashi 2000). Furthermore, TLRs 2 and 4 recognize extracellular matrix components released or modified during inflammation and tissue injury, such as hyaluronan, heparan sulfate, biglycan, and fibronectin extra domain A (Termeer 2002, Johnson 2002, Schaefer 2005, Okamura 2001).

#### *2.1.4.2 TLR activation and downstream signaling*

Upon ligand binding, most TLRs homodimerize or, in the case of some cell surface TLRs, heterodimerize (**Fig. 2**). Thus, TLR2:TLR1 heterodimer binds triacylated bacterial lipopeptides (Jin 2007), whereas TLR2:TLR6 heterodimer recognizes diacylated lipopeptides (Kang 2009). In addition to the presence of an appropriate ligand, activation of certain TLRs requires additional accessory molecules (Lee 2012). TLR4, the sensor of lipopolysaccharide (LPS) derived from cell walls of Gram-negative bacteria, offers a good example. LPS binds TLR4 only when complexed with the LPS-binding protein, and the co-receptor cluster of differentiation 14 (CD14) facilitates the transfer of this complex to TLR4 (Lee 2012). Finally, myeloid differentiation 2 (MD-2), a soluble protein that associates with TLR4, is essential for TLR4 activation by LPS and both MD-2 and TLR4 participate in LPS recognition (Lee 2012). Notably, CD14 binds also many other TLR ligands and facilitates their delivery to the TLRs (Lee 2012). Similarly, the scavenger receptor CD36 is required for binding of certain ligands to the TLR2:6 dimer (Lee 2012).

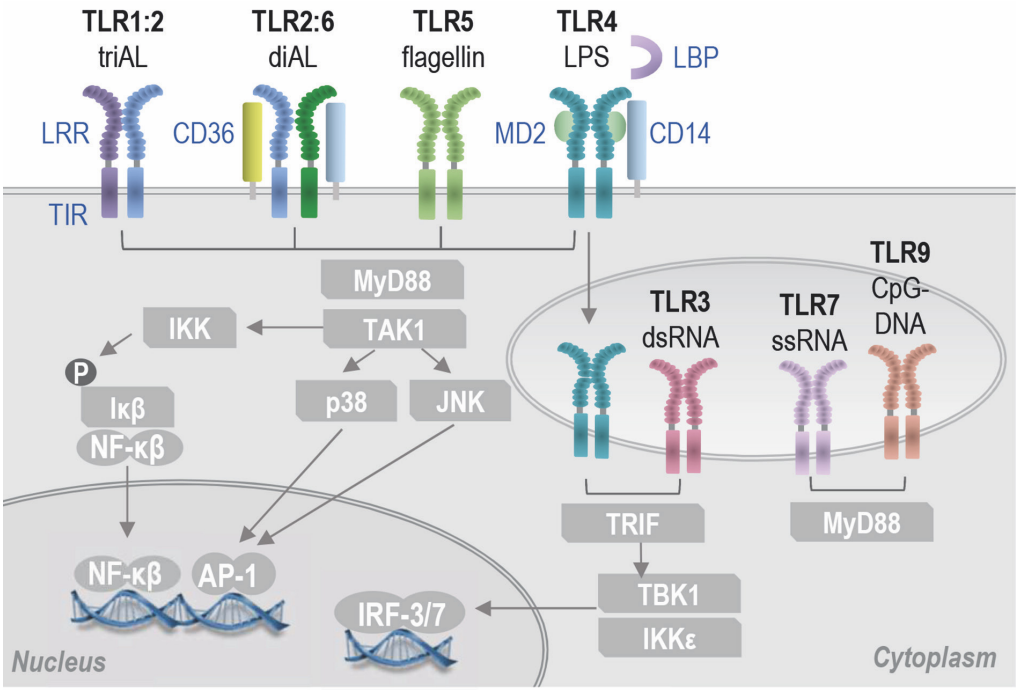


**Figure 2. TLR signaling.** Both the MyD88 and TRIF adapters initiate a TAK1-mediated cascade that results in activation of IKK, phosphorylation of IκB, and the nuclear translocation of NF-κB. In parallel, TAK1 activates the p38 and JNK MAPK cascades leading to AP-1 activation. The TRIF-mediated TAK1 signaling is omitted for clarity. In addition, TRIF triggers the activation of noncanonical IKKs, the TBK1 and IKKε, that mediate the phosphorylation and activation of IRF3 and IRF7. The TLR4 is unique among TLRs in that it triggers both an early MyD88-dependent response and a late TRIF-dependent response after receptor endocytosis. See the text for further details. AL, acylated lipopeptide; AP-1, activator protein 1; CD, cluster of differentiation; IκB, Inhibitor of κB; IKK, Inhibitor of κB kinase; IRF, interferon regulatory factor; JNK, c-Jun N-terminal kinases; LPS, lipopolysaccharide; LRR, leucine-rich repeat domain; MD-2, myeloid differentiation 2; MyD88, myeloid differentiation primary response 88; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; p38, p38 mitogen-activated protein kinases; TAK1, transforming growth factor β -activated kinase; TBK1, TRAF family member -associated NF-κB activator -binding kinase 1; TIR, Toll/IL-1 receptor domain; TRIF, TIR domain-containing adapter inducing interferon-β.

After TLR activation, the cytoplasmic TIR domains of TLRs recruit various TIR-containing adapter molecules that determine the activated downstream signaling cascades (Kawai 2010). The signaling adapter myeloid differentiation primary response 88 (MyD88) induces proinflammatory cytokine expression by activating the transcription factors NF-κB and activator protein 1 (AP-1). Transforming growth factor β -activated kinase (TAK1) is the central hub in MyD88-mediated

signaling. TAK1 triggers deactivation of the Inhibitor of κB (IκB) via activating the Inhibitor of κB kinase (IKK) complex, as well as activation of mitogen-activated protein kinase (MAPK) cascades resulting in AP-1 activation (Kawai 2010, Perkins 2007). Alternatively, some TLRs recruit the signaling adapter TIR domain-containing adapter inducing interferon-β (TRIF). TRIF triggers activation of the TAK1/NF-κB/AP-1 axis, as well as activation of interferon regulatory factor (IRF) 3 or 7 via the non-typical IKK complexes IKKε and TRAF family member -associated NF-κB activator -binding kinase 1 (TBK1) (Kawai 2010). NF-κB and AP-1 trigger the production of proinflammatory cytokines (e.g. IL-1, TNF-α, IL-6), whereas IRF3/7 mediates the production of type I interferons involved in antiviral responses.

### 2.1.5 Inflammatory mediators produced by macrophages

Macrophages produce a myriad of pro- and anti-inflammatory mediators. These include a large number of secreted protein mediators, the cytokines, as well as various lipid mediators. The key molecules and their functions during inflammatory responses are briefly introduced below.

The cytokines are further divided into several subgroups, including interleukins (IL), interferons (IFN), tumor necrosis factor (TNF) family, transforming growth factors (TGF), colony-stimulating factors, and chemokines. In response to an appropriate stimulus, macrophages are capable of releasing mediators from all these subgroups. Stimulation of macrophages with TLR ligands induces the synthesis of a key triad of pro-inflammatory cytokines: TNF-α, IL-1β, and IL-6. All three cytokines contribute systemically to the induction of acute-phase protein secretion by the liver, and, notably, IL-1β also induces fever (Cray 2009, Dinarello 2009). TNFα and IL-1β, in particular, are 'at the top of the food chain' in cytokine signaling networks. They amplify NF-κB activation and trigger the synthesis of countless other inflammatory mediators, including chemokines, adhesion molecules, lipid mediators, and other pro-inflammatory cytokines (Dinarello 2009, Turner 2014). Moreover, IL-1β promotes the activity of T helper (Th) cells, particularly the Th17 subset (Santarlasci 2013). A major function of IL-6 during inflammation is its crucial contribution to maturation of B cells into antigen-producing plasma cells (Hirano 1986). Furthermore, IL-12 and IL-18 released by macrophages promote the differentiation and IFN-γ production of Th1 cells, respectively (Hsieh 1993, Okamura 1995). Macrophages are also a major source of the widely studied chemokine IL-8, a strong chemoattractant for neutrophils (Turner 2014, Hammond 1995). Finally, the secretion of type I interferons by macrophages, triggered for example via various endosomal and cytoplasmic nucleic acid sensors, orchestrates antiviral processes in surrounding cells (Liu 2011). Thus, the interferon-inducible genes encode proteins that hinder viral entry to host cells, target viral components for degradation, and block budding of virions from the cell membrane.

On the other hand, macrophages produce potent anti-inflammatory cytokines, including IL-10 and TGF-β that function in resolution of inflammation, tissue repair, and maintenance of tolerance (Assoian 1987, Fiorentino 1991). IL-10 promotes differentiation of regulatory T cells (Groux 1997) and TGF-β has a wider role in regulation of the T cell functions (Li 2006). Both cytokines also act on macrophages themselves by dampening the synthesis of pro-inflammatory cytokines (IL-1α/β, TNF-α, IL-6) (Fiorentino 1991, Bogdan 1992). Moreover, macrophages also produce molecules specifically counteracting the effects of certain pro-inflammatory cytokines, such as IL-1 receptor antagonist (Matsushime 1991) and IL-18 binding protein (Corbaz 2002).

Lipid mediators of inflammation have been studied much less compared to cytokines, yet recently, some novel roles for lipid mediators have been discovered. Macrophages can produce a number of lipid mediators accross all the main subgroups (Yang 2011). Arachidonic acid is a polyunsaturated omega-6 fatty acid released from membrane phospholipids by phospholipase $A_2$ during inflammation. Further enzymatic reactions produce oxidized derivatives called eicosanoids, including prostaglandins and leukotrienes that promote vascular permeability, leukocyte chemotaxis, pain, and swelling locally at the inflamed site (Serhan 2007). Lipoxins, another class of arachidonic acid derivatives, and the newly discovered resolvins, protectins, and maresins, are critical for active resolution of inflammation (Serhan 2007, Buckley 2014). The latter are produced from omega-3 fatty acids.

Taken together, macrophages are equipped with an impressive arsenal of effector molecules that regulate all phases of the inflammatory response. These inflammatory mediators form a complex network or synergistic and opposing effects, as well as induction and counter-regulation among them. Depending on the mediator, the effects may spread systemically or be highly local, acting in an autocrine or paracrine manner.

### 2.1.6 Macrophage polarization during inflammation

Macrophage activation is defined as a transient induction or enhancement of a particular effector function in response to a stimulus (Adams 1984). The classical microbicidal and tumoricidal effector functions of macrophages are inducible by the Th1 cytokine interferon-γ, together with a microbial trigger (LPS) (Gordon 2003, Nathan 1983, Celada 1984, Dalton 1993). The discovery of an alternative mode of macrophage activation, inducible by the Th2 cytokines IL-4 or IL-13 (te Velde 1988, Stein 1992, McKenzie 1993, Doherty 1993, Doyle 1994), lead to the development of a highly influential concept of 'polarized' macrophage activation (Gordon 2003, Locati 2013). Mills and colleagues proposed the names 'M1' and 'M2' for the classically and alternatively activated macrophages, respectively, reflecting the associated Th1 and Th2 type immune responses (Mills 2000).

#### *2.1.6.1 Characteristics of M1- and M2-polarized macrophages*

The M1-polarized macrophages are characterized by production of high levels of pro-inflammatory cytokines (e.g. TNF-α, IL-1β, IL-6), nitric oxide, and reactive oxygen species, efficient phagocytosis and bacterial killing by respiratory burst, tumoricidal activity, as well as by the ability to promote Th1 and Th17 immune responses via production of IL-12 and IL-23 (Gordon 2003, Locati 2013). Conversely, the hallmarks of IL-4 or IL-13-elicited M2 macrophages are increased mannose receptor activity, enhanced expression of major histocompatibility complex II molecules, and attenuation of pro-inflammatory cytokine production (Gordon 2003, te Velde 1988, Stein 1992, McKenzie 1993, Doherty 1993, Doyle 1994). The mannose receptor, suppressed in M1 macrophages, is an endocytic receptor that recognizes both endogenous and microbial mannose-containing sugar moieties and is involved in the delivery of antigens into professional antigen-presenting cells (Gordon 2003, Martinez-Pomares 2012). Furthermore, M2-polarized macrophages are characterized by increased activity of arginase 1, an enzyme that converts arginine to ornithine that serves as a precursor for polyamines and collagen involved in cell growth and matrix production (Modolell 1995, Munder 1998). In contrast, M1 macrophages suppress the arginase 1 pathway and direct arginine to the synthesis of nitric oxide by the inducible nitric oxide synthase, thus promoting the cytotoxic and microbicidal effector functions (Modolell 1995, Munder 1998). Notably, also other cytokines, including IL-10, IL-21, and IL-33, have been described to induce M2-like homeostatic or anti-inflammatory macrophage polarization (Locati 2013).

The M1 polarization represents a stereotype of pro-inflammatory and microbicidal macrophage phenotype, whereas the M2 polarization is mainly associated with resolution of inflammation. M1-like macrophages are involved in resistance against intracellular pathogens, but mediate detrimental effects in autoimmune and inflammatory conditions, such as systemic lupus and rheumatoid arthritis (Sica 2012). Conversely, M2-like macrophages play important roles in host defence against parasite infections and contribute to allergies and asthma. However, it is important to note that the M1 and M2 phenotypes do not represent static subsets of macrophages, but rather, a reversible adaptation of the macrophages to their specific microenvironment. For example, a switch from M1-like to M2-like macrophage polarization is observed during transition from acute to chronic infection, which likely protects against excessive pro-inflammatory cytokine production (Sica 2012). In the setting of carcinogenesis, a similar M1-M2 switch and the associated suppression of M1-associated antitumor activity may contribute to tumor progression (Sica 2012).

#### *2.1.6.2 Beyond M1/M2: The dynamic continuum of macrophage polarization*

The M1/M2 paradigm has served as an invaluable roadmap in guiding the research and shaping our conceptions of innate immune function. However, as originally stated

by Mills and colleagues (Mills 2000) proposing the M1/M2 classification: *"--while useful for conceptualizing immune responses,* [the classification] *certainly could be an oversimplification. Instead, there may be a continuum of phenotypes between M-1 and M-2 macrophages."* Indeed, this firmly anchored dichotomous view of macrophage activation has often been interpreted without acknowledging its inherent restrictions (Martinez 2014). Analyses of tissue macrophages in disease have revealed mixtures of complex phenotypes poorly predictable from the *in vitro* models (Martinez 2014). To this end, the various 'omics' approaches offer tools to comprehensively characterize macrophage phenotypes and the underlying regulatory networks in an unbiased manner. For example, a large-scale transcriptomic analysis of primary human macrophages stimulated with 28 different stimuli identified a dynamic spectrum of expression signatures, comprising core signatures common to several activation states complemented by stimulus-specific additional modules (Xue 2014).

Other approaches include studying the effects of epigenetic modulation and noncoding microRNAs on macrophage polarization and plasticity. The histone demethylase Jumonji domain containing 3 is crucial in the epigenetic regulation of M2 polarization both *in vitro* and *in vivo,* and required for M2 macrophage -mediated host defence against helminth infection (Ishii 2009, Satoh 2010). Moreover, a histone methyltransferase, SET and MYND domain-containing 2, negatively regulates M1 polarization of macrophages by suppressing the expression of proinflammatory cytokines, major histocompatibility complex II molecules, and costimulatory molecules (Xu 2015). The microRNA miR-155 downregulates the expression of interleukin 13 receptor α1 and represses IL-13-induced gene expression in human macrophages, thus attenuating M2 polarization (Martinez-Nunez 2011). Conversely, miR-223 suppresses M1 polarization and drives M2 polarization of macrophages, which was protective against obesity-induced adipose tissue inflammation (Zhuang 2012).

Another emerging research field is the effect of macrophage energy metabolism on their inflammatory functions. A metabolic switch to aerobic glycolysis occurs during M1, but not during M2 activation of macrophages, resembling the well-established Warburg effect in cancer cells (Rodriguez-Prados 2010, Tannahill 2013, Warburg 1956). Moreover, the glycolytic switch was required for the synthesis of IL-1β, a key M1 cytokine (Tannahill 2013). Remarkably, this concept extends also to other innate and adaptive immune cells; the energy metabolism of pro- and anti-inflammatory subtypes are dominated by glycolysis and oxidative phosphorylation, respectively (O'Neill 2013).

These new approaches are rapidly reshaping the paradigm of macrophage activation and polarization, yet many study setups are still fundamentally based on the old M1/M2 paradigm. A comprehensive understanding of macrophage activation is emerging that highlights the dynamic and multidimensional nature of macrophage activation,

extending far beyond the M1/M2 dichotomy (Schultze 2015). The ultimate goal is to integrate the various aspects of regulation – transcriptional, post-transcriptional, epigenetic, and metabolic – into an overall model that will greatly deepen our understanding of macrophage biology and enable specific modulation of macrophage activation to combat inflammatory diseases.

## 2.2 THE INFLAMMASOME PATHWAY

The inflammasome is a key pro-inflammatory signaling pathway active in cells of the innate immunity. The term was coined by late Prof. Jürg Tschopp and his research group at the University of Lausanne, who discovered the pathway from monocyte-macrophages (Martinon 2002). The name refers to the structural and functional similarities of the inflammasome with an apoptotic signaling pathway, the apoptosome (Martinon 2009, Chai 2014). Both pathways involve cytoplasmic sensor molecules that upon activation assemble a large intracellular protein complex that functions as a caspase activation platform. Members of the NLR and PYHIN families serve as the sensor molecules initiating inflammasome complex assembly, which results in activation of an inflammatory caspase, the caspase-1 (Strowig 2012). Caspase-1 mediates the proteolytic maturation and secretion of the proinflammatory cytokines IL-1β and IL-18, as well as a highly proinflammatory form of cell death called pyroptosis (Strowig 2012).

Triggers of the inflammasome pathway include both microbial components and sterile endogenous danger signals. Accordingly, the inflammasome pathway is involved both in host defence and in chronic inflammatory diseases. Macrophages are the prototypical inflammasome pathway-expressing cells with high levels of inflammasome activity. Varying levels of inflammasome activity have been demonstrated also in other innate immune cells, including dendritic cells and neutrophils (Sharp 2009, Bakele 2014), and in certain non-immune cells, such as keratinocytes (Feldmeyer 2007). The first human diseases associated with impaired inflammasome pathway function were a group of rare autoinflammatory diseases called cryopyrin-associated periodic syndromes that are caused by mutations in one of the inflammasome sensor molecules, the NLRP3 (also known as CIAS1, cryopyrin, and NALP3) (Hoffman 2001, Aksentijevich 2002, Feldmann 2002). Today, the inflammasome pathway has been linked also to a growing number of common metabolic diseases involving chronic inflammation (Robbins 2014). Moreover, the inflammasome pathway has important roles in host defence against intracellular bacteria and viruses (Vanaja 2015), as well as in regulation of intestinal homeostasis (Chen 2014). The focus of this chapter will lie in the activation mechanisms of the inflammasome. Moreover, the NLRP3 inflammasome and its role as a mediator of sterile inflammation will be given a special emphasis.

### 2.2.1 The NLR and PYHIN family proteins: Initiators of inflammasome assembly

The human NLR and PYHIN families comprise 22 and 4 members, respectively (Ting 2008, Cridland 2012). Of these, three NLR proteins – NLRP1, NLRP3, and NLRC4 – and the PYHIN protein absent in melanoma 2 (AIM2) have been firmly established as cytoplasmic sensor/receptor molecules that initiate inflammasome complex assembly (Vanaja 2015). In addition, few other members of the NLR and PYHIN families, and even one protein outside these families (pyrin) have putative functions as initiators of inflammasome complex assembly (Vanaja 2015). Of note, the inflammasome complexes are named after the sensor/receptor molecule initiating their assembly, e.g. *the NLRP1 inflammasome*. The fourth letter after NLR in the nomenclature of NLR proteins signifies the N-terminal effector domain (e.g. NLR*C* for NLR family CARD domain containing and NLR*P* for NLR family pyrin domain containing) (Ting 2008).

Many NLR family proteins have also well-characterized functions independent of the inflammasome pathway. NLRC5 and NLRA/CIITA are nuclear transactivator/enhancer proteins essential for transcription of the antigen-presenting class I and class II human leukocyte antigen (HLA) molecules, respectively (Meissner 2010, Robbins 2012, Steimle 1993, Steimle 1994). Moreover, NLRC1/NOD1 and NLRC2/NOD2 act as cytoplasmic PRRs that mediate NF-κB activation (Inohara 1999, Ogura 2001). Conversely, NLRP6 and NLRP12 may suppress TLR-mediated NF-κB activation to regulate gut microbiota and homeostasis (Chen 2014). The PYHIN family was discovered as a cluster of interferon-inducible genes on mouse chromosome 1 (Ludlow 2005). Whereas the NLR family functions are focused on inflammation, the PYHINs serve mixed functions related also to DNA damage response, cell cycle regulation, and tumor suppression (Ludlow 2005).

The sensor molecules triggering inflammasome complex assembly show distinct, yet partially overlapping expression patterns. NLRP1 and NLRP3 proteins are expressed in various cultured immune cells, including monocytes, macrophages, neutrophils, and lymphocytes (Kummer 2007). Notably, NLRP3 protein is expressed at a low level in resting primary immune cells, but the expression is inducible by stimulation with TLR ligands (Kummer 2007, Bauernfeind 2009). In normal human tissues, NLRP1 staining was found in leukocytes of lymphoid organs and in alveolar macrophages (Kummer 2007). In contrast, NLRP3 stained negative in lymphoid tissues, implying inducible expression in immune cells also *in vivo*. Moreover, NLRP1 and NLRP3 proteins are differentially expressed in epithelial cells along the gastrointestinal and respiratory tracts, cervix, and bladder (Kummer 2007). NLRC4 mRNA was detected in human bone marrow, lymphoid organs, placenta, and brain (Poyet 2001), whereas

AIM2 mRNA was found in spleen, small intestine, testis, and peripheral blood leukocytes (DeYoung 1997).

Other crucial inflammasome pathway components include the adapter protein ASC (apoptosis-associated speck-like protein containing a caspase activation and recruitment domain) and the caspase-1 protease. ASC protein is widely expressed in human tissues, e.g. in epithelial cells of colon, tonsil, and skin, as well as in monocytes and alveolar macrophages (Masumoto 2001). Finally, caspase-1 mRNA is expressed in human bone marrow, lymph nodes, spleen, and placenta (Druilhe 2001). Taken together, the inflammasome pathway components are strongly expressed in immune cells and epithelial cells at barrier sites, in good agreement with the function of these receptors in immune surveillance.

## 2.2.2 Mechanisms of inflammasome assembly

The core components of inflammasome complexes variably express two functional domains from the death domain superfamily: the pyrin domain (PYD) and the caspase activation and recruitment domain (CARD) (Ting 2008, Cridland 2012, Park 2007b). Before the discovery of the inflammasome pathway, other death domain -containing proteins were known to assemble into diverse signaling complexes via homotypic domain interactions, resulting in the activation of NF-κB and apoptotic caspases (Park 2007b). Furthermore, the death domain superfamily family member Apaf-1 that assembles the apoptosome complex was found to bear an overall domain structure similar to NLRs, which provided a working model for studying NLR function (Martinon 2002, Poyet 2001). Apaf-1 binds cytochrome c via its WD40 repeat domain, which relieves an autoinhibitory conformation (Chai 2014). This enables the self-oligomerization of Apaf-1 via its nucleotide-binding and oligomerization domain into a ring-shaped septamer, which allows the recruitment of caspase-9 to Apaf-1 via CARD-CARD interaction. Indeed, similar mechanisms were later found to govern inflammasome assembly.

### *2.2.2.1 Domain structures of inflammasome complex components*

The inflammasome-forming NLRs, as well as AIM2, are cytoplasmic proteins maintained as inactive monomers in the absence of activating stimuli. Interaction with the chaperone heat-shock protein 90 and the ubiquitin ligase SGT1 is required for stabilization of the NLR monomers into an inactive but signaling-competent state (Mayor 2007). Moreover, the C-terminal leucine-rich repeat (LRR) domain of NLRs is required for maintenance of the protein in an autoinhibitory state (Ting 2008, Poyet 2001, Agostini 2004, Faustin 2007, Kofoed 2011) (**Fig. 3**). In addition to the LRR, the NLRs harbour a central nucleotide-binding and oligomerization domain (NOD) and an N-terminal death domain, either a PYD or a CARD, that mediates the recruitment of binding partners downstream NLR activation (Ting

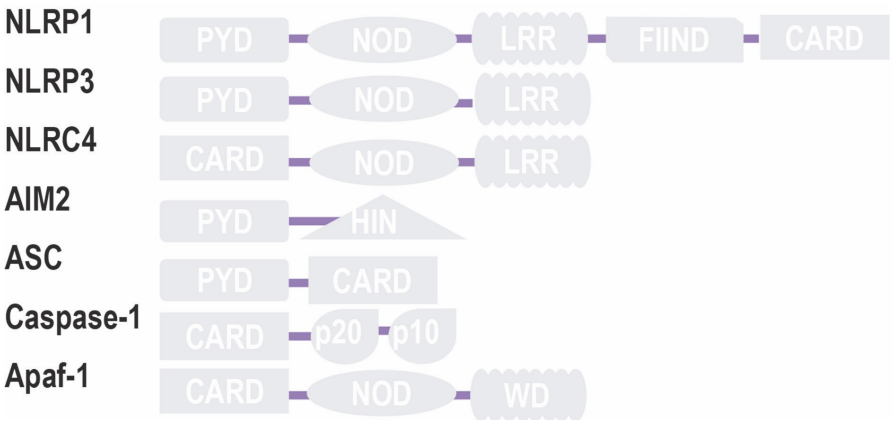


**Figure 3. Domain structures of the inflammasome complex components.** Domains are listed from N- to C-terminus. PYD, pyrin domain; CARD, caspase activation and recruitment domain; NOD, nucleotide-binding and oligomerization domain; LRR, leucine-rich repeat domain; FIIND, filamin interactin domain; HIN, hematopoietic interferon-inducible nuclear protein domain; WD, WD40 repeat domain. The p10 and p20 represent the catalytic subunits of caspase-1, connected by a short linker region that is cleaved during caspase-1 activation. The Apaf-1 scaffold protein mediating apoptosome assembly is shown for comparison. See the details in text.

2008). NLRP1 is unique among the NLR family in that it harbours two additional domains, a filamin interactin domain and a CARD, at the C-terminus distal to the LRR region. As discussed in detail in sections 2.2.4 and 2.2.5, the activation mechanisms of inflammasome-forming NLRs are rather complex and involve accessory NLR proteins that aid in ligand sensing. Similar to NLRP1/3, AIM2 harbours an N-terminal PYD that is followed by a C-terminal PYHIN family signature domain, the 'hematopoietic interferon-inducible nuclear protein domain' (HIN) (Cridland 2012). AIM2 directly binds to its activating ligand, cytoplasmic double-stranded DNA (Hornung 2009, Fernandes-Alnemri 2009, Bürckstümmer 2009, Roberts 2009). The HIN domain maintains AIM2 in an autoinhibitory state, which is relieved by the binding of DNA to HIN (Jin 2012). The ASC adapter comprises a PYD and a CARD domain, connected by a short flexible linker region of 23 amino acids (de Alba 2009). Finally, the caspase-1 protease is expressed as an inactive zymogen, pro-caspase-1, that comprises an N-terminal CARD and the p10/p20 catalytic subunits (Thornberry 1992).

*2.2.2.2 Inflammasome complex assembly*

Upon detection of an appropriate activator in the cytoplasm, the autoinhibitory state of the NLR or PYHIN receptor is relieved and a conformational change enables self-

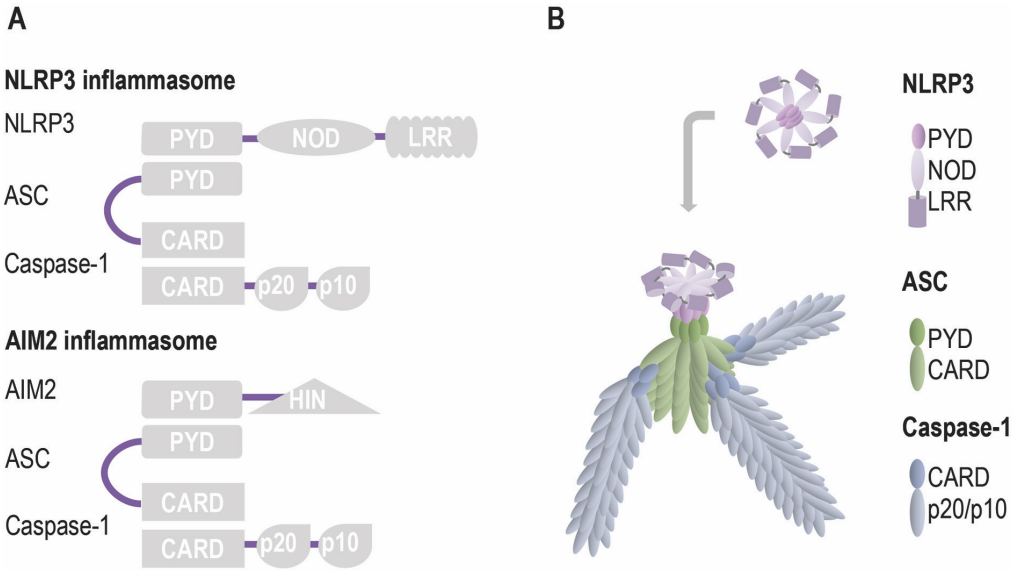


**Figure 4. Inflammasome complex assembly and structure.** (A) Schematic model of domain-domain interactions during inflammasome assembly. (B) A structural model of the NLRP3 inflammasome complex based on studies of purified inflammasome components. The AIM2 inflammasome complex is highly similar, except that rather than as a ring structure, the AIM2 oligomerization proceeds linearly along the DNA ligand. See the details in text.

oligomerization of the receptor and recruitment of downstream binding partners (**Fig. 4**). NLR oligomerization is mediated via the NOD domain and requires nucleotide binding (Faustin 2007, Duncan 2007, Lu 2005). The lack of oligomerization domain in AIM2 seems to be bypassed by the simultaneous binding of several AIM2 molecules on a multivalent DNA ligand to achieve oligomerization (Jin 2012). Activated NLR or PYHIN receptor then recruits caspase-1 either via direct CARD-CARD interaction, as in the case of NLRC4, or via recruitment of the ASC adapter to their PYD domains (**Fig. 4A**) (Martinon 2002, Hornung 2009, Fernandes-Alnemri 2009, Poyet 2001, Agostini 2004). After recruitment to the NLR or AIM2 oligomer, ASC forms complex multimeric structures via self-association and greatly amplifies the amount of available CARD domain interaction sites (de Alba 2009, Lu 2014). ASC seems to enhance even the function of the CARD-containing NLRC4 (Mariathasan 2004, Miao 2006, Zhao 2011). Recruitment of pro-caspase-1 to the nascent inflammasome complex then occurs via interaction of ASC CARD domains with caspase-1 CARD domains. Finally, proximity-induced autocleavage of the 45 kDa pro-caspase-1 into the 10 kDa and 20 kDa subunits forming the catalytically active enzyme takes place in the inflammasome complex (Poyet 2001, Wilson 1994).

#### *2.2.2.3 Structure of the inflammasome complexes*

The exact stoichiometries and structures of the various inflammasome complexes still remain a mystery. In agreement with the earliest studies (Masumoto 2001), the formation of a single perinuclear ~1 μm aggregate of ASC was later demonstrated upon activation of both NLR and AIM2 inflammasomes in countless reports. This "ASC speck" forms rapidly after inflammasome activation and colocalizes with other inflammasome components, suggesting that these structures represent the cytokine-processing unit (Fernandes-Alnemri 2009, Fink 2008, Baroja-Mazo 2014, Man 2014). The purified NLR receptors form ring-shaped oligomers reminiscent of the apoptosome (Faustin 2007, Halff 2012) (**Fig. 4B**). Moreover, structural analysis of purified inflammasome components suggested that a single, ordered stalk-like ASC polymer extends from the NLRP3 or AIM2 receptor oligomer, offering a nucleation point for multiple caspase-1 filament structures (Lu 2014) (**Fig. 4B**). In agreement, the NLRP3/ASC inflammasome complex formed in macrophage cytoplasm comprised a ~1-2 μm aggregate of fibrillar structures emanating from a single core (Baroja-Mazo 2014, Franklin 2014). However, it was recently proposed that smaller, oligomeric NLRP3/ASC assemblies spreading throughout the cytoplasm, rather than the large speck structure, would be the major sites of cytokine processing (Compan 2015). A further level of complexity is introduced by the simultaneous activation of several inflammasome-forming receptors during bacterial infection of macrophages, resulting in the colocalization of the receptors in a single speck structure (Man 2014).

### 2.2.3 Downstream effects of inflammasome activation

Caspase-1 triggers both major downstream effects of inflammasome activation: 1) the proteolytic maturation of the proforms of IL-1β and IL-18 into their biologically active, secreted form and 2) pyroptotic cell death associated with further pronflammatory effects.

#### *2.2.3.1 Proinflammatory cytokine secretion*

Caspase-1 cleaves pro-IL-1β and pro-IL-18 after an aspartic acid residue (Asp116 in IL-1β, Asp37 in IL-18) in a conserved recognition sequence, which removes an N-terminal propiece and yields the biologically active cytokine (Dinarello 2009, Thornberry 1992, Ghayur 1997). These cytokines lack the signal sequence that normally directs secreted proteins into the classical endoplasmic reticulum/Golgi-mediated secretory pathway. Thus, IL-1β and IL-18 are secreted via an unconventional mechanism that remains poorly defined, yet it is closely coupled to caspase-1 activity (Dubyak 2012). Notably, not only the mature cytokines, but also the procytokines and all inflammasome components are secreted from the cell in the process (Martinon 2002, Baroja-Mazo 2014, Franklin 2014). Over the years,

many mechanisms have been proposed, including specialized secretory lysosomes, exosomes, or autophagosomes, microvesicle shedding, or plasma membrane pore formation (Andrei 2004, Qu 2007, Dupont 2011, MacKenzie 2001, Pizzirani 2007, Fink 2006). Importantly, caspase-1 seems to be a more general mediator of unconventional protein secretion. A proteomic approach revealed 77 candidate molecules secreted via a caspase-1-dependent mechanism (Keller 2008). Among these, IL-1α and fibroblast growth factor 2 were verified as molecules secreted by a caspase-1-dependent mechanism, yet these targets were not cleaved by caspase-1.

The secretion of IL-1β and IL-18 is central for the role of the inflammasome pathway in various inflammatory diseases, whereas pyroptotic cell death is important for inflammasome-mediated host defence, as discussed below. Mature IL-1β binds to a heterodimer of IL-1 receptor I and IL-1 receptor accessory protein that is also activated by the close homolog IL-1α (Dinarello 2009). The signaling events triggered by this IL-1 receptor dimer closely resemble TLR signaling due to the shared TIR domain (see section 2.1.4). Thus, MyD88 recruitment to the receptor results in proinflammatory cytokine expression via NF-κB and p38/JNK MAPK cascades that activate AP-1. IL-1β triggers a broad range of inflammatory effects, such as nitric oxide-mediated vasodilatation and prostaglandin E2 production resulting in fever (Dinarello 2009). Moreover, IL-1β induces the expression of various adhesion molecules and chemokines, promoting extravasation of immune cells to the inflamed tissue site. IL-1β is also essential in differentiation of Th17 cells (Santarlasci 2013). Mature IL-18 signals by binding to a dimer formed by IL-18 receptor α and β chains, and, similar to IL-1 receptor 1, the receptor utilizes TIR domains to recruit MyD88 for downstream signaling via NF-κB and AP-1 (Dinarello 2009). IL-18 is best known as an inducer of interferon-γ production by T cells, thus contributing to Th1 type immune responses (Okamura 1995). However, IL-18 induces interferon-γ only in the presence of IL-12; when IL-12 is absent, IL-18 contributes to the development of Th2 type immune responses e.g. via induction of IL-4 (Dinarello 2009). The marked roles of IL-1β and IL-18 in inflammation are further underscored by the expression of specific negative regulators of IL-1 and IL-18 signaling: the IL-1 receptor antagonist (Matsushime 1991), the soluble IL-1 receptor II acting as a decoy (Colotta 1993), and IL-18 binding protein (Novick 1999).

#### *2.2.3.2 Pyroptotic cell death*

Pyroptotic cell death is an essential effector mechanism for inflammasome-mediated host defence, typically occurring after robust caspase-1 activation by microbial stimuli (Bergsbaken 2009). IL-1β and IL-18 are secreted also during inflammasome activation that involves pyroptosis, yet pyroptosis is the major effector mechanism of inflammasome-mediated clearance of intracellularly replicating bacteria *in vivo* (Miao 2010a). Nevertheless, also inflammasome-mediated cytokine secretion

contributes host defence, for example by controlling early virus replication *in vivo* (Rathinam 2010).

Pyroptosis is a form of programmed cell death that is distinguished from other forms of cell death primarily by caspase-1-dependence. Caspase-1-dependent plasma membrane pore formation during pyroptosis results in rapid cell swelling, osmotic lysis, and spillage of proinflammatory cytoplasmic contents (Fink 2006). Pyroptosis is independent of apoptotic caspases and both mitochondrial and nuclear integrity are preserved during pyroptotic cell death, further distinguishing it from apoptosis (Bergsbaken 2009). Furthermore, the caspase-1-mediated maturation and secretion of IL-1β and IL-18 is not required for execution of pyroptosis (Monack 2001, Le Feuvre 2002), and thus other caspase-1 targets initiate the pyroptotic cell death program. Caspase-1 digestome of THP-1 macrophage lysates identified 41 caspase-1 cleavage targets, of which only 30 % were shared with the crucial apoptotic effector caspase-3 (Shao 2007). Among these, major target groups were glycolytic enzymes, cytoskeletal components, RNA and protein synthesis machinery, chaperone proteins, as well as proteins involved in cellular trafficking. However, the crucial caspase-1 targets and mechanisms mediating pyroptosis remain to be elucidated.

### 2.2.4 The host defence inflammasomes: NLRP1, NLRC4, and AIM2

The NLRP1, NLRC4, and AIM2 inflammasomes are cytoplasmic pathogen sensors activated by conserved microbial structures, toxins, and bacterial/viral nucleic acids. Accordingly, these inflammasomes have important roles in host defence against bacteria, viruses, and parasites. The activators and activation mechanisms of the NLRP1, NLRC4, and AIM2 inflammasomes are introduced in this section and summarized in **Table 1**.

#### *2.2.4.1 NLRP1*

The NLRP1 inflammasome complex was first discovered from heat-activated cellular extracts of monocyte-macrophages (Martinon 2002). Muramyl dipeptide, a component of bacterial cell wall peptidoglycan, was subsequently shown to activate the NLRP1 receptor in human macrophages (Faustin 2007, Bruey 2007). However, direct binding of this ligand to NLRP1 could not be demonstrated (Reubold 2014). Instead, muramyl dipeptide may be bound by another cytoplasmic NLR, NOD2, that subsequently co-oligomerizes with NLRP1 to form an active inflammasome complex (Hsu 2008). The NLRP1 receptor is also required for IL-1β secretion in response to the intracellular parasite *Toxoplasma gondii* in a human monocyte cell line (Witola 2011). Similarly, *T. gondii* infection elicits a caspase-1 and ASC-dependent IL-1β response in mouse macrophages (Ewald 2014). The magnitude of IL-1β response to *T. gondii* was correlated with different NLRP1b alleles in different mouse strains, and caspase-1 was crucial for controlling the parasite burden

**Table 1.** Summary of the activators of the NLRP1, NLRC4, and AIM2 inflammasomes.

| Activators | Physiological relevance | References |
|---|---|---|
| **NLRP1 inflammasome** | | |
| Muramyl dipeptide of bacterial cell wall (human NLRP1) | Host defence, bacteria | (Faustin 2007, Bruey 2007, Hsu 2008) |
| *Bacillus anthracis* and anthrax lethal toxin (mouse NLRP1b) | Host defence, bacteria | (Fink 2008, Hsu 2008, Boyden 2006) |
| *Toxoplasma gondii* (human NLRP1 and mouse NLRP1b) | Host defence, parasites | (Witola 2011, Ewald 2014) |
| **NLRC4 inflammasome** | | |
| Flagellin proteins of flagellae (via mouse NAIP5 and NAIP6) | Host defence, bacteria | (Kofoed 2011, Miao 2006, Zhao 2011, Franchi 2006) |
| Rod proteins of type III secretion systems (via mouse NAIP2) | Host defence, bacteria | (Kofoed 2011, Zhao 2011, Miao 2010b) |
| Needle proteins of type III secretion systems (via mouse NAIP1 and human NAIP) | Host defence, bacteria | (Zhao 2011, Yang 2013) |
| Intracellularly replicating bacteria | Host defence, bacteria | (Mariathasan 2004, Miao 2010a) |
| **AIM2 inflammasome** | | |
| Synthetic dsDNA (poly-(dA:dT) | None | (Hornung 2009, Fernandes-Alnemri 2009, Bürckstümmer 2009, Roberts 2009) |
| Genomic DNA | Autoimmune/ autoinflammatory diseases? | (Fernandes-Alnemri 2009, Roberts 2009) |
| Bacterial DNA | Host defence, bacteria | (Fernandes-Alnemri 2009) |
| DNA viruses | Host defence, viruses | (Hornung 2009, Rathinam 2010) |
| Intracellularly replicating bacteria | Host defence, bacteria | (Rathinam 2010, Fernandes-Alnemri 2010, Saiga 2012) |

in infected mice (Ewald 2014). Finally, the mouse ortholog NLRP1b, but not human NLRP1, is activated in macrophages upon direct cleavage by the anthrax lethal toxin metalloprotease (Boyden 2006, Chavarria-Smith 2013). The access of this key virulence factor of *Bacillus anthracis* to macrophage cytoplasm was required for subsequent caspase-1 activation, target cytokine cleavage, and pyroptosis (Fink 2008). Interestingly, the IL-1β response of mouse macrophages to *B. anthracis* and anthrax lethal toxin is also dependent on NOD2 (Hsu 2008), implying a conserved NOD2-dependent activation mechanism of NLRP1 in mice and men. Beyond host defence,

NLRP1 polymorphisms have been associated with various autoimmune disorders, e.g. celiac disease and vitiligo, and identification of the underlying mechanisms will help to further elucidate NLRP1 function (Zhong 2013).

Recent studies have revealed a peculiar feature regarding NLRP1b-mediated activation of caspase-1 in mouse macrophages. NLRP1b harbours a CARD domain and can thus recruit caspase-1 directly for activation of caspase-1, bypassing the requirement for ASC adapter. In accord, activation of NLRP1b with anthrax lethal toxin in ASC-deficient macrophages elicits considerable amounts of pyroptosis and IL-1β release, showing only a mild dependence on ASC (Van Opdenbosch 2014, Guey 2014). However, the autoproteolytic cleavage of caspase-1 into the active p10/p20 subunits is abolished in lethal toxin-treated ASC-deficient macrophages. Remarkably, it was demonstrated that NLRP1b, but not NLRP3, NLRC4, or AIM2, was able to activate caspase-1 in its full-length form, without inducing caspase-1 autoproteolysis (Van Opdenbosch 2014, Guey 2014). This fundamentally new aspect in regulation of caspase-1 activity calls for further studies to deepen our understanding of caspase-1 activation mechanisms.

#### *2.2.4.2 NLRC4*

The interaction of NLRC4 with caspase-1 and the resulting caspase-1 activation was first discovered in overexpression studies of this Apaf1-related protein (Poyet 2001). The bacterial motor protein flagellin from *Salmonella typhimurium* was later identified as an activator of the NLRC4 receptor in cultured mouse macrophages (Mariathasan 2004, Miao 2006, Franchi 2006). Flagellin triggered IL-1β secretion by macrophages, as well as pyroptotic death of the cells. Later on, conserved rod and needle proteins of various bacterial type III secretion systems (Zhao 2011, Miao 2010b, Yang 2013), as well as flagellins from various pathogenic bacteria (Zhao 2011) were shown to trigger NLRC4 activation. Intriguingly, NLRC4 seems to function as an adapter molecule analogous to ASC function. The NLR family apoptosis inhibitory proteins (NAIPs) were identified as the sensor molecules that directly bind NLRC4 activators providing ligand specificity, and subsequently co-oligomerize with NLRC4 to activate caspase-1 (see details on NAIP ligand specificity in **Table 1**) (Kofoed 2011, Zhao 2011, Yang 2013). The NAIP/NLRC4 inflammasomes have a crucial role in limiting the replication of intracellular bacteria in macrophages *in vitro* and *in vivo*, which is dependent on inflammasome-mediated pyroptotic cell death, rather than on IL-1β and IL-18 secretion (Miao 2010a). Moreover, the NAIP/NLRC4 inflammasome contributes to discrimination between pathogenic and commensal bacteria by the intestinal phagocytes (Franchi 2012).

In 2014, the first reports mechanistically linking genetic variants of NLRC4 to human disease were recently published (Canna 2014, Romberg 2014). Two *de novo* mutations causing spontaneous hyperactivation of NLRC4 were discovered from

patients suffering distinct yet overlapping autoinflammatory diseases. The shared symptoms included periodic fever, high plasma levels of IL-1 family cytokines, and gastrointestinal pathologies (Canna 2014, Romberg 2014). The latter is in good agreement with the role of NLRC4 in regulation of intestinal homeostasis and recognition of bacteria that cause enteric inflammation (Mariathasan 2004, Franchi 2012).

#### *2.2.4.3 AIM2*

Four independent reports originally identified AIM2 as a cytoplasmic sensor of double-stranded DNA (dsDNA) that triggers inflammasome complex assembly (Hornung 2009, Fernandes-Alnemri 2009, Bürckstümmer 2009, Roberts 2009). The HIN domain of AIM2 directly binds the sugar-phosphate backbone of DNA in a sequence-independent, but length-dependent manner and the PYD domain recruits ASC and caspase-1 to form a functional inflammasome complex (Hornung 2009, Fernandes-Alnemri 2009, Bürckstümmer 2009, Jin 2012). The minimum length of DNA able to trigger AIM2-dependent IL-1β response is roughly 80 bp, implying that AIM2 utilizes the target DNA as an oligomerization platform (Jin 2012). Activation of the AIM2 inflammasome has been demonstrated during infection with various intracellularly replicating bacteria and DNA viruses. Thus, early control of mouse cytomegalovirus replication *in vivo* is dependent on AIM2-mediated IL-18 secretion (Rathinam 2010). Furthermore, AIM2-KO mice are highly susceptible to *M. tuberculosis* infection with greatly increased bacterial loads in the liver and the lungs, and markedly reduced IL-1β and IL-18 levels in bronchoalveolar lavage and serum (Saiga 2012). Finally, AIM2 had a major role in controlling bacterial loads during *F. tularensis* infection in mice (Fernandes-Alnemri 2010).

The AIM2 inflammasome can be activated by both genomic and pathogen-derived DNA (Fernandes-Alnemri 2009), which may imply a role in autoimmune or autoinflammatory diseases. Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by the presence of autoantibodies against self dsDNA, likely arising due to immune activation via defective clearance of apoptotic cells. Accumulating evidence suggests a role for AIM2 inflammasome activation in SLE. The mouse PYHIN protein p202 is a candidate SLE susceptibility factor and a negative regulator of AIM2-dependent caspase-1 activation (Roberts 2009). Moreover, AIM2 is activated by DNA derived from apoptotic cells and AIM2 deficiency dampens inflammation in a mouse model of SLE (Zhang 2013).

### 2.2.5 The NLRP3 inflammasome: Mediator of sterile inflammation

The inflammasome complexes described above serve a primary function in host defence against bacterial and viral pathogens. They respond to microbial components that enter the cytoplasm during infections via a direct interaction with the

inflammasome-activating stimulus, be it a simple receptor-ligand interaction or receptor modification by an active virulence mechanism. Microbial components and products, such as bacterial and viral RNA and the bacterial ionophore nigericin, featured also among the first activators identified for the NLRP3 inflammasome (Mariathasan 2006, Kanneganti 2006b, Kanneganti 2006a). Yet, increasingly, the NLRP3 inflammasome is studied in the context of sterile inflammation, which will also be the focus of this chapter.

#### *2.2.5.1 The concept of priming*

The caspase-1 target pro-IL-18 is expressed constitutively, but pro-IL-1β is absent in resting monocytes and macrophages (Puren 1999). Typically, the activators of the NLRP3 receptor are not capable of inducing the synthesis of pro-IL-1β. Importantly, also the NLRP3 receptor itself is expressed at a limiting level in resting macrophages, and induction of NLRP3 synthesis is an important checkpoint for NLRP3 inflammasome assembly (Bauernfeind 2009). Transcription of both NLRP3 receptor and pro-IL-1β can be induced by NF-κB activation, either via TLRs (TLRs 2, 3, 4, 7, 9) or by certain cytokines (TNF-α) (Bauernfeind 2009, O'Connor 2003, Franchi 2009). Yet, these stimuli are ineffective in triggering NLRP3 inflammasome assembly and the ensuing proteolytic maturation of the target cytokines. Thus, two separate signals are required for the NLRP3 inflammasome assembly and the release of biologically active cytokines: 1) a signal that induces the NF-κB-mediated transcription of NLRP3 receptor and pro-IL-1β, followed by 2) a signal that triggers NLRP3 receptor activation and inflammasome complex assembly, resulting in cytokine processing and secretion. The first, transcriptional signal is referred to as "*priming*", and the stimulation of TLR4 receptors with LPS is considered the canonical pathway for priming.

Recent studies have begun to uncover a role also for a post-translational modifications in the priming step of NLRP3 inflammasome activation (Elliott 2015). Thus, deubiquitination of the NLRP3 receptor (Juliana 2012), as well as ubiquitination and phosphorylation of the ASC adapter (Rodgers 2014, Hara 2013) were all necessary but not sufficient for activation of the NLRP3 inflammasome. However, both the priming signal and the inflammasome activation signal seemed to contribute to these post-translational modifications, and the exact pathways triggering them remain to be elucidated.

#### *2.2.5.2 Activators of the NLRP3 receptor*

Long before the discovery of the inflammasome pathway, high extracellular ATP concentration was known to trigger the release of IL-1β from LPS-primed macrophages via a mechanism that involved potassium efflux from the cells (Perregaux 1994). The ATP receptor $P2X_7$ was subsequently identified as the mediator of the

ATP-induced potassium efflux and IL-1β release (Ferrari 1997, Solle 2001). The $P2X_7$ receptor is an ATP-gated cation channel ($K^+$ out; $Ca^{2+}$ and $Na^+$ in) that also mediates the formation of a larger plasma membrane pore upon prolonged stimulation via an unknown mechanism (Dubyak 2012, Sung 1985, Qu 2011). Studies in ASC- and NLRP3-deficient mouse macrophages revealed ATP as one of the first endogenous triggers of NLRP3 inflammasome activation (Mariathasan 2006). Notably, in an *in vivo* model of sterile hepatic injury, ATP release from necrotic cells triggers NLRP3-mediated IL-1β secretion that initiates neutrophil recruitment to the site of injury (McDonald 2010).

Also monosodium urate crystals and calcium pyrophosphate dehydrate crystals formed in the joints of gout patients were recognized early on as sterile activators of the NLRP3 inflammasome (Martinon 2006). This fuelled the search for other crystalline and particulate inflammasome activators. Subsequently, the crystalline adjuvant alum, and silica crystals and asbestos particles causing fibrotic lung diseases were shown to trigger NLRP3 inflammasome in macrophages (Hornung 2008, Dostert 2008, Cassel 2008). Similarly, pathological protein aggregates, such as amyloid-β peptide deposited in the brain during Alzheimer's disease and the islet amyloid polypeptide forming pancreatic deposits in type II diabetes, were identified as NLRP3 inflammasome activators in macrophages and microglia (Halle 2008, Masters 2010). NLRP3 inflammasome assembly can be triggered also by extracellular matrix components released upon tissue injury. These include the glycosaminoglycan hyaluronan and the small leucine-rich repeat proteoglycan biglycan that are associated with inflammation in response to sterile renal or skin injury (Babelova 2009, Yamasaki 2009). **Table 2** lists examples of activators of the NLRP3 inflammasome, highlighting their heterogeneity in terms of structure and origin.

#### *2.2.5.3 Intracellular stress signals trigger NLRP3 activation*

As the list of the known activators of the NLRP3 receptor keeps on expanding, it is clear that the sheer diversity of these activators makes a direct interaction with the NLRP3 receptor an unlikely activation mechanism. According to the current paradigm, intracellular stress signals triggered by the activators indirectly mediate NLRP3 activation (Elliott 2015). Numerous studies have shown that the NLRP3-mediated IL-1β responses in macrophages variably depend on particle phagocytosis, phagosomal acidification, lysosomal destabilization, enhanced production of reactive oxygen species (ROS), and potassium efflux from the cells (Perregaux 1994, Hornung 2008, Dostert 2008, Cassel 2008, Halle 2008, Masters 2010, Cruz 2007, Lopez-Castejon 2010). Of these, potassium efflux seems to be the only signal universally required for NLRP3 inflammasome activation (Petrilli 2007, Munoz-Planillo 2013). A drop in the intracellular $K^+$ concentration has been directly demonstrated during stimulation with crystalline/particulate NLRP3 activators and with ATP (Perregaux

**Table 2.** Examples of activators of the NLRP3 inflammasome.

| Activators | Physiological relevance | References |
|---|---|---|
| ***Endogenous*** | | |
| Extracellular ATP | Sterile inflammation during tissue injury | (Mariathasan 2006, McDonald 2010) |
| Monosodium urate crystals | Sterile joint inflammation, gout | (Martinon 2006) |
| Calcium pyrophosphate dehydrate crystals | Sterile joint inflammation, pseudogout | (Martinon 2006) |
| Islet amyloid polypeptide oligomers | Sterile pancreatic inflammation, type II diabetes | (Masters 2010) |
| Fibrillar amyloid-β peptide | Sterile CNS inflammation, Alzheimer's disease | (Halle 2008) |
| Prion protein fibrils/aggregates | Sterile CNS inflammation, prion diseases | (Hafner-Bratkovič 2012) |
| Biglycan (proteoglycan) | Sterile inflammation during tissue injury | (Babelova 2009) |
| Hyaluronan (glycosaminoglycan) | Sterile inflammation during tissue injury | (Yamasaki 2009) |
| Endoplasmic reticulum stress | Metabolic stress –associated diseases | (Menu 2012) |
| Complement activation | Inflammatory diseases, host defence | (Laudisi 2013) |
| Cell swelling induced by hypo-osmolarity | Clinical use of hypertonic solutions to ameliorate inflammation | (Perregaux 1996, Compan 2012) |
| ***Exogenous*** | | |
| Pore-forming toxins (nigericin, maitotoxin) | Host defence, bacteria and protozoans | (Mariathasan 2006) |
| Ribotoxic ricin toxin | Lethal poison from the plant *Ricinus communis* | (Lindauer 2010) |
| Bacterial and viral RNA | Host defence, bacteria and viruses | (Kanneganti 2006b, Kanneganti 2006a) |
| Aluminum salt (alum) crystals | Adjuvant function in vaccines | (Hornung 2008) |
| Poly(lactide-co-glycolide) and polystyrene microparticles | Adjuvant function in vaccines | (Sharp 2009) |
| Silica crystals | Silicosis, lung inflammation and fibrosis | (Hornung 2008, Dostert 2008, Cassel 2008) |
| Asbestos fibres/particles | Asbestosis, lung inflammation and fibrosis | (Dostert 2008) |
| Hemozoin crystals (derived from heme) | Host defence, blood-feeding parasites incl. malaria | (Dostert 2009, Shio 2009) |
| Nanoparticles used in cosmetics | Skin inflammation, lung inflammation | (Yazdi 2010) |

1994, Munoz-Planillo 2013). Nevertheless, the $K^+$ efflux is a signal shared with other inflammasome complexes and likely plays a permissive, rather than a decisive role in NLRP3 inflammasome activation (Fink 2008, Fernandes-Alnemri 2010). Similar to $K^+$ efflux, cellular $Cl^-$, $Na^+$, and $Ca^{2+}$ fluxes, as well as changes in cell volume coupled to various regulatory mechanisms triggering fluxes of ions and water, have been linked with NLRP3 activation (Perregaux 1996, Compan 2012, Perregaux 1998, Schorn 2010, Murakami 2012, Rabolli 2014).

Both crystalline/particulate NLRP3 activators and ATP cause lysosomal destabilization and the release of lysosomal contents into the cytoplasm (Hornung 2008, Halle 2008, Masters 2010, Lopez-Castejon 2010). A single lysosomal protease, cathepsin B, markedly contributed to the IL-1β response induced by crystals and particles. Furthermore, cathepsin B inhibition blocked $K^+$ efflux during NLRP3 activation, suggesting that cathepsin B release from lysosomes may act upstream $K^+$ efflux (Munoz-Planillo 2013). Notably, the inhibitor CA-074-Me widely used to block cellular cathepsin B activity also inhibits the closely related cathepsin L (Montaser 2002). Indeed, genetic ablation of either cathepsin B or L attenuated NLRP3-mediated IL-1β responses, yet not for all activators (Halle 2008, Munoz-Planillo 2013, Duewell 2010). These data suggest redundant roles for lysosomal cathepsins in NLRP3 inflammasome activation.

Cellular ROS production is enhanced by crystalline/particulate NLRP3 activators and by ATP, and general inhibitors of ROS often block NLRP3-mediated IL-1β secretion (Dostert 2008, Cassel 2008, Cruz 2007). However, the interpretation is complicated by the inhibitory effects of these compounds on the transcription of NLRP3 receptor and pro-IL-1β (Bauernfeind 2009, van de Veerdonk 2010, Bauernfeind 2011). Intriguingly, thioredoxin-binding protein, released from the ROS-detoxifying protein thioredoxin upon ROS production, was suggested to act as a direct NLRP3-activating ligand (Zhou 2010). However, the results could not be repeated in another study (Masters 2010). The major cellular sources of ROS in macrophages are the mitochondrial respiratory chain and the phagosomal NADPH oxidase involved in microbial killing (Terada 2006). Knocking out NADPH oxidase components has yielded controversial results in mouse macrophages regarding a role in NLRP3 inflammasome activation (Hornung 2008, Dostert 2008). However, the data from NADPH oxidase -defective patient monocytes confirmed that the NADPH oxidase is dispensable for NLRP3 activation (van de Veerdonk 2010). Conversely, the role of mitochondrial ROS (mitROS) is now intensively studied.

Most, but not all, NLRP3 activators trigger mitROS production, and scavenging of mitROS diminishes NLRP3 activation (Elliott 2015). Induction of mitROS by respiratory chain inhibitors or by blocking the autophagic clearance of damaged mitochondria induces spontaneous NLRP3 activation in macrophages and further

sensitizes them to NLRP3 activation by ATP and crystals (Zhou 2011, Nakahira 2011). Intriguingly, a partial loss of mitochondrial membrane potential was associated with elevated mitROS and NLRP3 activation, whereas a full collapse did not trigger mitROS or IL-1β release (Zhou 2011). Thus the level of mitochondrial stress and damage may define the decision between inflammasome activation and apoptosis. Finally, NLRP3, ASC, mitochondria, and endoplasmic reticulum markers colocalized in the perinuclear region during inflammasome activation (Zhou 2011, Misawa 2013).

Taken together, potassium efflux, enhanced mitROS formation, and lysosomal destabilization may all contribute to activation of the NLRP3 inflammasome (**Fig. 5**). The relative importance of these stress signals, as well as the detailed mechanisms ultimately triggering NLRP3 receptor activation, remain elusive. The NLRP3-activating stress signals may act in parallel or in a sequence, and, most likely, two or more signals are required for triggering NLRP3 activation. Intriguingly, all three stress signals have been implicated also in activation of the apoptosome complex, again highlighting the close connection between these pathways (Gross 2011).

#### *2.2.5.4 Role in autoinflammatory syndromes and chronic inflammatory diseases*

Cryopyrin-associated periodic syndromes (CAPS) are a group of three rare, inherited, autoinflammatory syndromes caused by mutations in the NLRP3 receptor (Hoffman 2001, Aksentijevich 2002, Feldmann 2002). These include familial cold autoinflammatory syndrome, Muckle–Wells syndrome, and neonatal onset multisystem inflammatory disease (Aksentijevich 2011). The term *autoinflammatory* distinguishes these disorders of the innate immune system from antibody and T cell-mediated *autoimmune* diseases. The shared symptoms of CAPS include recurrent episodes of fever, arthritis, and rashes. Mutations underlying CAPS cause NLRP3 hyperactivation, resulting in uncontrolled, spontaneous secretion of inflammasome target cytokines by monocytes (Aksentijevich 2002, Dowds 2004). These findings rapidly led to successful treatment of CAPS patients with the recombinant human IL-1 receptor antagonist, anakinra, showing dramatic improvement of the symptoms (Hawkins 2003, Goldbach-Mansky 2006).

Today, the NLRP3 inflammasome has been mechanistically linked to a wide range of chronic inflammatory diseases, including gout, asbestosis, silicosis, obesity-induced inflammation and type II diabetes, as well as Alzheimer's disease (Robbins 2014, Zhong 2013). The NLRP3 inflammasome contributes to joint inflammation in a mouse model of gout (Amaral 2012), and the inflammatory and fibrotic responses to asbestos or silica in the lungs are dependent on the NLRP3 inflammasome (Dostert 2008, Cassel 2008). Furthermore, knock-out (KO) mouse models have established a role for the NLRP3 inflammasome in the development of obesity-induced adipose tissue inflammation and insulin resistance (Vandanmagsar 2011, Stienstra 2011).

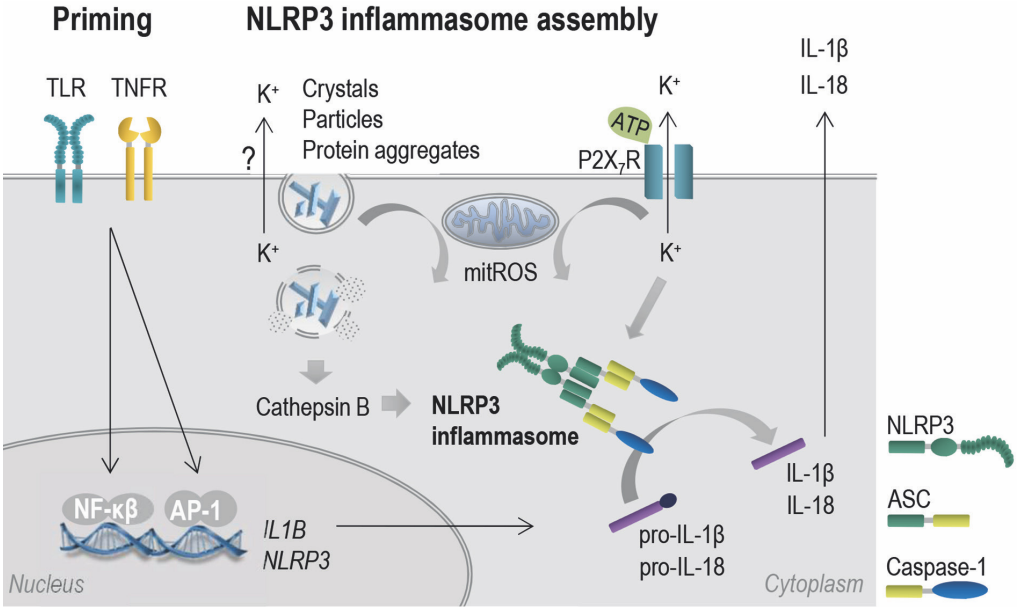


**Figure 5. Proposed activation mechanisms of the NLRP3 inflammasome.** See the text for details. AP-1, activator protein 1; ATP, adenosine triphosphate; mitROS, mitochondria-derived reactive oxygen species; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; $P2X_7R$, $P2X_7$ receptor; TLR, Toll-like receptor; TNFR, TNF-α receptor.

Strikingly, caspase-1 activation was demonstrated in the brain of Alzheimer's disease patients, and NLRP3- or caspase-1-deficiency in a mouse model of Alzheimer's disease was highly protective against loss of spatial memory (Heneka 2013). Finally, polymorphisms in the gene encoding the NLRP3 receptor are associated with several autoimmune diseases, including psoriasis, type I diabetes, and celiac disease (Zhong 2013). Taken together, the NLRP3 inflammasome is emerging as a major regulator of sterile inflammation that contributes to a broad range of diseases associated with chronic inflammation, metabolic disturbances, and ageing.

## 2.3 ATHEROSCLEROSIS – A CHRONIC INFLAMMATORY DISEASE

Atherosclerosis is the underlying cause of myocardial infarction and stroke, the leading causes of death worldwide. It is a slowly progressing disease of the large arteries strongly associated with lifestyle and aging. Focal development of lipid-rich, inflamed deposits, called atherosclerotic 'plaques' or 'lesions', along the arterial tree is the hallmark of disease pathogenesis. The prominent risk factors for atherosclerosis and cardiovascular disease are age, male sex, hypertension, smoking, obesity associated with impaired glucose tolerance or overt diabetes, high LDL cholesterol, low high density lipoprotein (HDL) cholesterol, and high triglycerides (Fruchart 2004). In

addition, high-sensitivity C-reactive protein, a liver-derived general marker of low grade inflammation, is used clinically in cardiovascular risk assessment (Koenig 2013). Thus, atherosclerosis is a highly multifactorial disease affected by a wide range of metabolic variables, defined as the total 'cardiometabolic risk' of a person (Despres 2008).

Atherosclerotic plaque development, or 'atherogenesis', in the arterial wall begins from *tunica intima*, a layer comprising the luminal endothelium and the underlying connective tissue (Stary 1992). The intima is further divided into a subendothelial *proteoglycan-rich layer* that contains scattered tissue macrophages and smooth muscle cells (SMCs) of the synthesizing phenotype, and a deeper *musculoelastic layer* rich in collagen, elastic fibers, and SMCs of the contractile phenotype (Stary 1992). An internal elastic lamina separates the intima from *tunica media*, a highly ordered structure of contractile SMCs layered within elastic fibers and collagen. Finally, an outer layer of loose connective tissue, called *tunica externa* or *adventitia*, surrounds the media. The adventitia hosts lymphatics and small blood vessels, *vasa vasorum*, essential for flux of nutrients and metabolic waste to and from the arterial wall (Nakano 2005). Notably, the healthy intima is avascular and thus dependent on the adventitial lymphatics and blood vessels.

## 2.3.1 Lipoproteins and the initiation of atherosclerotic lesion development

Lipoproteins are the major carriers of circulating neutral lipids, notably cholesteryl esters and triglycerides. The main function of lipoproteins is to provide a convenient package for transportation of the hydrophobic lipids to peripheral tissues for use as a source of energy or as structural components. A diet rich in cholesterol and saturated fats results in elevated levels of circulating lipoprotein-carried cholesterol, which in turn has a definitive role in development of atherosclerosis. Lipoproteins come in many different shapes, sizes, and lipid/protein compositions, and they can be divided into subclasses based on their buoyant densities. Of these, the subclasses of LDL and HDL play crucial, yet opposing roles in atherogenesis.

### *2.3.1.1 Retention of native LDL in the arterial intima*

LDL is a globular 20-25 nm particle packing cholesteryl esters and triglycerides in its core, wrapped in a phospholipid/cholesterol monolayer that embeds a large multifunctional protein, the apolipoprotein (apo)B-100 (Hevonoja 2000). Liver secretes very low density lipoprotein (VLDL), and the removal of triglycerides from circulating VLDL into peripheral tissues generates first intermediate density lipoprotein (IDL) (Brown 1986). Further removal of triglycerides from IDL generates the cholesterol-enriched LDL that is cleared back to the liver via LDL receptors (LDLR) (Brown 1986).

Elevated plasma levels of LDL promote selective accumulation of LDL particles into the arterial walls at branch points and curvatures of the arterial tree. The altered hemodynamic forces at these sites induce changes in the phenotype of the vascular endothelial cells and loosening of the endothelial tight junctions, as well as adaptive thickening of the intima (Stary 1992, Tabas 2015). These changes increase the entry of LDL into the subendothelial intima and the transit distance of LDL to adventitial lymphatics (or back to the vessel lumen), rendering the sites highly susceptible to atherosclerotic lesion development (Kovanen 1990). Once in the intima, LDL accumulates in the extracellular matrix via ionic interactions between clusters of positively charged residues on apoB-100 and negatively charged matrix proteoglycans (Camejo 1990). Abundant accumulation of LDL in the intimal matrix is detectable by electron microscopy already 2 hours after bolus injection of human LDL into rabbits (Nievelstein 1991). These observations led to the formulation of the 'response-to-retention theory' of atherosclerotic lesion development (Williams 1995). The theory postulates that the earliest event in lesion development is the subendothelial retention of native LDL in the extracellular matrix proteoglycans of the intima. The positions of the key amino acid residues in apoB-100 mediating the LDL-proteoglycan interactions were later identified (Boren 1998). Moreover, these residues were mutated in a landmark study by Skålén *et al.*, demonstrating their crucial role in the LDL-proteoglycan interaction and in the initiation of atherosclerotic lesion development in mice (Skålén 2002).

#### *2.3.1.2 Modification of LDL triggers massive lipid uptake and inflammation*

Once trapped within the matrix proteoglycans, the LDL particles become exposed to oxidative, lipolytic, and proteolytic modifications, eliciting fusion and aggregation of the particles and further hindering their exit from the intima (Hevonoja 2000, Öörni 2000). Moreover, LDL modification enables accelerated cellular uptake of LDL by creating recognition sites for 'scavenger receptors' (SR), such as SR-A1, SR-B1, and CD36 (Canton 2013). The SRs form a heterogeneous group of PRRs that were originally identified based on their affinity for modified lipoproteins, but are now known to mediate the clearance of diverse endogenous and microbial ligands (Canton 2013). Native LDL is internalized by cells via the LDLR pathway (Brown 1980). The ensuing influx of cholesterol suppresses LDLR expression and cholesterol biosynthesis, so preventing accumulation of excess cellular cholesterol. In contrast, the uptake of modified LDL by SRs is not downregulated by cellular cholesterol content, allowing the massive accumulation of cholesteryl esters stored as cytoplasmic droplets within macrophages and other SR-expressing cells (Goldstein 1979, Brown 1979). This morphological transformation of macrophages into lipid droplet-filled 'foam cells' is the hallmark of early atherosclerotic lesions (Stary 1994).

Modified LDL species trigger also proinflammatory activation of resident

arterial wall cells. In this context, oxidized LDL (oxLDL), detected in plaques *in situ* (Haberland 1988, Palinski 1989), has been the subject of intensive research. It was recently shown that binding of copper-oxidized LDL to the SR CD36 triggers the formation of a heterotrimeric CD36:TLR4:TLR6 complex that signals NF-κB activation and synthesis of chemokines and pro-IL-1β in macrophages (Stewart 2010). CD36 recognizes protein adducts of oxidized phospholipids on the surface of copper-oxidized LDL (Miller 2011). Furthermore, oxidation by 12/15-lipoxygenase, an enzyme present in atherosclerotic lesions, generates 'minimally modified LDL' that elicits unconventional TLR4 signaling and moderate cytokine and chemokine secretion by macrophages (Miller 2012). The TLR4 signaling is mediated by cholesteryl ester hydroperoxides generated on the LDL particle (Miller 2011). Of note, the proinflammatory effects of oxidatively modified LDL and the oxidized components thereof are not limited to macrophages; these species can also trigger the expression of chemokines (e.g. monocyte chemotactic protein 1 (MCP-1)), growth factors (e.g. M-CSF), and adhesion molecules (e.g. vascular cell adhesion molecule 1 (VCAM-1)) on cultured human aortic endothelial cells and SMCs (Cushing 1990, Rajavashisth 1990, Leitinger 1999).

Taken together, LDL, the major etiological agent of atherosclerosis, exerts its effects on arterial wall cells by promoting both lipid accumulation and inflammatory activation, the key drivers of plaque development. The importance of LDL in atherogenesis is underscored by the success story of LDL-lowering statins in the treatment of atherosclerosis and cardiovascular disease. Statins block cholesterol biosynthesis in the liver by inhibiting the rate-limiting enzyme of the pathway, 3-hydroxy-3-methylglutaryl coenzyme A reductase (Brown 1986). This results in enhanced expression of LDL receptors in the liver, promoting more efficient clearance of LDL from circulation.

#### *2.3.1.3 HDL promotes reverse cholesterol transport from macrophage foam cells*

HDL comprises a heterogeneous group of 7-13 nm lipoprotein particles originating from the liver or the small intestine (Toth 2013). The major protein component of HDL particles, apoAI, lacks the positive charge clusters found in apoB-100. This allows the HDL particles to diffuse more freely through the intima, facilitating their exit through the lymphatics in media and adventitia. Importantly, on their way through the intima, the HDL particles accept excess cholesterol from the macrophage foam cells and transport it via circulation back to the liver for excretion in bile. The process is called 'reverse cholesterol transport' and it is the major antiatherogenic role assigned to HDL (Rosenson 2012). The transfer of cholesterol from lipid-loaded macrophages to HDL is mediated actively via ATP-binding cassette transporters A1 and G1 on the donor cell membrane, and via passive bidirectional transfer through SR-B1 and diffusion (Adorni 2007, Larrede 2009). Finally, the HDL is taken up by

the liver via direct SR-B1-mediated uptake or indirectly via cholesteryl ester transfer protein -mediated transfer to apoB-containing lipoproteins (Rosenson 2012).

Notably, HDL particles have also numerous anti-inflammatory, antioxidant, and antithrombotic effects that are particularly important for the functioning of the vascular endothelium (Rosenson 2013). Despite the wealth of data showing antiatherogenic functions of HDL, clinical trials have failed to show improved cardiovascular outcomes by pharmacological treatments that elevate plasma concentration of HDL cholesterol (Toth 2013). These unexpected negative results have led to the conclusion that, rather than concentration of HDL cholesterol, the antiatherogenic functionality of the HDL particles is the key factor underlying their protective effects against cardiovascular disease, a concept now at the focus of HDL research (Rosenson 2013).

## 2.3.2 Stages of atherosclerotic lesion development

The highly complex process of atherosclerotic plaque development, occurring over decades in humans, has made the study of this process challenging. The consecutive morphological stages of atherosclerotic lesion development were detailed in a series of seminal papers published in the 1990's by a committee set up by the American Heart Association (AHA) (Stary 1992, Stary 1994, Stary 1995). The resulting, widely used AHA classification of lesions to types I-VI is based on careful examination of autopsy samples from patients of different ages and disease stages (**Fig. 6**).

### *2.3.2.1 Early stages of atherosclerotic lesion development*

The subendothelial retention of LDL in the intima precedes the appearance of first isolated macrophage foam cells (Williams 1995), which, in turn, is considered the earliest stage of lesion development designated as type I lesion (Stary 1994). As discussed in section 2.3.1, modification of the LDL particles *in situ* in the arterial wall drives the SR-mediated uptake of LDL into resident macrophages, as well as inflammatory activation of the macrophages and other arterial wall cells. These early events promote increased expression of leukocyte adhesion molecules on the arterial endothelium and the recruitment of immune cells, chiefly monocytes, into the intima. The recruited monocytes differentiate into lipid-scavenging macrophages, generating multiple layers of foam cells that form a 'fatty streak', or type II lesion (Stary 1994). Cholesterol in the fatty streaks accumulates almost exclusively as intracellular cholesteryl esters in the foam cells, with very little extracellular lipid. Moreover, the intimal SMCs markedly contribute to lipid uptake and foam cell formation, and switch from a contractile to synthetic phenotype with enhanced production of extracellular matrix components (Stary 1994). Continued lipid accumulation yields scattered extracellular lipid deposits in the deep musculoelastic intima, and small cholesterol monohydrate crystals begin to appear in the lesions

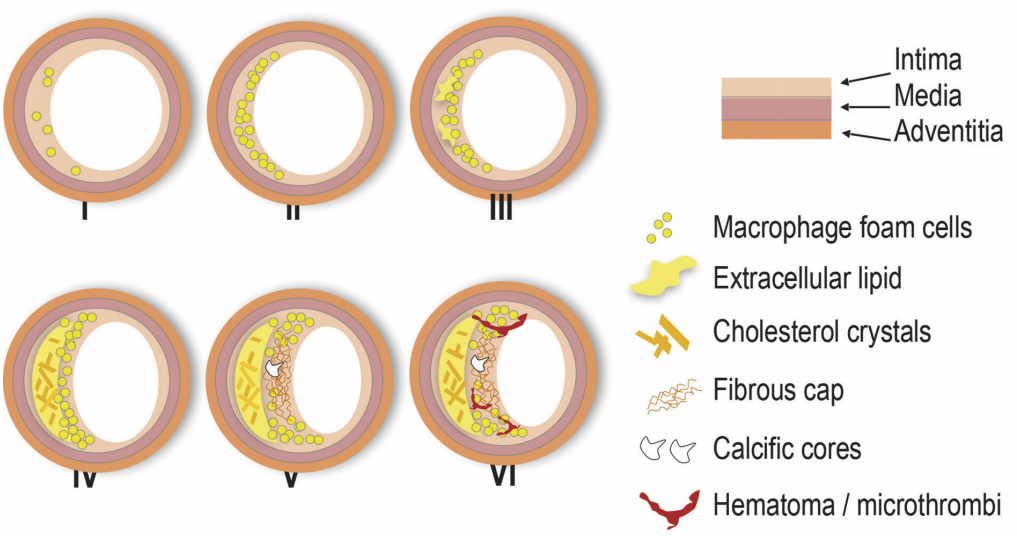


**Figure 6. Stages of atherosclerotic lesion development according to the classification by the American Heart Association.** A schematic view of lesion development in a cross-section of an artery. The vessel lumen is represented by the white space in the center. Note the adaptive intimal thickening at the lesion-prone site already in the initial type I lesion. Type I-III lesions are clinically silent early lesions, whereas type IV-VI represent advanced plaques that may cause clinical manifestations. Adapted from the reports by the Committee on Vascular Lesions of the Council on Arteriosclerosis of the American Heart Association (Stary 1994, Stary 1995). See the text for details.

(Stary 1994, Small 1988). These type III lesions are the last in the series of clinically silent early lesions and also the precursor for more advanced, symptomatic lesions.

*2.3.2.2 Advanced stages of atherosclerotic lesion development*

Advanced, type IV-VI lesions are defined as lesions causing marked disruption of the normal structural organization of the arterial wall (Stary 1995). The hallmark of a type IV lesion is the appearance of a large, continuous pool of extracellular lipid, 'the lipid core', in the deep intima. This is accompanied by a dramatic increase in the number and size of cholesterol crystals, found in the core areas of virtually all type IV lesions (Small 1988). Crystallization can be predicted from the plaque lipid composition; type IV lesions are oversaturated in free cholesterol, overwhelming the solubilizing capacity of phospholipids in cellular membranes and extracellular lipid pools (Small 1988). Cholesterol crystals are readily nucleated within lysosomes of cholesterol-loaded cultured macrophage foam cells (Tangirala 1994, Kellner-Weibel 1999). Moreover, in human atherosclerotic lesions with an emerging lipid core, cholesterol crystals frequently appear within the lipid deposits of deep intima that

is sparse in cells (Bocan 1986). Thus, nucleation of cholesterol crystals *in vivo* may occur both intra- and extracellularly.

SMC proliferation and matrix production leads to the formation of a fibrous cap between the lipid core and endothelium, which is considered to exert a protective effect against plaque rupture (Stary 1995). Fibrotic responses in the presence or absence of a lipid core, accompanied by calcification and stenosis of the vessel lumen, characterize type V lesions. Finally, the appearance of leaky neovessels within the intima and various endothelial defects at the luminal surface of advanced type IV/V lesions may cause haemorrhage and microthrombi that characterize type VI lesions (Stary 1995). The advanced plaques may narrow the luminal space and restrict the blood flow, or rupture into the vessel lumen, causing a large thrombus and acute complications. Myocardial infarction is caused by an occluding thrombus in the coronary artery tree, whereas stroke is typically caused by a non-occluding thrombus at a carotid artery that results in cerebral embolism.

The local microenvironment in the advanced lesions becomes progressively hostile. The growing plaque mass hinders diffusion of oxygen to the plaque core, creating a hypoxic zone in the deep intima (Leppänen 2006, Sluimer 2008). Hypoxia promotes anaerobic glycolytic metabolism in the arterial wall cells, resulting in increased glucose consumption and lactate production in the deep intima (Leppänen 2006). Moreover, macrophage activation involves a switch to glycolytic metabolism even under normoxia and thus, macrophages promote increased production of metabolic acid and lactate independently of hypoxia (Rodriguez-Prados 2010, Tannahill 2013, Roiniotis 2009). Indeed, advanced human carotid plaques exhibit marked deviations from the normal physiological pH of 7.4 (Naghavi 2002). The local acidosis of extracellular fluids in the plaques reaches approximately pH 6.8-6.0, as measured by pH electrodes and pH-sensitive fluorescent dyes (Naghavi 2002).

## 2.3.3 Role of immune cells and inflammatory mediators in plaque development

### *2.3.3.1 The immune cell population in atherosclerotic lesions*

A dynamic population of innate and adaptive immune cells exists in the arterial wall throughout the stages of atherosclerotic lesion development. Recent studies have shown that the areas of adaptive intimal thickening develop a much denser subendothelial network of resident macrophages/dendritic cells compared to the adjacent areas (Randolph 2014). This implies that also enhanced immune surveillance may contribute to the high susceptibility of these sites to atherosclerosis. The continued recruitment of immune cells to the developing lesion greatly amplifies the initial inflammatory reaction and profoundly alters the metabolism of the resident arterial wall cells. Monocyte-derived macrophages are the most abundant

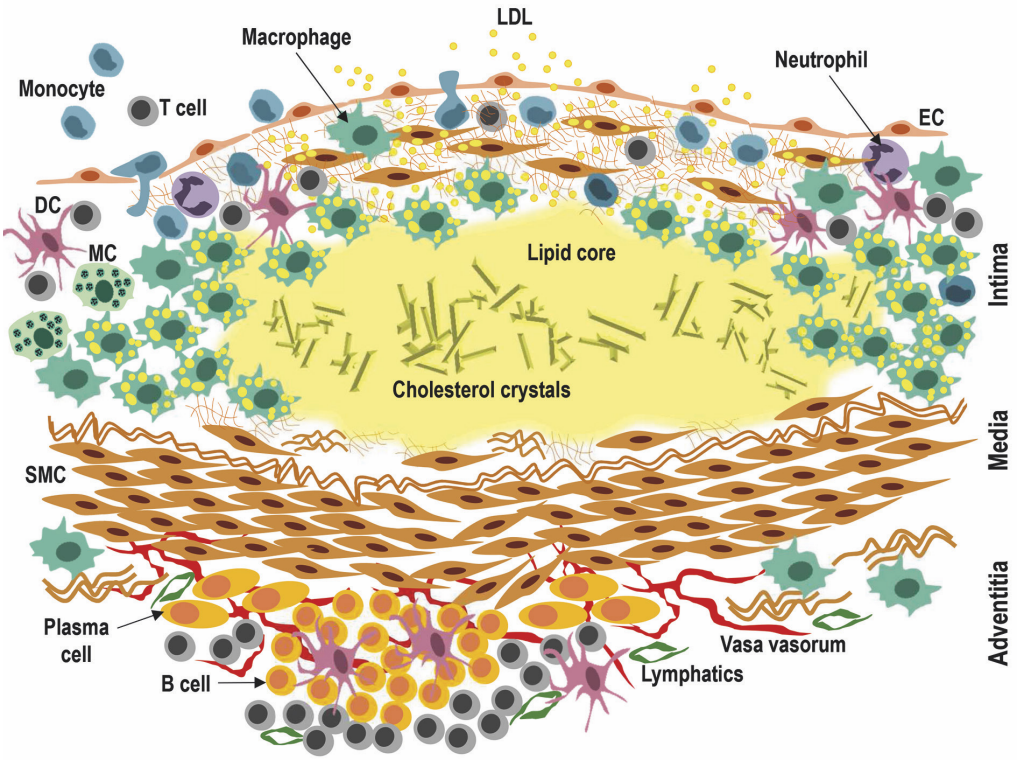


**Figure 7. Organization of immune cells in atherosclerotic lesions.** Macrophages and other immune cells are particularly abundant at the plaque shoulder regions flanking the lipid core. Both macrophages and smooth muscle cells are transformed into lipid-loaded foam cells in the intima. The lipid core consists of cell remnants and lipid droplets released from the dying cells, modified and aggregated lipoproteins, and cholesterol crystals. The media adjacent to advanced plaques becomes disordered and secretes chemokines that contribute to the formation of tertiary lymphoid organs in the adventitia. EC, endothelial cell; SMC, smooth muscle cell; DC, dendritic cell; MC, mast cell.

immune cells in atherosclerotic plaques and the nexus between lipid accumulation and inflammation. Furthermore, the plaques harbour also abundant T cells and dendritic cells, as well as some mast cells and neutrophils (Hansson 2011) (**Fig. 7**).

Notably, immune cell infiltrates are found not only within the intimal plaque, but also in the adventitia adjacent to advanced human plaques (Houtkamp 2001). These adventitial infiltrates have an organized structure resembling lymphoid follicles, implying local maturation of B cells and generation of humoral immune responses (Houtkamp 2001). The development of these so-called tertiary lymphoid organs (TLO) is closely linked with chronic inflammation in various diseases, yet their functions remain poorly defined (Mohanta 2014). In aged atherosclerotic mice, the TLOs comprise activated B cell follicles, a separate T cell area, antigen-presenting

cells, and plasma cells, implying local activation and proliferation of adaptive immune cells (Mohanta 2014, Grabner 2009). Furthermore, medial SMCs underlying the intimal plaque were activated to secrete chemokines required in the TLO formation, suggesting the existence of a defined relay mechanism of inflammatory signals from the intima to the adventitia.

#### *2.3.3.2 Triggers of inflammation in atherosclerotic plaques*

Oxidized LDL has been suggested as the key agent triggering inflammatory responses, as well as foam cell formation, in the arterial wall (Hansson 2011, Miller 2011). However, dietary supplementation with antioxidants has failed to show protection against cardiovascular disease in numerous clinical trials (Tinkel 2012). One proposed explanation is that the critical role of oxLDL could be restricted to early stages of atherogenesis, rendering antioxidants powerless in patients with established atherosclerosis (Tedgui 2006). Nevertheless, only the advanced plaques present symptoms in humans. Heat shock proteins and other danger signals released from dying cells, diabetes-associated advanced glycation end products, and obesity-associated adipokines, among others, have been proposed as potential secondary drivers that maintain the chronic inflammation in the plaques (Tedgui 2006). Also defective resolution of inflammation plays a major role (Tabas 2010). Thus, plaque inflammation is exacerbated by the failure to suppress further immune cell recruitment, to clear apoptotic cells, and to induce immune cell egress. Notably, microbes and their components, such as bacterial DNA, are found in the arterial intima, where they could conceivably trigger inflammation (Lehtiniemi 2005, Ott 2006). However, based on experiments in germ-free mice, microbial presence (bacterial, viral, and fungal) is dispensable for lesion development, and treatment of patients with antibiotics showed no protection from cardiovascular events (Tedgui 2006, Wright 2000). Taken together, despite intensive research in the area, the mechanisms triggering and maintaining chronic inflammation during atherogenesis remain incompletely understood.

#### *2.3.3.3 Cytokines and chemokines*

The inflammatory activation in human plaques is marked by the immunoreactivity for numerous inflammatory cytokines, including for example IFN-γ, TNF-α, IL-1α/β, IL-2, IL-6, IL-8, IL-10, IL-18, and TGF-β (Frostegard 1999, Ait-Oufella 2011). In general, pro- and anti-inflammatory cytokines exert pro- and antiatherogenic effects, respectively, in cultured cells and animal models, affirming the major role of inflammation in lesion development (Tedgui 2006, Ait-Oufella 2011). Furthermore, a wide selection of chemokines and their receptors are implicated in atherogenesis (e.g. MCP-1/CCR2, fractalkine/$CX_3$CR1, RANTES/CCR5), and the vast majority of these mediate monocyte trafficking into the lesions (Rolin 2014).

Certain cytokines, including IL-1α/β, can further induce their own production and the production several other inflammatory mediators to amplify the inflammatory response (Dinarello 2009). Furthermore, cytokines can directly activate newly recruited immune cells and regulate their polarization state in the lesions (Ait-Oufella 2011). Importantly, proinflammatory cytokines trigger diverse responses in the vascular wall cells, including oxidative stress, decreased expression of cholesterol efflux receptors (IL-1β, IFN-γ), increased expression of scavenger receptors (TNF-α, IFN-γ), induction of leukocyte adhesion molecules and chemokines (IL-1α/β, TNF-α, IFN-γ, IL-18), suppression of antithrombotic properties of the endothelium (IL-1α/β, TNF-α), loosening of endothelial tight junctions (IFN-γ, TNF-α), suppression of SMC collagen synthesis (IFN-γ), production of matrix-degrading enzymes (IL-1α/β, TNF-α, IL-18), induction of neovessel formation (IL-1β, TNF-α), and, ultimately, apoptosis (IL-1α/β, TNF-α, IFN-γ) (Tedgui 2006, Ait-Oufella 2011, Gerdes 2002). Anti-inflammatory cytokines tend to counteract these effects. For example, IL-10 and TGF-β inhibit the production of matrix-degrading enzymes and promote the expression of cholesterol efflux receptors, and IL-10 also blocks neoangiogenesis as well as reduces the production of proinflammatory cytokines (Tedgui 2006, Ait-Oufella 2011).

#### *2.3.3.4 Other effectors contributed by immune cells*

The immune cells are not only a major source for proinflammatory mediators, but release proteolytic and lipolytic enzymes and ROS that modify lipoproteins, promoting foam cell formation (Öörni 2000). Furthermore, macrophages, mast cells, and neutrophils secrete numerous proteases that degrade extracellular matrix (e.g. chymase, tryptase, cathepsins, and metalloproteases) and weaken the fibrous cap, promoting plaque rupture (Newby 2014). Indeed, immune cell infiltrates with macrophages and activated mast cells are prominent at the immediate site of plaque rupture or erosion (Moreno 1994, Kovanen 1995, Kaartinen 1998, Willems 2013, Howard 2015). Moreover, their presence correlates with the severity of cardiovascular symptoms and predicts future cardiovascular events. Finally, immune cells contribute to the production of lipid mediators that modulate vascular tone, platelet aggregation, and leukocyte adhesion and migration. Lesional macrophages are a prominent source of the proatherogenic eicosanoid leukotriene B4, acting as a chemoattractant for monocytes and T cells (Hansson 2011). Also activated mast cells produce eicosanoids, including prostaglandin D2 and leukotriene B4 (Boyce 2007).

#### *2.3.3.5 Macrophages and innate immune signaling in atherosclerosis*

Majority of our knowledge related to the functional role of inflammation in atherogenesis is derived from mouse models. The models most frequently used are the LDLR-KO mice (Ishibashi 1993), resembling human familial hypercholesterolemia,

and the apoE-KO mice (Zhang 1992) exhibiting impaired clearance of VLDL and chylomicrons by the liver. Homozygous LDLR- and apoE-KO mice exhibit moderate increases in plasma cholesterol and a shift in the lipoprotein profile from HDL to high levels of VLDL, IDL, and LDL. These mice spontaneously develop early atherosclerotic plaques in the aortic arch, yet a high-fat high-cholesterol diet (HFD) induces a rapid development of more advanced lesions in both mouse models.

M-CSF-null mice exhibit severely diminished populations of blood monocytes and tissue macrophages (Wiktor-Jedrzejczak 1992). Crossing these mice with apoE-KO mice resulted in 86 % reduction in atherosclerotic lesion area, despite a 3-fold higher plasma cholesterol in the M-CSF-null/apoE-KO mice (Smith 1995). These findings were later confirmed in a similar study (Qiao 1997). These data verified an essential role for macrophages in atherogenesis. In agreement, genetic ablation of the chemokine MCP-1 or its receptor CCR2 - the major pathway of monocyte recruitment into the arterial wall - markedly reduces atherogenesis in mouse models (Gu 1998, Boring 1998). The polarization of macrophages in atherosclerotic plaques seems highly adaptive to the immediate microenvironment (Chistiakov 2015). Thus, macrophages display a spectrum of different phenotypes, and M1- and M2-like phenotypes may localize to distinct niches.

Numerous studies have reported a role for innate immune activation in atherogenesis. TLR expression is low in healthy human arterial walls, yet strong TLR2 and TLR4 staining was found in macrophages and endothelial cells of atherosclerotic plaques (Xu 2001, Edfeldt 2002). Moreover, marked reductions in atherosclerotic lesion area and macrophage numbers were demonstrated both in TLR2/LDLR-dKO and in TLR4/apoE-dKO mice (Mullick 2008, Michelsen 2004). Intriguingly, deficiency in the essential LPS co-receptor, CD14, had no effect on atherogenesis in apoE-KO mice, implying that microbial ligands were not required for lesional TLR4 signaling (Bjorkbacka 2004). Moreover, deficiency in any single component of the CD36:TLR4:TLR6 signaling complex, inducible in macrophages by oxLDL, markedly reduced plaque area and inflammatory markers in apoE-KO mice (Sheedy 2013). Finally, deficiency in the Toll/IL-1 signaling adapter MyD88 strongly reduced lesion area, as well as macrophage numbers and chemokine expression in the lesions of apoE-KO mice (Michelsen 2004, Bjorkbacka 2004). A severely impaired chemokine production in response to IL-1β or IL-18 was further demonstrated in MyD88-KO mouse macrophages (Bjorkbacka 2004). Collectively, these data suggest an important role for innate immune responses in atherosclerosis.

#### *2.3.3.6 Inflammasome target cytokines*

In atherosclerotic plaques, macrophages are without a doubt the most prominent cells competent in inflammasome signaling and robust cytokine secretion. IL-1β

immunoreactivity was detected in macrophage foam cells and endothelial cells of human coronary artery and carotid plaques, but not in healthy arteries (Frostegard 1999, Galea 1996). The amount of IL-1β in the vessel wall correlated with the severity of coronary atherosclerosis (Galea 1996). Similarly, immunoreactivity for IL-18 was detected in macrophages of human carotid plaques, but not in healthy aorta (Gerdes 2002, Mallat 2001a). Moreover, the cleaved, active form of IL-18 and abundant IL-18 receptor α were found in the plaques, but not in healthy aorta. Finally, IL-18 mRNA was more abundant in unstable compared to stable plaques (Mallat 2001a).

Both IL-1α and IL-1β signal through the IL-1 receptor I that is blocked by IL-1 receptor antagonist (IL-1Ra) (see section 2.2.3). Subcutaneous administration or overexpression of IL-1Ra, as well as deficiency in IL-1 receptor I reduces atherosclerotic lesion area in apoE-KO mice by ~50-80 % (Elhage 1998, Merhi-Soussi 2005, Chi 2004). Both IL-1α and IL-1β contribute to the effects of IL-1 signaling in atherosclerosis, as deficiency in either cytokine markedly reduces lesion development in apoE-KO mice (Kirii 2003, Kamari 2011). Thus, both IL-1α/apoE-dKO and IL-1β/apoE-dKO mice showed ~50 % reduction in lesion area, and similar results were obtained when apoE-KO mice were transplanted with IL-1α- or IL-1β-KO bone marrow (Kamari 2011). The attenuation of atherogenesis in IL-1β-KO mice correlated with reduced aortic expression of VCAM-1 and MCP-1 (Kirii 2003). Importantly, treatment of rheumatoid arthritis patients with IL-1Ra markedly improved vascular and left ventricular function, as assessed by endothelium-dependent dilation of the brachial artery and by echocardiography, respectively (Ikonomidis 2008). Moreover, the plasma levels of IL-1 downstream targets, IL-6 and the vasoconstrictor endothelin-1, were reduced after IL-1Ra treatment.

Also IL-18 exhibits clear proatherogenic effects. Neutralization of IL-18 activity by IL-18 binding protein markedly reduced lesion development in aorta of apoE-KO mice (Mallat 2001b). Moreover, there was a striking change in the plaque composition towards increased stability, as assessed by increased collagen content and reduced immune cell infiltration. Likewise, lesion development was significantly attenuated in IL-18/apoE-dKO mice, despite elevated cholesterol levels caused by the IL-18 deficency (Elhage 2003). Moreover, the lesions of IL-18/apoE-dKO mice displayed reduced markers of IFN-γ activity, linking IL-18 with induction of IFN-γ *in vivo*. A very recent publication by Wang *et al.* gave a new perspective to IL-18 signaling in atherosclerosis (Wang 2015). It was shown that the IL-18 receptor interacts with Na-Cl co-transporter SLC12A3 strongly induced in atherosclerotic arterial wall, and that knocking out both IL-18 receptor and SLC12A3, but not either one of the molecules alone, attenuates atherosclerosis in apoE-KO mice (Wang 2015). The effect was attributed to receptor deficiency in bone marrow –derived leukocytes, and the expression of either IL-18 receptor or SLC12A3 was sufficient to trigger cellular

IL-18 signaling and IL-18-mediated cytokine production in macrophages and T cells (Wang 2015). Thus, cellular ion fluxes and cell volume regulation involved in NLRP3 inflammasome activation (Perregaux 1994, Perregaux 1996, Compan 2012, Petrilli 2007, Munoz-Planillo 2013, Perregaux 1998, Schorn 2010, Murakami 2012, Rabolli 2014) may also contribute to downstream signaling by the inflammasome target cytokine IL-18.

#### *2.3.3.7 Adaptive immune responses in atherosclerosis*

Majority of the T cells found in human atherosclerotic plaques are $CD4^+$ T helper cells, and a significant proportion of them bear markers of activation (class II HLA, IFN-γ) (Hansson 1989). Moreover, human plaques, but not peripheral blood, harbour $CD4^+$ T cell clones that proliferate and secrete the classical Th1 cytokine IFN-γ when exposed to monocytes presenting oxLDL-derived antigens (Stemme 1995). These data suggest the presence of a local oxLDL-driven autoimmune response in human plaques. In agreement, adoptive transfer of oxLDL-reactive $CD4^+$ T cells to apoE-KO mice accelerates atherosclerosis (Zhou 2006). Moreover, IFN-γ receptor/apoE-dKO mice have strongly reduced lesion development, implying a proatherogenic role for T cell activation (Gupta 1997). Dendritic cells readily migrate from atherosclerotic plaques to the draining lymph nodes, where they present plaque-derived antigens to naïve T lymphocytes to activate them (Hansson 2011). In apoE-KO and LDLR-KO mice, dendritic cells significantly contribute to T cell activation and generation of Th1 responses via MHC II-restricted antigen presentation (Gautier 2009, Sage 2014).

OxLDL elicits also humoral immune responses that exert atheroprotective effects. Autoantibodies reactive to oxLDL are found in human and rabbit plasma, and immunization with oxLDL or native LDL reduces atherogenesis in animal models (Hansson 2011, Palinski 1989, Palinski 1995). Both T cell-dependent IgG antibodies and T cell-independent natural IgM antibodies are produced against oxLDL (Hansson 2011). Moreover, splenectomy aggravates atherogenesis, and transfer of splenic B cells from old apoE-KO mice with established atherosclerosis into young apoE-KO mice attenuates lesion development via elevating oxLDL antibody titers (Caligiuri 2002).

Surprisingly, lymphocyte-deficient Rag-1- or Rag-2-null mice crossed with apoE- or LDLR-KO mice suggest only a minor or transient role for T and B cells in atherogenesis (Dansky 1997, Daugherty 1997, Song 2001). In these experiments, atherosclerotic lesion area is transiently reduced in early fatty streak lesions of mice on chow or HFD, but the difference is lost upon continued HFD resulting in more advanced lipid core lesions. In line with these data, proinflammatory Th1 type responses and IFN-γ production in the lesions dominate in chow-fed apoE-KO mice, whereas a clear switch to antibody-associated Th2 type responses and IL-4

production is found in apoE-KO mice on HFD (Zhou 1998). Moreover, attenuation of Th1 responses in atherosclerotic mice markedly reduces early lesion development and boosts the production of antiatherogenic oxLDL antibodies (Laurat 2001, Buono 2005). Thus, the effect of T cells on atherogenesis is dependent on disease stage and T helper cell polarization. Whereas the Th1 subset has a clear proatherogenic role, studies on the Th2 and Th17 subsets have yielded mixed results (Hansson 2011). Conversely, regulatory T cells (Tregs) that suppress the activity of effector T cells have been shown to exert atheroprotective effects (Hansson 2011). Depletion of both the naturally arising $CD4^+CD25^+$ Treg population and the Tregs positive for the lineage marker FOXP3 promotes atherosclerotic plaque development, albeit via different mechanisms (Ait-Oufella 2006, Klingenberg 2013).

In contrast to the combined depletion of T and B cells, the specific depletion of B cells in LDLR-KO mice moderately increases atherogenesis, in line with the protective effects of oxLDL antibodies (Major 2002). However, more recent data suggests that similar to T cells, B cells may display both pro- and antiatherogenic effects depending on the B cell subset. Thus, depletion of mature B2 cells using a CD20 antibody reduces atherogenesis via limiting T cell responses (Ait-Oufella 2010). Conversely, adoptive transfer of B1 cells into splenectomized apoE-KO mice is atheroprotective (Kyaw 2011). The critical determinant in B1 cell-mediated atheroprotection is the production of natural IgM antibodies against oxLDL. In agreement, the depletion of B2 cells preserved anti-oxLDL IgM levels while reducing only the anti-oxLDL IgG (Ait-Oufella 2010).

To conclude, both innate and adaptive immune responses have a marked impact on the development of atherosclerotic lesions. Whereas innate immune activation seems to exert mainly proatherogenic effects, the effects of adaptive immune responses strongly depend on the balance between different T and B cell subsets (**Fig. 8**). OxLDL and its various components have gained much attention as the potential autoantigens triggering innate and adaptive immune responses in atherosclerosis, yet many other potential autoantigens have been proposed (Shah 2014). While passive immunization with anti-oxLDL antibodies has failed to show atheroprotective effects in clinical studies, active vaccination against LDL is a promising immunomodulatory strategy to combat atherosclerosis (Shah 2014). However, a major challenge in designing practical prototypes of atherosclerosis vaccines is the identification of the specific antigens on LDL that trigger atheroprotective immunity (Shah 2014). Other current approaches to target atherosclerotic inflammation in clinical trials include a more generalized anti-inflammatory treatment with low-dose methotrexate (Everett 2013), as well as neutralization of specific inflammatory mediators strongly associated with atherogenesis (Ridker 2011). The outcomes of these first large phase III clinical trials will lead the way in determining whether inflammation, alongside hyperlipidemia, truly is a feasible treatment target for atherosclerosis and cardiovascular disease.

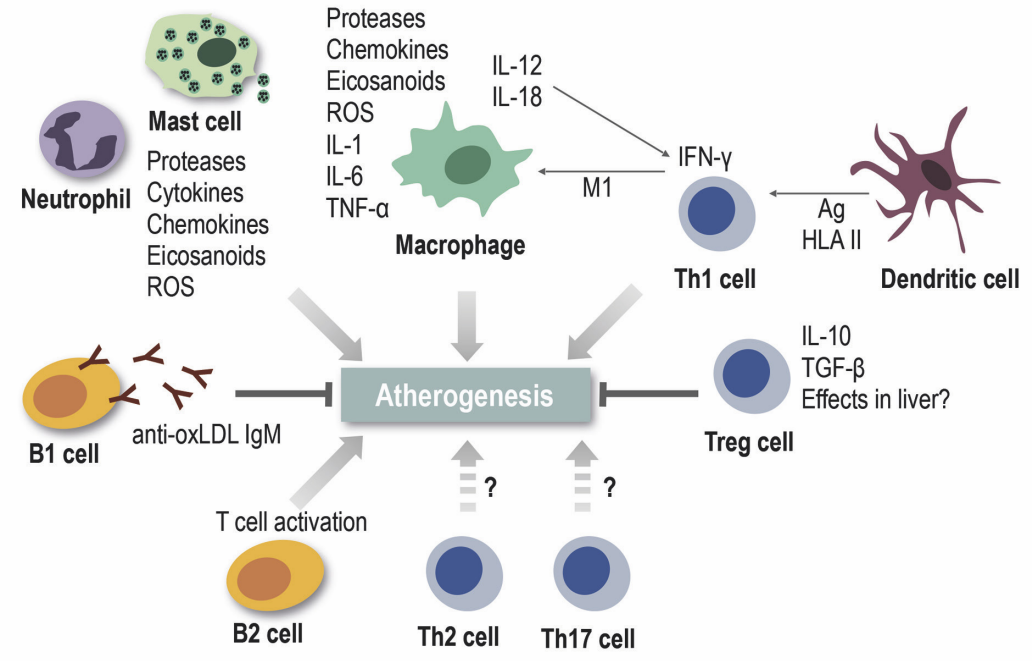


**Figure 8. Summary of immune cell involvement in atherosclerosis.** The solid arrows denote proatherogenic effects, the T-shaped lines denote antiatherogenic effects, and the dashed arrows indicate mixed results. See the details in text. Ag, antigen; HLA II, type II human leukocyte antigen; IFN, interferon; IL, interleukin; ROS, reactive oxygen species; TGF-β, transforming growth factor β; TNF-α, tumor necrosis factor α.

# 3 AIMS OF THE STUDY

Inflammation is today recognized as a major driving force in atherogenesis, yet our understanding of the specific mechanisms triggering and maintaining inflammation in the arterial wall is incomplete. The purpose of this study was to examine the potential role of the inflammasome pathway in the pathogenesis of atherosclerosis. The proatherogenic effects of the inflammasome-controlled cytokines, IL-1β and -18, are well documented, but the triggers for secretion of these cytokines in atherosclerotic plaques have remained unknown. Macrophages are the canonical inflammasome pathway-competent cells, and also the key immune cells in atherosclerotic lesions. Thus, cultured human macrophages offered a highly relevant cell model for the screening of candidate inflammasome activators associated with atherogenesis. In particular, we were interested in the NLRP3 inflammasome emerging as a mediator of sterile inflammatory responses to endogenous danger signals. Furthermore, a unique collection of human coronary artery specimens with varying stages of atherosclerosis was available at the Wihuri Research Institute. This specimen collection offered the opportunity to study the expression of the inflammasome pathway in coronary arterial wall, a site most crucial to the acute complications of atherosclerosis.

The specific aims of this study were:

1) to identify atherosclerosis-associated endogenous danger signals that activate the inflammasome pathway and interleukin-1β and -18 secretion in cultured human macrophages,

2) to characterize in detail the molecular mechanisms mediating the inflammasome activation, and

3) to study the expression pattern of inflammasome pathway components and regulators in the coronary arterial wall, and to identify potential changes in the expression pattern during progression of coronary atherosclerosis.

# 4 MATERIALS AND METHODS

The materials and methods used in this study are summarized in **Table 3**. For a detailed description of each method, the reader is referred to the original publications in this thesis (I, II, III) and to additional references or source materials, as indicated.

**Table 3.**

| Method | Original publications | Reference /source |
|---|---|---|
| **Cell culture and tissue samples** | | |
| Human peripheral blood monocytes | I | (Nakanishi 2009) |
| Human monocyte-derived macrophages | I, II, III | (Nakanishi 2009) |
| Monocytic human THP-1 cell line | I, II | (Tsuchiya 1980) |
| Mouse bone marrow-derived macrophages | II | |
| Frozen and formalin-fixed paraffin-embedded human coronary artery specimens | III | |
| **Antibody-based techniques** | | |
| Enzyme-linked immunosorbent assays (ELISA) | I, II, III | R&D Systems |
| Western blotting | I, II, III | (Burnette 1981) |
| Immunohistochemical stainings | III | |
| **Fluorescence-based techniques** | | |
| Imaging of lysosomes in live cells using the acridine orange dye | I | (Traganos 1994) |
| Imaging of cathepsin B activity in live cells using the substrate z-Arg-Arg-cresyl violet | I | (Van Noorden 1997) |
| Confocal fluorescence/reflection microscopy to detect intracellular cholesterol crystals | I | (Hornung 2008) |
| Cathepsin B/L activity measurement using the substrate z-Phe-Arg-AMC | II | (Barrett 1980) |
| Recording of intracellular pH changes in live cells using the fluorescent probe BCECF-AM | II | (Nordstrom 2012) |
| **Manipulation and analysis of gene expression** | | |
| Gene knock-down by small interfering RNAs | I, II | (Elbashir 2001) |
| Quantitative real-time RT-PCR | I, II, III | (Heid 1996) |
| $RT^2$ Profiler Human Inflammasomes PCR array for human coronary RNA | III | Qiagen |
| **Miscellaneous** | | |
| Preparation of cholesterol crystals | I, II, III | (Flynn 1979) |
| Preparation of monosodium urate crystals | II | (Scanu 2010) |
| Design of acidic and basic culture media | II | (Eagle 1971) |
| Thin layer chromatography | I, II | (Lähdesmäki 2009) |
| Cytotoxicity assay (lactate dehydrogenase) | II, III | Roche |
| Limulus amebocyte lysate endotoxin assay | I, II, III | Lonza |
| **Statistical analyses** | | |
| Mann-Whitney U test, Wilcoxon test, t-test | I, II, III | |

# 5 RESULTS AND DISCUSSION

## 5.1 IDENTIFICATION OF ATHEROSCLEROSIS-ASSOCIATED ACTIVATORS OF THE INFLAMMASOME PATHWAY (I, II, UNPUBLISHED DATA)

### 5.1.1 Candidate activators of the inflammasome pathway in atherosclerotic plaques (I, II)

When we first set out to find potential inflammasome pathway activators from atherosclerotic lesions, cholesterol crystals (CHC) stood out as the strongest candidate. During the preceding years, several reports had identified endogenous or exogenous crystalline substances as triggers of the NLRP3 inflammasome in macrophages (Martinon 2006, Hornung 2008, Dostert 2008). In particular, we were intrigued by the gout-associated monosodium urate crystals, the first endogenously formed crystals shown to trigger a sterile NLRP3-mediated inflammatory response (Martinon 2006). CHC have been acknowledged as a hallmark of advanced atherosclerotic lesions since the very earliest studies on atherosclerosis (Virchow 1863). However, they were considered as an inert material in the lesions and largely ignored in the mechanistic studies of disease pathology.

Interest in another candidate activator of the inflammasome pathway arose from the earlier studies on extracellular acidosis and lipoprotein modification at the Wihuri Research Institute (Lähdesmäki 2009). Elevated lactate concentrations and local acidification of the extracellular fluids have been demonstrated in the cores of advanced human and rabbit atherosclerotic plaques (Leppänen 2006, Naghavi 2002). In fact, local acidosis is a common characteristic of many acute and chronic inflammatory sites (Menkin 1956, Cummings 1966, Treuhaft 1971, Hunt 2000). Despite the clear connection between inflammation and local acidosis, studies on the effects of low pH on immune cell function are rather limited (Lardner 2001). Notably, during acute inflammation, macrophages are resistant to the acidification of the inflammatory exudate and are thus able to adapt to survival and function under acidic environment (Menkin 1956).

### 5.1.2 Cholesterol crystals and extracellular acidosis trigger NLRP3 inflammasome activation in macrophages (I, II, unpublished data)

We began our studies by crystallizing pure cholesterol from ethanol (study I) and by designing a custom RPMI-based cell culture medium covering the pH range 7.5-6.0 with a single buffer system (study II). We treated human peripheral blood monocytes, primary human monocyte-derived macrophages (MDMs), mouse bone marrow–derived macrophages (BMDMs), and phorbol-12-myristate-13-acetate

(PMA)-differentiated human THP-1 macrophages with these two candidate inflammasome activators. Resting primary monocytes and macrophages express low levels of NLRP3 receptor and no pro-IL-1β, and the transcriptional induction of both molecules is required for NLRP3 inflammasome assembly and secretion of mature IL-1β (Bauernfeind 2009, Puren 1999). In contrast, PMA-differentiation induces stable expression of pro-IL-1β (Fenton 1988), as well as markedly enhances the expression of NLRP3 receptor (unpublished data) in THP-1 macrophages. Secretion of the mature 17 kDa form of IL-1β was used as the primary readout for inflammasome activity, as detected by IL-1β (p17)-specific ELISA or by Western blotting.

We found that neither CHC nor acidic culture medium triggered IL-1β secretion in resting primary human monocytes or MDMs (study I, Fig. 2A-B; study II, stated in text). However, when the cells were costimulated (study I) or primed for 4 h (study II) with the TLR4 ligand LPS, both CHC and acidic media at pH 6.5-6.0 triggered dose-dependent secretion of mature IL-1β (study I, Fig. 2A-B; study II, Fig. 1A). Similarly, acidic pH triggered IL-1β secretion in LPS-primed mouse BMDMs (Supplemental Fig. S2A). LPS rapidly induced the synthesis of pro-IL-1β and NLRP3, but not NLRP1, in human MDMs, whereas CHC had little effect (study I, Fig. 3A-C). Thus, both CHC and acidic environment specifically induced the inflammasome-mediated cleavage step of pro-IL-1β in LPS-primed human MDMs, enabling IL-1β secretion. As expected, the THP-1 macrophages showed strong, dose-dependent secretion of IL-1β in response to CHC and low pH without the requirement for LPS priming (study I, Fig. 2C; study II, Fig. 1B). Also IL-18 secretion was induced by low pH (study II, Fig. 1C). Notably, the effect of acidic pH was fully reversible, as IL-1β secretion was not sustained after incubation of the cells at low pH, followed by return to physiological pH (study II, Fig. 5D).

The caspase-1 inhibitor z-YVAD-fmk markedly attenuated CHC- and low pH-induced IL-1β secretion in human MDMs and THP-1 macrophages (study I, Fig. 4; study II, Fig. 3A, E, and H). Furthermore, caspase-1 activation was directly demonstrated by Western blotting in culture supernatants of low pH-treated THP-1 macrophages that displayed the active 10 kDa caspase-1 subunit at pH 6.5-6.0 (study II, Fig. 1D). As described in section 2.2.3, robust activation of caspase-1, particularly during bacterial infection, may cause a rapid caspase-1-dependent pyroptotic cell death. However, CHC and low pH only slowly triggered cell death of human MDMs, as determined by lactate dehydrogenase release into the culture medium (unpublished data). For example, over 95 % and ~80 % of LPS-primed human MDMs remained viable after 8 and 24 h treatment with a high 2 mg/ml concentration of CHC, respectively. Compared to MDMs, the THP-1 macrophages were more susceptible to cell death in response to inflammasome activation. Nevertheless, stimulation of THP-1 macrophages with acidic medium triggered the release of mature IL-1β at

earlier time points than the release of lactate dehydrogenase (study II, Supplemental Fig. S2B and C). Thus, IL-1β was actively secreted, rather than passively released upon cell death.

We subsequently silenced the expression of the NLRP3 receptor in THP-1 macrophages using a combination of two small interfering RNAs (siRNAs) (study I, Fig. 6A; study II, Fig. 4C). As a result, IL-1β secretion in response to CHC (study I, Fig. 6B) and low pH (study II, Fig. 4D) was completely abolished, whereas TNF-α secretion was not significantly reduced (study II, Fig. 4E). Thus, CHC and extracellular acidosis were identified as novel endogenous danger signals that trigger an innate immune response via the NLRP3 inflammasome.

Both the active and inactive forms of IL-1β and caspase-1 were secreted by THP-1 macrophages in response to CHC and acidic medium (study I, Fig. 2C inset; study II, Fig. 1D). Similarly, we observed low pH-induced secretion of ASC by Western blotting (unpublished data). It was recently shown that the whole speck-like NLRP3/ASC inflammasome complex is secreted from mouse macrophages as an intact complex that continues pro-IL-1β processing in extracellular fluids (Baroja-Mazo 2014, Franklin 2014). Moreover, proteases secreted by neutrophils and mast cells in human atherosclerotic lesions (proteinase 3, cathepsin G, chymase) can process IL-1β extracellularly into an active form (Newby 2014, Coeshott 1999, Hazuda 1990, Mizutani 1991). Thus, multiple mechanisms may propagate local production of active IL-1β in plaques, promoting chronic inflammation.

### 5.1.3 Initial events in NLRP3 inflammasome activation by cholesterol crystals and extracellular acidosis (I, II)

CHC and extracellular acidosis are chemically and physically very different entities that both act as NLRP3-activating danger signals. We next explored the underlying mechanisms enabling NLRP3 inflammasome activation by these stimuli. While cholesterol crystals are subjected to phagocytosis by macrophages (McConathy 1989), changes in extracellular pH could affect a wide range of cellular functions via triggering changes in the behaviour of various biomolecules.

#### *5.1.3.1 Phagocytosis of cholesterol crystals*

Phagocytosis is required for NLRP3 activation by other crystalline and particulate stimuli (Hornung 2008, Dostert 2008) and we thus explored phagocytosis as the potential first step in CHC-induced inflammasome activation. CHC are phagocytosed by cultured macrophages and the cells store some of the crystal-derived free cholesterol as cholesteryl esters (McConathy 1989). We thus utilized the cellular cholesteryl ester content as a marker of CHC phagocytosis. Inhibition of CHC phagocytosis by cytochalasin D strongly reduced both intracellular cholesteryl

ester accumulation and IL-1β secretion in THP-1 macrophages (study I Fig. 1A and B; Fig. 4). Of note, more and more cell-associated crystals accumulated in macrophages over time, suggesting that the cells are not able to eliminate the CHC by dissolving them. To directly demonstrate phagocytosis of CHC, we employed confocal reflection microscopy (Hornung 2008). The ordered crystal structure reflects the laser beam back to the detector, which allowed us to visualize CHC in the intracellular space, confined within the cholera toxin subunit B-labeled plasma membrane (study I, Fig. 1C). In the presence of cytochalasin D, the CHC remained extracellular. Taken together, the phagocytotic uptake of CHC is required for CHC-induced activation of the NLRP3 inflammasome.

#### *5.1.3.2 Acidic environment causes intracellular acidification*

Changes in the extracellular pH could, in principle, affect virtually any biomolecule at the cell surface or in the surrounding matrix to trigger cellular signaling events. In the context of NLRP3 inflammasome activation, acidic pH could conceivably induce cellular potassium efflux and/or other disturbances in intracellular ion concentrations. Direct sensors of acidosis, the acid-sensing ion channels and the proton-sensing G-protein coupled receptors, could modulate cellular ion fluxes directly or via second messengers (Deval 2015, Okajima 2013). However, the expression and functionality of these molecules in macrophages remains largely unexplored (Okajima 2013, Friese 2007). Intriguingly, the NLRP3-activating bacterial toxin nigericin is an ionophore that mediates electroneutral $K^+$ efflux compensated by $H^+$ influx and intracellular acidification (Mariathasan 2006, Perregaux 1994). The $K^+$ efflux, rather than $H^+$ influx, is essential for IL-1β secretion. However, the counterbalancing cation influx plays an important role, as an electrogenic ionophore inducing only $K^+$ efflux is not sufficient to trigger IL-1β maturation and secretion (Perregaux 1994). We thus hypothesized that intracellular acidification (i.e. influx of $H^+$) was an essential step in NLRP3 inflammasome activation induced by acidic environment.

To monitor changes in intracellular pH ($pH_i$), we performed live cell imaging using the cell-permeable, fluorescent pH indicator dye BCECF-AM (Nordstrom 2012). We showed that at extracellular pH ($pH_e$) of 7.5, the $pH_i$ in THP-1 macrophages was ~7.2 (study II, Fig. 5C), in line with earlier studies (Bidani 1995, Baldini 2003). A drop in $pH_e$ to 6.5 caused a decrease in $pH_i$ to 6.85 within 10 min, which was sustained after 3 h of incubation (study II, Fig. 5C and Supplemental Fig. S5B). We subsequently blocked the major regulators of macrophage $pH_i$, the V-type $H^+$ ATPase and $Na^+/H^+$ exchangers (Swallow 1993). These ion pumps/exchangers extrude metabolic acid from the cells and their inhibition by bafilomycin A1 and 5(N-ethyl-N-isopropyl)amiloride results in gradual acidification of macrophage $pH_i$ (Bidani 1995, Baldini 2003). Exposure of human MDMs or THP-1 macrophages to these agents enhanced IL-1β secretion at $pH_e$ 6.5 and after prolonged exposure,

triggered IL-1β secretion at $pH_e$ 7.5 (study II, Fig. 5A and B, Supplemental Fig. S5A). These data suggested that a drop in $pH_i$ was indeed required for inflammasome activation at acidic environment.

Intriguingly, a marked drop in cytosolic pH is an early event in the intrinsic pathway of apoptosis (Gottlieb 1996, Matsuyama 2000). Thus, acidosis in the cytosol precedes cytochrome c release from the mitochondria and enhances the subsequent cytochrome c-mediated activation of apoptotic caspases in the cytoplasm. Cytochrome c triggers caspase activation via the apoptosome complex that exhibits marked similarity with the inflammasome complexes in structure, function, and regulation (Chai 2014, Gross 2011). Similar to potassium efflux, ROS, and lysosomal damage, acidification of the cytosol may thus be a conserved signaling event in the assembly of the apoptosis-associated and the inflammation-associated caspase-activating platforms (Gross 2011).

### 5.1.4 Intracellular stress signals mediate the activation of the NLRP3 inflammasome by cholesterol crystals and extracellular acidosis (I, II, unpublished data)

After defining the early steps involved in the exposure of macrophages to CHC or acidosis, we dissected the roles of the intracellular stress signals previously shown to mediate NLRP3 inflammasome activation (described in section 2.2.5). Thus, following phagocytosis of many crystalline/particulate NLRP3 activators, lysosomal destabilization and release of lysosomal enzymes, cathepsin B in particular, into the cytoplasm signals activation of the NLRP3 receptor. Similarly, enhanced production of mitochondrial ROS and potassium efflux from cells contribute to activation of the NLRP3 receptor, with ensuing assembly of the NLRP3 inflammasome.

#### *5.1.4.1 Lysosomal destabilization and cathepsin B*

Silica crystals and fibrillar amyloid-β trigger NLRP3 activation via lysosomal swelling, permeabilization, and release of cathepsin B into the cytoplasm (Hornung 2008, Halle 2008). We thus studied lysosomal integrity in THP-1 macrophages treated with CHC or pH 6.5 medium. Acridine orange stain is trapped into acidic organelles, where the low pH induces stacking of the dye molecules and a shift from green to red fluorescence (Traganos 1994). A typical red, punctate cytoplasmic staining corresponding to lysosomes was seen in untreated macrophages, and the red signal was markedly diminished in CHC-treated cells, but not in cells treated with acidic medium (study I, Fig. 5; study II, Supplemental Fig. S3A). Similarly, visualization of cellular cathepsin B activity with a fluorescent substrate showed loss of the punctate lysosomal pattern in CHC-treated cells (study I, Fig. 5). Instead, large swollen vesicle structures with active cathepsin B and an overall decrease in cellular cathepsin B activity were observed, implying release into the cytoplasm with

ensuing enzyme inactivation by neutral pH. Finally, CHC-induced IL-1β secretion was reduced by ~70 % in the presence of the cathepsin B inhibitor CA-074-Me (study I, Fig. 4). As CA-074-Me inhibits both cathepsins B and L, both proteases may be involved in the CHC-induced inflammasome activation (Montaser 2002). Notably, the expression and activity of cathepsins B and L is increased in the arterial wall macrophages during atherogenesis (Chen 2002, Liu 2006).

The normal lysosomal pH is ~4.8 and thus, it was not surprising that the mild extracellular acidosis lacked effect on these organelles. However, CA-074-Me weakly inhibited low pH-induced caspase-1 activation and IL-1β secretion (study II, Fig. 3C, H and I). Additionally, release of active cathepsin B by macrophages was enhanced in acidic environment (study II, Supplemental Fig. S3B). Conceivably, the lysosomal permeabilization could have been at a stage too early to detect after 3 h at pH 6.5, despite the clear IL-1β response (study II, Supplemental Fig. S3A). This would also imply that the lysosomal permeabilization was a relatively late, secondary effect in low pH-induced inflammasome activation. Notably, human MDMs constitutively secrete cathepsins, and increased cathepsin B activity in the acidic medium could also reflect the slower inactivation of these proteases at acidic pH (Punturieri 2000).

#### *5.1.4.2 Reactive oxygen species (ROS) formation*

The general ROS inhibitor N-acetyl-L-cysteine (NAC) markedly reduces IL-1β secretion in response to various NLRP3 activators (Dostert 2008, Hewinson 2008). NAC reacts directly with e.g. hydroxyl radical and hydrogen peroxide and is also a precursor for the antioxidant glutathione (Aruoma 1989). We tested a wide range of NAC concentrations and treatment modes both in human MDMs and THP-1 macrophages. However, NAC consistently lacked any effect on CHC- or low pH-induced IL-1β secretion in human MDMs and in THP-1 macrophages (study II, Fig. 3B, unpublished data). In agreement, extracellular and intracellular acidosis reduces superoxide levels in LPS- and PMA-treated macrophages (Bidani 1995, Swallow 1993). Interestingly, CHC-induced IL-1β secretion is partially blocked in NF-E2-related 2 (Nrf2)-deficient macrophages (Freigang 2011). Nrf2 is a potent mediator of antioxidant responses, but CHC-induced ROS production or other mechanistic aspects were not explored in the study. Thus, the involvement of ROS or ROS-detoxifying responses in CHC-induced inflammasome activation warrants further studies.

#### *5.1.4.3 Potassium efflux*

Potassium efflux is an essential prerequisite for the activation of NLRP3 inflammasome by diverse stimuli (Petrilli 2007, Munoz-Planillo 2013). We showed that the CHC- and low pH-induced IL-1β response was highly dependent on $K^+$ efflux, both in THP-1 macrophages (study I, Fig. 4; study II, Fig. 3D and H) and

in human MDMs (study II, Fig. 3F). This was demonstrated by adding 130 mM KCl to the culture medium, which balances the extracellular and intracellular $K^+$ concentrations and thereby abolishes the $K^+$ gradient. The release of active cathepsin B into the cytoplasm has been linked to $K^+$ efflux during NLRP3 inflammasome activation (Munoz-Planillo 2013), which may underlie also the CHC-induced $K^+$ efflux. Conversely, low pH is more likely to modulate the activity of cell membrane ion channels. Notably, pH modulates the activity of the ATP-gated ion channel $P2X_7$ that is known to trigger activation of the NLRP3 inflammasome (Liu 2009). However, acidic extracellular pH strongly inhibits the $P2X_7$ receptor-mediated ion currents, rather than enhances them. In agreement, preincubation of macrophages at neutral pH with the $P2X_7$ receptor antagonists oxidized ATP (Murgia 1993) or KN-62 (Baraldi 2003) did not block the low pH-induced IL-1β secretion (study II, Fig. S4A and B). Thus, we explored other candidate $K^+$-conducting channels.

PMA-differentiated THP-1 macrophages express at least two types of outward $K^+$ currents: small conductance 'SK' and large conductance 'maxi-K' type $Ca^{2+}$-activated $K^+$ channels (DeCoursey 1996, Kim 1996). Both SK and maxi-K currents are blocked by tetraethylammonium, and SK channels are additionally blocked by $Cs^+$ and $Ba^{2+}$. We found significant inhibition of low pH-mediated IL-1β secretion by each of these three nonselective $K^+$ channel blockers in THP-1 macrophages, whereas 4-aminopyridine showed no effect (study II, Fig. 3G). In agreement with our results, THP-1 monocytes express a 4-aminopyridine-sensitive delayed rectifier $K^+$ current that is lost upon PMA-differentiation of THP-1 macrophages (DeCoursey 1996). As the activation of the NLRP3 inflammasome involves intracellular $Ca^{2+}$ signaling (Elliott 2015), the $Ca^{2+}$-sensitive $K^+$ channels mediating the SK or the Maxi-K type currents in THP-1 macrophages could indeed be involved in low pH-induced NLRP3 activation. Earlier studies found no effect by the maxi-K channel blocker paxilline on NLRP3-mediated caspase-1 activation (Petrilli 2007). However, the SK channels remain unexplored in this context.

#### *5.1.4.4 Summary of NLRP3 activation mechanisms by cholesterol crystals and low pH*

Taken together, we identified two novel atherosclerosis-associated danger signals that trigger a strong inflammatory response via activating the NLRP3 inflammasome in human macrophages (**Fig. 9**). The proatherogenic effects of IL-1β and -18 have been widely documented (see section 2.3.3), yet the factors triggering their secretion in the plaques remained unknown. CHC are a danger signal directly arising from the defective lipid metabolism in the arterial wall and crystallization of cholesterol may thus link pathological lipid accumulation to chronic inflammatory responses. Phagocytosis of CHC, lysosomal permeabilization, release of cathepsin B to cytoplasm, and $K^+$ efflux were all essential for CHC-induced IL-1β secretion

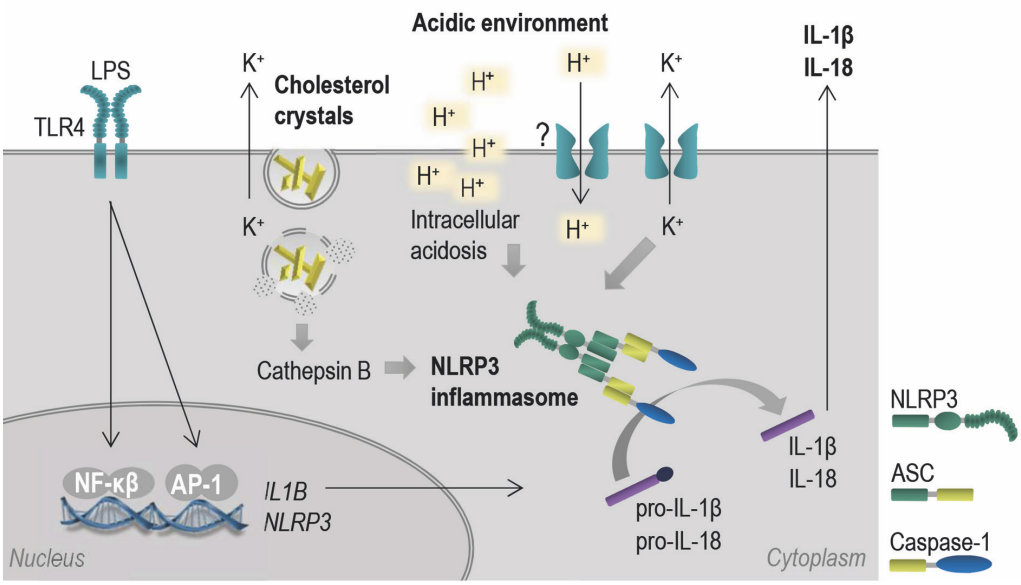


**Figure 9. Summary of NLRP3 activation mechanisms by cholesterol crystals and acidic environment.** See the text for details. AP-1, activator protein 1; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; LPS, lipopolysaccharide; TLR, Toll-like receptor.

(study I). While we were finalizing the manuscript, a group led by Prof. Eicke Latz published a study describing a highly similar mechanism of CHC-induced NLRP3 inflammasome activation in mouse macrophages (Duewell 2010). Thus, CHC caused marked lysosomal damage and the CHC-induced IL-1β secretion was markedly reduced in cathepsin B or L-deficient cells, and abolished in NLRP3- and ASC-deficient cells. As described in chapter 5.2, this highly influential work also explored the effects of knocking out NLRP3 inflammasome components in mouse models of atherosclerosis.

Conversely, extracellular acidosis is a danger signal related to hypoxia and the chronic activation of macrophages and other immune cells in the arterial wall, causing a build-up of metabolic acid similar to other inflammatory sites (Öörni 2015). Acidic environment caused a drop in the intracellular pH of macrophages, which was sufficient to trigger the inflammasome-mediated cytokine response (study II). Moreover, $K^+$ efflux was crucial for low pH-induced NLRP3 activation. It seems conceivable that the efflux of $K^+$ is triggered to compensate for the electrogenic influx of $H^+$ at acidic environment, yet this remains to be proven. Our data suggests that the $K^+$ efflux is not mediated by nonspecific loss of membrane integrity, but, instead, via opening of as yet unidentified $K^+$ channels at the plasma membrane.

## 5.2 EXPRESSION OF THE INFLAMMASOME PATHWAY IN HUMAN ARTERIAL WALL (III)

The first study to explore the effects of knocking out NLRP3 inflammasome components in atherogenesis provided persuasive evidence suggesting a proatherogenic role for this pathway (Duewell 2010). LDLR-KO mice were reconstituted with NLRP3- or ASC-KO bone marrow, or with IL-1α/β-dKO bone marrow, and fed a HFD for 8 weeks. Lesion area at the aortic sinus was in each case reduced by ~70 % compared to mice receiving wild type bone marrow. Serum levels of IL-18 were also significantly decreased. In a study published a year later, apoE-KO mice were crossed with NLRP3-, ASC-, or caspase-1-KO mice and no alterations in plaque size or composition were observed after 11 weeks of HFD (Menu 2011). Two further studies utilizing apoE/caspase-1-dKO mice and LDLR-KO mice reconstituted with caspase-1-KO bone marrow showed decreased atherosclerotic lesion area and reduced IL-1β locally in the plaques and in plasma (Usui 2012, Hendrikx 2015). The underlying causes for these contrasting results remain unclear, but differences in the HFD compositions may have played a role. Thus, the three studies showing attenuation of atherosclerosis featured 0.15-0.20 % cholesterol in the HFD (Duewell 2010, Usui 2012, Hendrikx 2015), while the report showing no changes featured an 8-fold higher concentration (1.25 %) (Menu 2011). The apoE-KO mice used in the latter study are also inherently more hypercholesterolemic than LDLR-KO mice (Ishibashi 1993, Zhang 1992). Thus, an overwhelmingly high plasma cholesterol level driving rapid and massive lipid accumulation could have masked any inflammation-associated effects.

Meanwhile, data on the role of the inflammasome pathway in human atherosclerosis remains scarce. In stark contrast to the rapid lesion development in mice, human lesions develop over decades and the advanced plaques acquire much more complex and varied morphologies. This prompted us to study the expression of inflammasome pathway components and regulators in the atherosclerotic human arterial wall.

### 5.2.1 Pathway-focused expression analysis of the inflammasome pathway in human coronary arteries (III)

To analyse inflammasome pathway expression in human arterial wall, we utilized a human coronary artery specimen collection established earlier at the Wihuri Research Institute. RNA was isolated from frozen coronary artery specimens obtained from 10 males, age 49-65 years, exhibiting marked coronary atherosclerosis (sample details in study III, Supplemental Table 1). The specimens were collected either during heart transplantation surgery or from candidate heart transplants that did not fulfil the criteria for clinical use. Two cross-sectional coronary segments were retrieved from each patient for RNA isolation (total n=20): a control sample from an early,

clinically silent lesion (AHA type I-III; see section 2.3.2) and a sample from an advanced atherosclerotic plaque (AHA type IV-VI) (Stary 1994, Stary 1995). All arterial wall layers were included in the samples. The setup allowed the comparison of early and advanced disease status in a single patient, i.e. the samples had an identical genetic background and had been exposed to the same local tissue environment and risk factors of atherosclerosis. The expression analysis of the human coronary RNA samples was carried out using a commercial, thoroughly validated quantitative PCR (qPCR) array targeting 88 inflammasome pathway-related genes. The target genes included the core components and regulators of the inflammasome complexes, as well as other NLR family proteins, IL-1 family cytokines, and molecules related to proinflammatory cytokine expression and signaling (full listing in study III, Supplemental Table 2).

The results of the pathway-focused qPCR array analysis are shown in Table 1 of Study III. The significant changes in gene expression are shown as fold changes of expression in the advanced coronary plaques (AHA IV-VI) compared to the early lesions (AHA I-III). Twelve target genes were significantly upregulated in the pair-matched comparison of the samples, whereas no genes were significantly downregulated. The fold changes were moderate, which was expected due to the experimental setup. Indeed, at advanced stages of atherosclerosis, inflammatory cell infiltrates are highly prevalent in plaques throughout the coronary tree (Mauriello 2005). Thus, also the areas free of gross plaques are, to varying degrees, exposed to an inflammatory microenvironment. Nevertheless, the stringent comparison to an internal control area from the same coronary tree eliminated several confounding factors related to inter-person comparisons. These include differences in age (truly atherosclerosis-free coronary artery specimens can be retrieved only from young children, whereas advanced plaques develop in adults), genetic background, lifestyle, medication, and in details of the coronary sample collection procedure.

Among the target genes significantly upregulated in advanced coronary plaques, the most consistent changes were found in the expression levels of caspase-1, IL-18, p38δ MAPK, baculoviral IAP repeat containing 3 (BIRC3), and IL-1Ra (study III, Table 1). Furthermore, the universal inflammasome adapter ASC was upregulated, and, together with caspase-1 and IL-18, formed a prominent signature of upregulated inflammasome pathway core components in the advanced coronary lesions. The increased expression of IL-1Ra in advanced plaques compared to early lesions further implied ongoing IL-1 signaling in the arterial wall. Moreover, p38 MAP kinases are intimately involved in pro-IL-1β synthesis and in IL-1β and IL-18 signaling (Arthur 2013, Lee 2004). Finally, the ubiquitin ligase BIRC3 was recently linked with regulation of inflammasome activity (Labbe 2011, Vince 2012).

The inflammasome-forming AIM2 dsDNA sensor was likewise significantly upregulated in advanced coronary plaques compared to early lesions (study III, Table 1), in agreement with a recent immunohistochemical study in carotid plaques (Hakimi 2014). AIM2 expression is potently induced by interferon-γ, a proatherogenic Th1 cytokine found in atherosclerotic lesions (DeYoung 1997, Frostegard 1999, Gupta 1997). Moreover, bacterial DNA has been demonstrated in human coronary lesions and could serve as an AIM2 activator (Lehtiniemi 2005, Ott 2006) alongside genomic DNA released from dying cells. In addition to the PYHIN family member AIM2, the NLR family proteins known to assemble inflammasomes (NLRP1, NLRP3, NLRP6, NLRC4 (Strowig 2012)) were all expressed in the coronary artery wall, and of these, NLRP6 was significantly upregulated in advanced coronary plaques (study III, Supplemental Fig. 1C and Table 1). Also the class II major histocompatibility group transactivator (CIITA/NLRA), essential for expression class II HLA molecules and thus crucial for antigen presentation (Steimle 1993, Steimle 1994), was upregulated in advanced plaques. Finally, we found upregulation of cathepsin B involved in NLRP3 activation, in agreement with upregulation and activation of cathepsin B in advanced aortic lesions of mice (Chen 2002).

All IL-1 family cytokines were expressed at detectable levels (IL-1α only barely) in the coronary lesions, in the order of increasing expression IL-1α < IL-1β < IL-1Ra < IL-18 < IL-33 (study III, Supplemental Fig. 1A). Moreover, among the caspases targeted by the qPCR array (caspases 1, 5, and 8), caspase-1 was the most abundantly expressed (study III, Supplemental Fig. 1B). Among the NLR and PYHIN family target genes, NAIP, a ligand sensor for the NLRC4 inflammasome (Yang 2013), was by far the most highly expressed, matched by the ASC adapter protein (study III, Supplemental Fig. 1C). NLRC4, NLRC5, NLRP1, NLRP3, and NOD1 were expressed at an intermediate level; AIM2, CIITA, NLRP6, NLRP9, and NLRX1 were expressed at a low level; finally, the expression of NLRP4, NLRP5, NLRP12, and NOD2 was virtually undetectable.

Taken together, we have defined here the set of inflammasome pathway components expressed in human coronary artery wall. Furthermore, we showed upregulation of several core components of the inflammasome pathway, including ASC, caspase-1, and IL-18, in advanced coronary plaques compared to early lesions from the same coronary trees, implying increased activity of the pathway in progression of atherosclerosis.

### 5.2.2 Immunohistochemical detection of the NLRP3 inflammasome in human coronary arteries (III)

As the NLRP3 inflammasome had been implicated in atherosclerotic lesion development in mouse models, we further studied the expression of the NLRP3 inflammasome in human atherosclerotic lesions by immunohistochemistry. Similar to the setup used in the qPCR array analysis, we compared areas with early coronary

lesions to advanced coronary plaques in the same set of 10 male subjects. Formalin-fixed paraffin-embedded coronary specimens, rather than frozen specimens, were utilized owing to their superior morphological features. Adjacent sections were stained with antibodies against the NLRP3 receptor, the ASC adapter, the caspase-1 protease, and the macrophage marker CD163.

Representative results of the immunohistochemical stainings are presented in Fig. 1A-C of Study III. Negative control stainings with corresponding nonimmune immunoglobulin preparations showed no staining in the coronary arteries or appendix (study III, Supplemental Fig. 2A-B). The early coronary lesions exhibited scattered groups of intimal, as well as abundant adventitial, $CD163^+$ macrophages (study III, Fig. 1A). Occasional $ASC^+$ and caspase-$1^+$ cells with macrophage morphology were found in the intima and the adventitia of the early lesions, but $NLRP3^+$ cells were extremely rare. In advanced coronary plaques, abundant intimal $CD163^+$ macrophage foam cells were found around the lipid cores of the lesions (study III, Fig. 1B and C). In addition, extracellular CD163 staining was frequent within and around the acellular lipid core, reflecting macrophage cell death. $NLRP3^+$, $ASC^+$, and caspase-$1^+$ foam cells were found around the lipid core, with a staining pattern closely resembling that of CD163. Strikingly, also extracellular NLRP3 staining was found within and around the lipid core, closely resembling the extracellular staining pattern of CD163 in adjacent sections (study III, Fig. 1B and C, Supplemental Fig. 2C). The strongest staining for the NLRP3 receptor was observed within large lipid cores exhibiting abundant cholesterol crystals and macrophages, whereas NLRP3 was absent in fibrocalcific plaques with less pronounced macrophage infiltration (study III, Supplemental Fig. 2C and D). Thus, the expression of the NLRP3 receptor was associated with an inflammatory plaque phenotype and with the presence of cholesterol crystals, an established activator of the NLRP3 inflammasome.

Similar to our results, other studies have found caspase-$1^+$ macrophages near the lipid cores of human atherosclerotic plaques, where they colocalized with apoptotic and glycolytic markers, as well as with IL-1β (Geng 1995, Folco 2014). Of note, enhanced glycolysis at the sites of caspase-1 expression implies metabolic acid production and exposure of the macrophages to fluctuations in pH. Interestingly, the expression pattern of the AIM2 receptor in human carotid arteries (Hakimi 2014) markedly differs from that of NLRP3, ASC, and caspase-1 in human coronary arteries observed in Study III. Thus, AIM2 is strongly expressed in healthy carotid arteries, in the endothelial cells of luminal endothelium and vasa vasorum, as well as in vascular smooth muscle cells. Moreover, in carotid plaques, both smooth muscle cells and macrophages surrounding the plaque lipid cores express AIM2 (Hakimi 2014). The strong expression of AIM2 in non-immune cells of the healthy arterial wall implies functions divergent from the NLRP3 receptor that was exclusively expressed in macrophages and only in advanced plaques with a marked lipid core.

### 5.2.3 p38δ MAPK – a novel effector in NLRP3 inflammasome activation (III)

The p38δ MAPK was among the qPCR array targets most consistently upregulated in advanced human coronary plaques compared to early lesions (study III, Table1). Moreover, p38δ was the only MAPK upregulated in the panel of 8 MAPKs in the array, which also included p38β and p38γ MAPKs (study III, Supplemental Table 2).

#### *5.2.3.1 The functions of p38δ MAPK in inflammation remain largely unknown*

The p38 MAPKs are serine/threonine protein kinases that are activated by upstream MAPK kinases 3, 4, and 6 via dual phosphorylation of conserved threonine and tyrosine residues (Cuadrado 2010). The p38 MAPK family plays an important role in TLR-mediated proinflammatory cytokine expression, including pro-IL-1β synthesis, as well as in signaling elicited by the IL-1 and IL-18 receptors (Arthur 2013, Lee 2004). Thus, the p38 MAPKs contribute to inflammasome priming and to downstream signaling by the inflammasome target cytokines. However, the role of p38 MAPKs in inflammation and in proinflammatory cytokine expression has been mainly attributed to the ubiquitous p38α isoform (Arthur 2013, Cuadrado 2010). The other p38 isoforms (β, γ, and δ) have more restricted expression patterns, implying specialized functions (Arthur 2013, Cuadrado 2010). Very few studies have explored the role of p38δ MAPK in the context of inflammation. However, the p38δ MAPK is expressed in various primary human immune cells, with abundant expression in human macrophages (Hale 1999, Smith 2006). Importantly, it was recently shown that p38γ and p38δ MAPKs contribute to LPS-induced endotoxic shock and to collagen-induced arthritis *in vivo* in mice, suggesting that these p38 isoforms have significant roles in inflammation (Risco 2012, Criado 2014).

In addition to TLR ligands and proinflammatory cytokines, the p38 MAPKs are activated by various physicochemical stressors, such as UV irradiation and hyperosmolar shock (Cuadrado 2010). Conceivably, the p38 MAPKs could be activated also by the intracellular stress signals associated with NLRP3 inflammasome activation. In line with this reasoning, the prototypical NLRP3 activator ATP was reported to trigger p38 MAPK activation, though the isoform(s) were not identified (Donnelly-Roberts 2004, Noguchi 2008). We thus tested the hypothesis that p38 MAPKs, and specifically the p38δ MAPK, could be directly involved in triggering NLRP3 inflammasome assembly.

#### *5.2.3.2 The p38δ MAPK is activated in macrophages by NLRP3 activators*

We stained p38δ MAPK in sections of human coronary artery plaques and found strong expression in intimal macrophage foam cells, as well as extracellular staining in the lipid core (study III, Fig. 3A and Supplemental Fig. 4A). The staining pattern

closely resembled that of the macrophage marker CD163 and the NLRP3 receptor in adjacent sections (study III, Fig. 1A and C). We subsequently studied p38 MAPK activation by Western blotting in cultured primary human MDMs stimulated with 5 mM ATP to activate the NLRP3 inflammasome. Extracellular ATP triggered a rapid, transient phosphorylation of p38 MAPKs at 1 min of stimulation, both in unprimed and LPS-primed MDMs (study III, Fig. 3B). Thus, the induction of p38 MAPK activation was independent of cytokine signaling, and it immediately preceded the appearance of mature IL-1β in the culture medium of LPS-primed cells (study III, Fig. 3C).

We next explored the specific p38 isoforms activated during ATP stimulation. The p38α and p38δ were the major p38 MAPK isoforms expressed in human MDMs (study III, Supplemental Fig. 4B). This is in agreement with earlier studies showing abundant p38α and p38δ, but no p38β and p38γ at the protein level in human MDMs (Hale 1999, Smith 2006). Thus, we detected the dually phosphorylated (Thr180/Tyr182), active forms of p38α and p38δ MAPKs (p-p38α and p-p38δ) from cell lysates by specific ELISAs. ATP triggered the activation of p38δ, but not p38α, peaking at 1 min of stimulation (study III, Fig. 3D and Supplemental Fig. 4C). The ATP-induced p38δ activation was dependent on the $P2X_7$ receptor that mediates the ATP-triggered activation of the NLRP3 inflammasome (study III, Fig. 5D). Similarly, the ATP-induced phosphorylation of total p38 MAPKs is $P2X_7$-dependent (Donnelly-Roberts 2004). Importantly, also cholesterol crystals (CHC) triggered activation of p38δ, but not p38α, in unprimed and LPS-primed human MDMs (study III, Fig. 4A and B; Supplemental Fig. 5A and B). The p38δ activation by CHC displayed slower kinetics that closely paralleled IL-1β secretion in LPS-primed cells. Thus, two structurally and functionally very different NLRP3 activators shared the capability to activate the p38δ MAPK. Moreover, ATP and CHC induced peak levels of p-p38δ that were highly similar in magnitude to those induced by the canonical p38 MAPK activators, LPS and IL-1β (study III, Fig. 4C).

Unexpectedly, we could not detect activation of p38α by LPS or IL-1β in human MDMs (study III, Fig. 4C), despite their well-established role in triggering p38α activation in various cell types (Arthur 2013, Cuadrado 2010). In contrast, an earlier report in human MDMs showed that LPS induces the activation of both p38α and p38δ, resulting in similar enhancements in the kinase activity of both isoforms in an ATF2 phosphorylation assay (Hale 1999). Despite the differing culture protocols, the relative expression patterns of the four p38 MAPK isoforms in human MDMs were highly consistent between the studies (study III, Supplemental Fig. 4B) (Hale 1999). However, Hale *et al.* unexpectedly found that majority of the LPS-activated p-p38δ was in Tyr182-monophosphorylated form (Hale 1999). Given that in Study III, we used ELISAs that detect only the conventional Thr180-Tyr182 dually phosphorylated p38α and p38δ, it is possible that LPS triggered some p38α activation

via monophosphorylation. However, in the study by Hale *et al.*, the antibodies against mono-phospho-p38 and dual-phospho-p38 were raised against a phospho-peptide from aa 171-186 of p38α (Hale 1999). Despite the conserved phosphorylation sites, the corresponding peptide sequence in p38δ differs by 3 amino acids (NCBI Protein BLAST sequence alignment p38δ NP_002745.1 vs full-length isoforms 1 and 2 of p38α NP_001306.1 and NP_620581.1). Thus, the failure of the dual-phospho-p38α antibody to recognize the LPS-activated p38δ could have been due to poor affinity of the antibody for dual-phospho-p38δ, rather than due to true monophosphorylation (Hale 1999). To conclude, further studies are needed to account for the variable p38α activation by LPS observed in these two studies.

#### *5.2.3.3 Activation of p38δ MAPK is required for NLRP3-mediated IL-1β secretion*

The available inhibitors of p38 MAPKs are restricted to a group of pyridinyl imidazole compounds that block p38α/β, and the newer, chemically distinct compound BIRB-796 that blocks all four p38 isoforms (Bain 2007). We chose to use the pyridinyl imidazole SB203580 (SB) and BIRB-796 (BIRB), both highly specific for their p38 MAPK targets among 80 screened kinases (Bain 2007). By using these inhibitors in parallel, we were able to distinguish the contribution of the two major p38 isoforms, p38α and p38δ, in human MDMs. Thus, the p38α/β inhibitor SB will target p38α, whereas the p38α/β/γ/δ inhibitor BIRB will target both p38α and p38δ.

We showed that BIRB, but not SB, dose-dependently inhibited ATP-induced secretion of mature IL-1β from LPS-primed human MDMs (study III, Fig. 5A). The inhibitors were added after LPS priming to allow unhindered synthesis and accumulation of pro-IL-1β. Moreover, the p38 MAPKs regulate cytokine transcription and translation, but not protein stability (Lee 1994, Baldassare 1999). Accordingly, BIRB did not affect intracellular pro-IL-1β protein levels (study III, Fig. 5B). The Western blots also indicated that ATP-induced secretion of pro-IL-1β was unaffected by BIRB, suggesting that the IL-1β secretion machinery remains intact (study III, Fig. 5B and Supplemental Fig. 6A). Furthermore, both BIRB and SB were functional in human MDMs, as confirmed by inhibition of LPS-induced TNF-α secretion (study III, Fig. 5C). As we could not detect p38α activation by LPS in human MDMs, the inhibition of TNF-α secretion by SB could be mediated by p38β. Although much less expressed compared to p38α and p38δ, the p38β mRNA showed a 2-fold induction by LPS priming uniquely among the p38 isoforms (study III, Supplemental Fig. 4B). In agreement with our data, also earlier studies failed to show inhibition of ATP-induced IL-1β secretion by p38α/β MAPK inhibitors (Donnelly-Roberts 2004, Michel 2006).

We further confirmed that BIRB, but not SB, completely inhibited the ATP-induced p38δ MAPK activation (study III, Fig. 5D). Moreover, BIRB did not affect the expression of NLRP3 receptor or ASC (study III, Supplemental Fig. 6C and

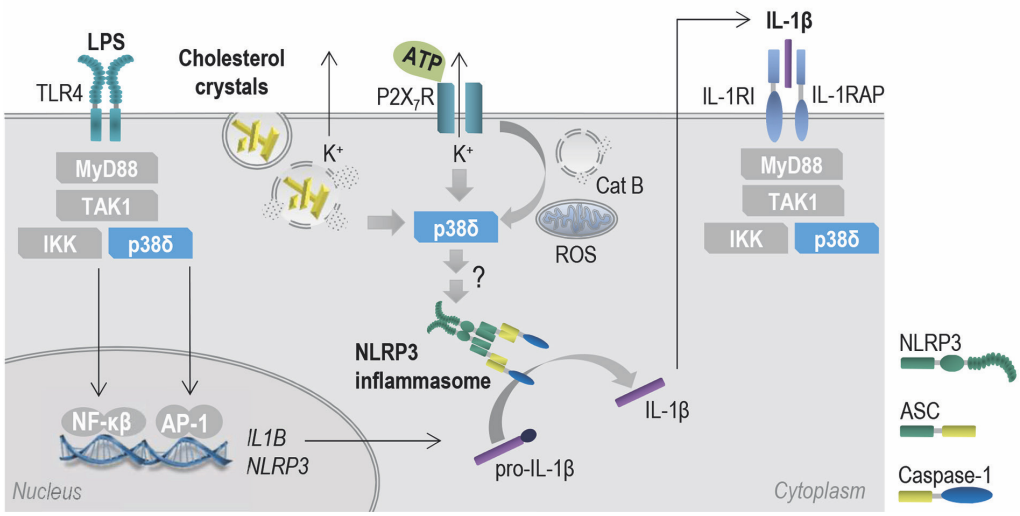


**Figure 10. The p38δ *MAPK contributes to several steps in activation of the NLRP3 inflammasome in primary human macrophages.*** See the details in text. AP-1, activator protein 1; CatB, cathepsin B; IL-1RI, IL-1 receptor I; IL-1RAP, IL-1 receptor accessory protein; IKK, Inhibitor of κB kinase; LPS, lipopolysaccharide; ROS, reactive oxygen species; MyD88, myeloid differentiation primary response 88; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; $P2X_7R$, $P2X_7$ receptor; TAK1, transforming growth factor β -activated kinase; TLR, Toll-like receptor.

D). These data strongly suggested the involvement of p38δ MAPK in activation of the NLRP3 inflammasome. To elucidate the activation mechanism of p38δ MAPK during NLRP3 inflammasome activation, we blocked the intracellular stress signals required for NLRP3 activation. High extracellular potassium concentration, the general ROS inhibitor NAC, and the cathepsin B inhibitor CA-074-Me all markedly attenuated the peak p-p38δ MAPK levels after 1 min ATP stimulation (study III, Fig. 6A; Supplemental Fig. 7A-D). Similarly, inhibition of potassium efflux blocked cholesterol crystal-induced p38δ MAPK activation (study III, Fig. 6B).

Taken together, the p38δ MAPK is activated by both receptor- and phagocytosis-dependent activators of the NLRP3 inflammasome and may integrate several intracellular stress signals to trigger NLRP3 inflammasome activation in human MDMs. The p38δ MAPK was also activated during inflammasome priming and IL-1β signaling in human MDMs. These data suggest the involvement of p38δ MAPK during several steps of NLRP3 inflammasome activation, revealing a novel proinflammatory role for this poorly characterized p38 isoform (**Fig. 10**). Importantly, the p38δ MAPK and NLRP3 inflammasome components are abundantly expressed by macrophage foam cells surrounding the lipid cores in human coronary atherosclerotic

plaques. These macrophages are in close contact with cholesterol crystals and with dying macrophages/SMCs in the lipid core, and thus frequently exposed also to extracellular ATP and other danger signals released upon cell death. Thus, the p38δ MAPK-NLRP3 inflammasome signaling elicited by CHC and ATP is a relevant candidate pathway as a mediator of sterile inflammation in atherosclerotic lesions. Finally, these findings contribute new insight into the elusive activation mechanism of the NLRP3 inflammasome. Intriguingly, phosphorylation of ASC via a pathway involving spleen tyrosine kinase and c-Jun N-terminal kinases is required for ASC speck formation during NLRP3 inflammasome activation (Hara 2013), and the activation of the NLRC4 inflammasome is likewise regulated by phosphorylation of the NLRC4 receptor (Qu 2012). Thus, regulation by kinase signaling is emerging as a novel theme in inflammasome activation.

## 5.3 THE INTERPLAY OF PRIMING AND INFLAMMASOME ACTIVATION IN ATHEROSCLEROTIC LESIONS (II, III, UNPUBLISHED DATA)

It was recently shown that oxLDL primes the transcription of NLRP3 receptor and pro-IL-1β in mouse macrophages via the CD36:TLR4:TLR6 signaling complex (Sheedy 2013). Importantly, hypercholesterolemia in apoE-KO mice upregulates the NLRP3 receptor and pro-IL-1β in peritoneal macrophages and aortic wall also *in vivo* via the same complex. Moreover, oxLDL-induced crystallization of cholesterol in lysosomes triggers the NLRP3 inflammasome, resulting in a low level IL-1β response in unprimed mouse macrophages (Duewell 2010, Sheedy 2013). Indeed, crystallization of cholesterol, caspase-1 activation, and IL-1β immunoreactivity are markedly attenuated in lesions of CD36/apoE-dKO mice, accompanied by reduced lesion size (Sheedy 2013). Thus, a picture is emerging where LDL oxidation provides the priming signal, and both intracellular and extracellular CHC can subsequently trigger the NLRP3 inflammasome (**Fig. 11**). However, we could not detect any secretion of IL-1β by unprimed human MDMs during a prolonged 24-48 h incubation with a high concentration (200 μg/ml) of oxLDL (unpublished data). Thus, differences may exist in the responsiveness of mouse and human macrophages to oxLDL in the context of inflammasome priming and activation. Interestingly, recent studies at our research group (Satu Lehti and Katariina Öörni, unpublished results) and by our collaborators (Estruch 2015) suggest that primary human monocytes and macrophages respond more strongly to other types of LDL modification. In addition, atherosclerotic lesions harbour several microbial and endogenous TLR ligands that may contribute to the inflammasome priming step (Zimmer 2015).

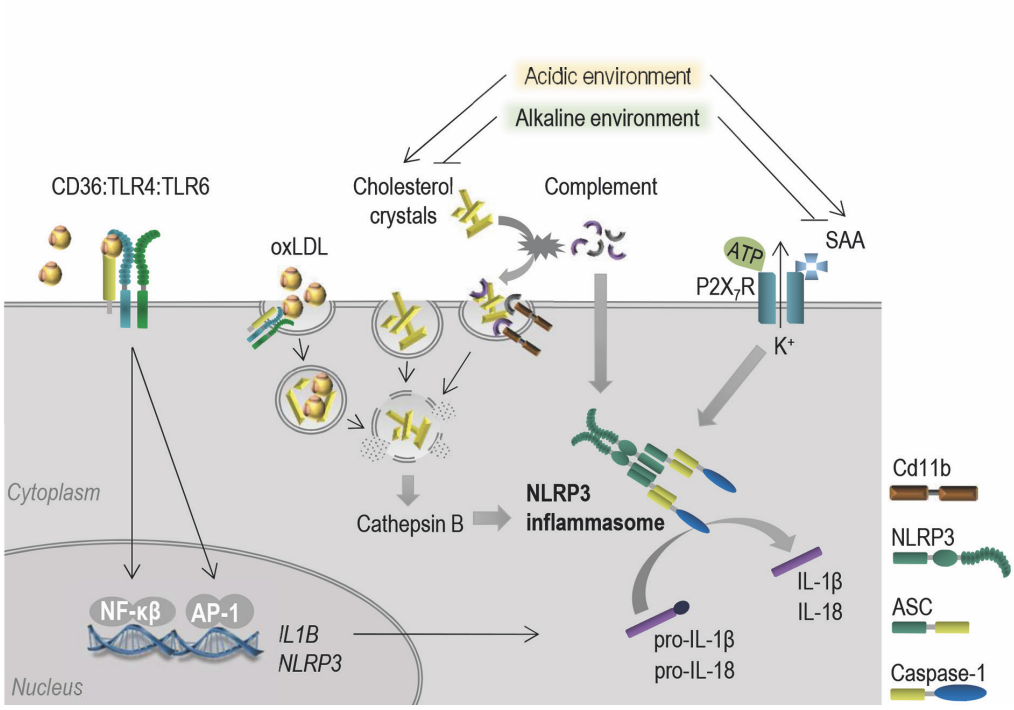


**Figure 11. The interplay between priming and activation of the NLRP3 inflammasome in atherosclerotic plaques.** See the text for details. AP-1, activator protein 1; CD, cluster of differentiation; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; oxLDL, oxidized low density lipoprotein; $P2X_7R$, $P2X_7$ receptor; SAA, serum amyloid A; TLR, Toll-like receptor.

Cholesterol crystals (CHC) and oxLDL are the prominent, lipid-derived danger signals in atherosclerotic plaques. They coexist in the lesions with several other activators of the NLRP3 inflammasome, including extracellular acidosis (study II), ATP (Mariathasan 2006), the acute phase protein serum amyloid A (SAA) (Niemi 2011), activated complement (Laudisi 2013), and certain components released from the extracellular matrix (Babelova 2009, Yamasaki 2009). Various modes of interaction can be envisioned between these dangers signals, yet the area remains largely unexplored. Samstad *et al.* reported complement activation by CHC in whole blood (Samstad 2014). The subsequent CHC opsonisation by complement augments CHC phagocytosis and inflammasome activation in human monocytes, revealing a bidirectional interaction between these signals. We explored the effects of acidic environment on NLRP3 inflammasome activation by CHC and SAA. The mildly acidic environment of pH 7.0 triggered a modest IL-1β response in THP-1 macrophages (study II, Fig. 1B and D). However, when we treated the cells with CHC and SAA at pH 7.0, we found significant synergy between these signals, as indicated by the amplified IL-1β response (study II, Fig. 6A and B). Thus, local acidosis may

substantially potentiate NLRP3 inflammasome activation by other danger signals by creating a sensitizing microenvironment for macrophages (**Fig. 11**).

Conversely, antagonism may also occur between the NLRP3-activating danger signals. Acidosis likely attenuates ATP-induced inflammasome activation, owing to the severe inhibition of ATP-induced $P2X_7$ receptor ion currents at low pH (Liu 2009). However, SAA triggers NLRP3 activation via the $P2X_7$ receptor at neutral pH (Niemi 2011), which is at odds with the observed synergy demonstrated between low pH and SAA in triggering IL-1β secretion (study II, Fig. 6B). The exact mode of interaction between SAA and the $P2X_7$ receptor is not known and it is possible that this interaction is pH-independent. Moreover, SAA interacts with several receptors, including the SR CD36 that mediates cellular uptake of SAA (Baranova 2010). CD36 acts as a receptor also for oxLDL and amyloid-β, and is involved in the transformation of these ligands in lysosomes into NLRP3-activating crystals and fibrils, respectively (Sheedy 2013). Similarly, SAA is transformed into pathological fibrillar aggregates upon endocytosis by human monocytes (Magy 2007). Thus, hypothetically, SAA could activate the NLRP3 inflammasome via an alternative CD36-mediated pathway at low pH.

During our studies on acidic pH, we became likewise interested in the effects of alkaline pH. Unexpectedly, the deviation from physiological pH towards more alkaline values did not trigger NLRP3 inflammasome activation, but rather blocked it (study II, Fig. 7). The IL-1β response to monosodium urate crystals, SAA, and CHC was sharply reduced at pH 8.0-8.5, especially in THP-1 macrophages (study II, Fig. 7A-C and E). The interaction of SAA with TLRs was unaffected by alkaline environment (study II, Fig. 7D). Similarly, phagocytosis of CHC was not blocked at pH 8.0 and the expression of NLRP3, ASC, and pro-IL-1β remained unchanged in alkaline environment (study II, Fig. 7F and Supplemental Fig. S6C). Interestingly, the lipid-rich areas of human carotid plaques tend to be acidified, whereas extensively calcified areas exhibit marked alkalinisation (Naghavi 2002). This type of macrocalcification stabilizes atherosclerotic plaques by reducing inflammation, and inhibition of NLRP3 inflammasome activity by alkaline calcium salts could conceivably contribute to this process (Naghavi 2002, Pugliese 2015). In line with this reasoning, NLRP3 immunoreactivity was absent in fibrocalcific lesions with low numbers of macrophages and large calcific cores (study III, Supplemental Fig. 2D). On the other hand, microcalcification is associated with inflamed plaques, implying differing roles for calcification depending on the calcification mechanism (Pugliese 2015).

The activation of the NLRP3 inflammasome may shape the local immune response in the plaques via several mechanisms. We found significant upregulation of CIITA, a regulatory protein essential for class II HLA molecule expression, in advanced

coronary plaques compared to early lesions (study III, Table 1) (Steimle 1993, Steimle 1994). As CIITA is an NLR family protein, we became interested in the possible connection between antigen presentation and NLRP3 inflammasome activation. We found strong downregulation of CIITA mRNA by CHC and low pH in human MDMs, independently of the inflammasome-mediated cytokine response (study III, Fig. 2A and B). This was reflected in significant downregulation of HLA-DRA and HLA-DRB expression by CHC (study III, Fig. 2C and D). Interestingly, CHC could suppress CIITA via p38 MAPK activation, as p38 MAPK contributed to the inhibition of IFN-γ-inducible class II HLA expression by LPS (Yao 2006). Surprisingly, CHC and low pH downregulated also NLRP3 receptor mRNA in unprimed, but not in LPS-primed MDMs (study III, Supplemental Fig. 3A). These observations highlight the strict regulation of NLRP3-mediated sterile inflammatory responses in macrophages. Firstly, endogenous activators of NLRP3 suppress receptor expression in the absence of an appropriate priming stimulus. Secondly, when both priming and inflammasome activation occur, the coupling of this innate immune response to adaptive immunity may be weakened due to attenuated expression of CIITA and HLA class II antigen-presenting molecules.

# 6 CONCLUSIONS & FUTURE PERSPECTIVES

This thesis focused on studying the mechanisms that drive inflammation during atherogenesis. Despite the well-established role of inflammation in atherosclerotic plaque development, there are currently no treatment strategies for atherosclerosis and cardiovascular disease (CVD) that would directly target the inflammatory process. Nevertheless, statins have both LDL-lowering and anti-inflammatory effects, although the existence of direct anti-inflammatory effects remains debated (Bonetti 2003, Shaw 2009). A large prospective cohort study demonstrated that reduction of high-sensitivity C-reactive protein (hsCRP) levels by rosuvastatin treatment significantly reduced cardiovascular event rates even in apparently healthy people with low LDL cholesterol (Ridker 2009). This landmark study provided persuasive evidence supporting a key role for inflammation in the pathogenesis of atherosclerosis, independent of LDL cholesterol levels. Furthermore, the study sparked a wide interest towards finding a more targeted anti-inflammatory therapy for the treatment of atherosclerosis and CVD (Ridker 2014).

In order to develop novel anti-inflammatory treatment strategies, a detailed mechanistic understanding of the immunopathogenesis of atherosclerosis is vital (Libby 2015, Libby 2011). Innate immunity has an essential role in atherogenesis through the intimate involvement of macrophages both in lipid accumulation and inflammation in the arterial wall. This thesis explored a major innate immune signaling pathway, the inflammasome, in the context of atherosclerosis. We characterized two novel atherosclerosis-associated activators of the inflammasome pathway, cholesterol crystals and local extracellular acidosis, that elicited a strong inflammatory response in cultured human macrophages (studies I and II). These endogenous danger signals activated the NLRP3 inflammasome that is now widely implicated as a mediator of sterile inflammation in metabolic diseases (Robbins 2014). Once formed, cholesterol crystals are highly persistent in atherosclerotic lesions and thus a good candidate as a mediator of chronic inflammation. Moreover, even a very mildly acidified environment sensitized the macrophages to cholesterol crystals and other NLRP3 activators, implying a general role in amplification of NLRP3-mediated inflammation.

In Study III, we performed a comprehensive analysis of inflammasome pathway-related gene expression in human coronary artery samples. A signature of three inflammasome core components – ASC, caspase-1, and IL-18 – was consistently upregulated in advanced atherosclerotic plaques compared to early lesions from the same coronary trees. Moreover, we found upregulation of p38δ MAPK, a poorly characterized isoform of the stress- and cytokine-activated p38 MAPK family, in advanced coronary plaques when compared to early lesions. Immunohistochemical stainings showed that the p38δ MAPK localized in lesional macrophage foam cells

together with NLRP3, ASC, and caspase-1. In a series of experiments in cultured human macrophages, we identified the p38δ MAPK as a novel mediator of NLRP3 inflammasome activation by cholesterol crystals and ATP. Taken together, the studies presented in this thesis revealed the existence of potent endogenous triggers of the NLRP3 inflammasome and IL-1β/IL-18 secretion in atherosclerotic lesions, and identified the p38δ MAPK as a novel effector in NLRP3-mediated sterile inflammation.

Cholesterol crystals were dismissed for decades as an inert, terminal form of cholesterol accumulation in the arterial wall. Results by our group (study I) and by Duewell *et al.* (Duewell 2010) challenged this view by showing that rather than an end point, the crystallization of cholesterol represents a transformation of this lipid into a proinflammatory danger signal. Moreover, Duewell *et al.* revealed the appearance of cholesterol microcrystals already at the earliest stages of lesion development in mouse aortic wall (Duewell 2010). Similarly, small cholesterol crystals occur already in human fatty streaks, yet a sharp increase in free cholesterol and cholesterol crystals is observed upon transition from the clinically silent type III lesion to the potentially symptomatic type IV lesion (Stary 1995, Small 1988). Thus, a critical concentration of free cholesterol may tip the balance strongly towards crystal nucleation and drive the transition of the lesion from a benign stage to a pathological one. Supporting this notion, clinical trials with inhibitors of the acyl–coenzyme A:cholesterol acyltransferase (ACAT) showed accelerated coronary and carotid atherosclerosis, as well as increased cardiovascular event rates (Nissen 2006, Meuwese 2009). The ACAT inhibitors block esterification of cellular cholesterol, the hallmark of foam cell formation. Thus, the data suggest an overall cardioprotective effect for cholesterol esterification, and, conversely, a pathogenic role for accumulation of free cholesterol that promotes crystal nucleation.

Along these lines, drugs that could inhibit cholesterol crystallization or dissolve cholesterol crystals from the arterial wall could be beneficial in reducing arterial wall inflammation and atherosclerotic plaque development. A promising candidate drug is 2-hydroxypropyl-β-cyclodextrin that redirects excess cholesterol from lysosomes into the cytoplasm for esterification and thus reduces lysosomal crystallization of cholesterol (Walenbergh 2015). Administration of this drug to LDLR-KO mice on high-fat diet decreased lysosomal cholesterol accumulation and crystallization in liver Kupffer cells (specialized macrophages), while increasing the amount of cytoplasmic cholesterol ester droplets (Walenbergh 2015). Further studies are needed to elucidate the mechanism of action behind these effects and to determine whether 2-hydroxypropyl-β-cyclodextrin has similar effects in macrophage foam cells of atherosclerotic plaques.

The p38 MAPKs, and p38α in particular, have been linked to various stages of cardiovascular pathology in a plethora of studies in cultured cells and animal models (Martin 2015). Of note, these studies were focused almost exclusively on the p38α and p38β isoforms, while p38γ and p38δ remain unexplored. The activation of p38α/β has been associated with endothelial dysfunction, atherosclerosis, platelet activation, thrombosis, ischemic heart injury, and with pathological post-infarction remodeling and fibrosis in the heart (Martin 2015). These observations have led to clinical trials with the p38α/β inhibitor losmapimod that was shown to significantly improve vascular function of atherosclerotic patients after 1-3 months of treatment (Cheriyan 2011, Elkhawad 2012). Thus, losmapimod improved endothelial function and reduced both systemic inflammation (hsCRP) and local inflammatory activity in atherosclerotic segments of carotids and aorta. Encouraged by these results, a large phase III clinical trial exploring the effect of losmapimod on cardiovascular outcomes in patients with acute coronary syndrome (the LATITUDE-TIMI 60 trial) was initiated in 2014, with an aim to recruit > 20 000 patients (O'Donoghue 2015).

Despite these promising developments, there are some concerns related to targeting the p38 MAPK pathway for suppressing atherosclerotic inflammation. The primary target of drug development, p38α MAPK, promotoes the development of ischemic heart injury but is also required for the cardioprotective effects of ischemic preconditioning (Sicard 2010, Kumphune 2010). Moreover, endothelium- or macrophage-specific knock-out of p38α had no effects, or even aggravating effects on atherogenesis in mouse models (Kardakaris 2011, Seimon 2009). Lastly, clinical studies with inhibitors targeting p38α in rheumatoid arthritis have faced problems with adverse effects, perhaps related to the diverse functions of this ubiquitous isoform (Genovese 2009). The p38α MAPK is essential for embryonal development in mice, whereas knock-out animals of the other p38 isoforms are fully viable and fertile (Cuenda 2007). Taken together, p38 isoforms beyond p38α, including the p38δ MAPK associated with inflammasome activation, may offer alternative targets for the anti-inflammatory treatment of CVD.

The value of hsCRP in cardiovascular risk prediction has been validated in numerous prospective studies in human populations, yet it is considered mainly a marker rather than a causal agent in CVD (Koenig 2013). As a more direct measure of ongoing inflammatory signaling, the plasma levels of certain proinflammatory cytokines, including IL-6 and IL-18, have been identified as independent predictors of cardiovascular risk (Ridker 2000, Blankenberg 2002, IL6R Genetics Consortium Emerging Risk Factors Collaboration 2012, Kaptoge 2014). Indeed, targeting of specific key proinflammatory cytokines has emerged as a potential therapeutic strategy to reduce cardiovascular events (Ridker 2014).

Notably, IL-1β was recently chosen as the target cytokine in a large phase III clinical trial exploring cardiovascular outcomes, the Canakinumab Anti-inflammatory Thrombosis Outcomes Study (CANTOS) (Ridker 2011). The decision was based on the growing body of evidence linking the balance between IL-1 and IL-1Ra with the pathogenesis of atherosclerosis and CVD (Ridker 2011). Notably, IL-1β is also a highly potent inducer of IL-6 *in vivo*, and strategies blocking IL-1 signaling are effective in reducing the systemic levels of IL-6 (Dinarello 2009). The drug under evaluation in CANTOS is a monoclonal IL-1β-neutralizing antibody, canakinumab, previously approved for the treatment of e.g. autoinflammatory syndromes. The CANTOS study enrolled a total of 10 065 post-myocardial infarction patients with persistent elevation of hsCRP to evaluate the efficacy of anti-inflammatory treatment on recurrent cardiovascular events (enrollment data by trial chairman, Dr. Paul M. Ridker, at the 12th World Congress on Inflammation, August 2015, Boston, Massachusetts, USA). A smaller pilot study of CANTOS has already been published, demonstrating efficient lowering of hsCRP and IL-6 by canakinumab in the absence of major effects on blood lipids (Ridker 2012). The results of the CANTOS study (estimated completion 2017) are eagerly awaited, as this is the first large-scale outcome trial directly assessing the inflammatory hypothesis of atherosclerosis and atherothrombosis.

Beyond blocking cytokine signaling, there is a growing interest towards developing drugs that block the activity of the inflammasome pathway. Several small-molecule drugs have been shown to inhibit inflammasome activation. The first compound identified was the type II diabetes drug glyburide that blocks the activation of the NLRP3 inflammasome, but not NLRP1 or NLRC4 inflammasomes (Lamkanfi 2009). The NF-κB inhibitory drugs parthenolide and Bay 11-7082 function by inactivating caspase-1 by alkylation and by blocking the ATPase activity of the NLRP3 receptor, respectively (Juliana 2010). Moreover, Coll *et al.* reported a compound that interferes with the oligomerization of ASC and thus blocks the activation of the ASC-dependent NLRP3 and AIM2 inflammasomes at micromolar concentrations (Coll 2011). In 2015, the same group reported another, much more potent compound, MCC950, that selectively inhibits NLRP3 inflammasome activation at nanomolar concentrations, without affecting the activation of NLRP1, NLRC4, and AIM2 inflammasomes (Coll 2015). Importantly, MCC950 was active *in vivo* in mouse models of multiple sclerosis and CAPS, and *ex vivo* in monocytes of CAPS patients (Coll 2015).

Although the above-mentioned compounds have not yet been tested in the context of atherosclerosis, a plant-derived compound called arglabin was recently shown to attenuate atherogenesis in mice via inhibition of the NLRP3 inflammasome (Abderrazak 2015). The suggested mechanism of action was an indirect inhibition of NLRP3 inflammasome activity via enhancement of autophagy, yet arglabin

also markedly reduced the levels of total cholesterol and triglycerides in plasma. This first demonstration of the beneficial effects of NLRP3-blocking drugs in atherosclerosis will no doubt encourage further studies with other inflammasome inhibitors under development. Indeed, the interest in the inflammasome pathway as a mediator of inflammation in atherosclerosis has rapidly expanded since the first reports on cholesterol crystal-mediated NLRP3 activation. Importantly, the NLRP3 inflammasome contributes not only to inflammation in atherosclerotic plaques, but also mediates obesity-induced inflammation in the adipose tissue and liver, and thus contributes to the development of insulin resistance and type II diabetes (Vandanmagsar 2011, Stienstra 2011). As obesity and type II diabetes are major risk factors for the development of atherosclerosis and CVD, the NLRP3 inflammasome is a compelling therapeutic target in the treatment of these closely linked metabolic disorders that represent a major global health threat.

# 7 ACKNOWLEDGEMENTS

This study was carried out at the Wihuri Research Institute during the years 2009-2015. I wish to sincerely thank the Jenny and Antti Wihuri Foundation for providing the excellent research facilities throughout this project, first at the iconic Salus Hospital in Kaivopuisto, and later on at the Biomedicum Helsinki research centre. Especially the years spent at Salus were truly memorable, coming to work each day to breathe in the thick history of the building, quietly greeting the Rytkönen sisters and Antti Wihuri in the lobby. I wish to warmly acknowledge the former and current directors of the Wihuri Research Institute, Professor Petri Kovanen and Professor Kari Alitalo, for welcoming me to work at the institute.

This thesis work was financially supported by the Magnus Ehrnrooth Foundation, the Finnish Cultural Foundation, the Finnish Foundation for Cardiovascular Research, the Paulo Foundation, the Oskar Öflund Foundation, the Orion Research Foundation, the Biomedicum Helsinki Foundation, the Finnish-Norwegian Medical Foundation, and the Maud Kuistila Foundation. I wish to warmly thank the foundations for their support.

I had the privilege to be guided by three excellent supervisors in this thesis project: Professor Petri Kovanen, Docent Katariina Öörni, and Professor Kari Eklund. Backed up by this solid trio, one could always feel confident that a solution will be found. Kari, I still remember the first talk you gave at our institute on 'the inflammasome', a some sort of peculiar inflammatory machine activated by crystals (yes, crystals!) found in rheumatic joints. I was immediately intrigued by the concept and inspired by your positive outlook and enthusiasm in science throughout the years. It has been a great pleasure working with you and I very much look forward to our future projects. Kati, you are an incredible one person think tank expertly and calmly solving any problem that happens to shake the people around you, be it a crisis of scientific, existential, or mundane nature. Thank you for sharing your expertise and for all the encouragement, guidance, and mentoring during these years, and of course, for our fabulous conference trips! Petu, I wish to express my deepest gratitude for sharing the incredible amount of knowledge, expertise, enthusiasm, and 'Wihuri spirit' that you have accumulated over the years. We certainly share the passion for linguistic detail, and I have learned a great deal from our reflections and debates on scientific writing. Your guidance has been invaluable throughout the thesis work.

I wish to express my gratitude to the members of the thesis advisory committee, Docent Vesa Olkkonen and Docent Hanna Jarva; thank you for all the valuable feedback, comments, and support throughout the thesis project. The pre-examiners of

the thesis, Professor Mika Rämet and Professor Seppo Nikkari, are warmly thanked for their constructive feedback generously contributed amidst the summer holidays.

I am deeply grateful for all my co-authors. With Katariina Nurmi, we have worked our way through the inflammasome pathway, side by side, one IL-1β ELISA after another, and shared the ups and downs of science along the way. I wish to warmly thank Docent Sampsa Matikainen and Elina Välimäki, our collaborators at the Finnish Institute of Occupational Health, and Katri Niemi at the 'Wihuri inflammasome team' for all the exchange of ideas and inflammasome troubleshooting over the years. Tommy Nordström and Professor Karl Åkerman from the Institute of Biomedicine/Physiology of the University of Helsinki are warmly acknowledged for their expertise in cellular pH imaging. I am most grateful for Mikko Mäyränpää for sharing his expertise in atherosclerotic plaque development, Jani Lappalainen for introducing me to the world of qPCR, and Jarno Tuimala for his expertise in statistics. A warm thank you also for Montserrat Estruch Alrich and Pauli Ohukainen for our collaborations.

I wish to warmly thank all the lovely people working in the Wihuri team throughout the years. I have been privileged to be surrounded by such bright and lively coworkers. Thank you for all your help and support, for all the discussions on science, life, and whatnot over the years – and for joyously sharing the dance floor after a sophisticated glass(es) of wine(s). Maija and Mari, 'excellent technical assistance' can't even begin to cover it, you are the heart and soul of our institute. Your expertise and true interest in being a part of the scientific process have been invaluable, not to forget your sense of humor and the so-let's-get-this-done-today-then attitude. A special thank you also for the countless times you patiently followed me into the cold room to point out whatever boxes and parcels lay right under my nose. Laura, thank you for taking care of so many practical matters over the years, I always enjoyed visiting your office upstairs. Su, I have enjoyed seeing you become firmly attached to our research group and to Finland over the years and I wish you all the best in your future career. I warmly thank my roommates at Biomedicum – Maisa, Kata M, Kata N, Satu, and Reija. It was nice to quite literally 'bump into to you' every day, and even though it was not always quiet, our room certainly was *the* place to be at!

I owe my deepest gratitude to my family and friends for all the love and support you have given me. Päivi, Terhi, and Anski, we have shared so much over the years. It is once again my turn to throw a graduation party; sometimes it feels that everything just goes around in circles, but I have to admit I'm very fond of those circles! Vilma, my fellow biochemist and partner in crime, it's been a long journey from the old days in the Viikki labs. You have graced me with your company at lunch hour in Biomedicum, and we have gracefully forgotten all about Biomedicum on many other cheerful occasions. Mahesh, long lost to science (for a good reason), I miss our long

aimless walks and hope we can catch up one day. Katja-Maria, Leo, Heini, Virpi, and the 'SINK people' - as all of you, I am deeply moved by dancing and sharing that with you has been the secret ingredient that keeps me going.

Mom, dad, you have been incredibly supportive throughout my studies, be it offering encouragement against my self-criticism or dragging my futon to Stockholm and back. I am deeply grateful for this. Many thanks to mom also for designing the final layout of this thesis. Tapsa, Päivi, I'm so lucky to have doubly the amount of moms and dads around to support me! Ari, I've been noticing lately a spark for science and invention growing in you, take good care of that spark and it will take you far. Pirjo and Hannu, thank you for our lively discussions on life, the thesis, the sheep, and the like during the past years; your home is truly a haven. Finally, I wish to express my deepest gratitude to Otto. As always, you have patiently supported me in all imaginable ways. We have worked on our theses side by side, or lately laptop against laptop on the kitchen table, 'The Vaasankatu Brain and Heart Study'.

Helsinki, June 2015

Kristiina Rajamäki

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