Peptidoglycan in atherosclerotic plaque formation and vulnerability

Door M.M. Oude Nijhuis

Peptidoglycan in atherosclerotic plaque formation and vulnerability
Manon Oude Nijhuis

Peptidoglycan in atherosclerotic plaque formation and vulnerability
Oude Nijhuis, Manon M.
Utrecht, Universiteit Utrecht, Faculteit Geneeskunde
Proefschrift Universiteit Utrecht, met een samenvatting in het Nederlands

ISBN-10: 90-393-4262-8
ISBN-13: 978-90-393-4262-6

Lay-out: Niels Oude Nijhuis
Photograph cover: Kevin Cobb
Printed by Ponsen en Looijen BV

Peptidoglycan in atherosclerotic plaque formation and vulnerability

Peptidoglycaan in atherosclerotische plaque vorming en kwetsbaarheid

(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht
op gezag van de rector magnificus, prof.dr. W.H. Gispen,
ingevolge het besluit van het college voor promoties
in het openbaar te verdedigen op
dinsdag 20 juni 2006 des ochtends te 10.30 uur

door

Manon Marieke Oude Nijhuis
geboren op 31 maart 1979 te Almelo, Nederland

Promotoren:

Prof. Dr. G. Pasterkamp
Prof. Dr. J.D. Laman

Co-promotor:

Dr. D.P.V. de Kleijn

Het onderzoek dat aan dit proefschrift ten grondslag ligt is mogelijk gemaakt door een subsidie van de Nederlandse Hartstichting (NHS-2001B077).

Dit proefschrift werd mogelijk gemaakt met financiële steun van het Interuniversity Cardiology Institute of the Netherlands (ICIN).

Het verschijnen van dit proefschrift werd ook mede mogelijk gemaakt door steun van de Nederlandse Hartstichting en de Prof. R.L.J. van Ruyvenstichting.

If we knew what it was
we were doing,
it would not be called
research, would it?

Albert Einstein

Content

Abbreviations

ABPI	ankle-brachial pressure index
APC	antigen-presenting cell
ApoE-/-	Apolipoprotein E-deficient
CABG	coronary artery bypass surgery
CAD	coronary artery disease
CARD	caspase recruitment domain
CATERPILLER	CARD, transcription enhancer, R-(purine-) binding, pyrin, lots of LRRs
CD	Crohn's disease
CHD	coronary heart disease
CI	confidence interval
CMV	cytomegalovirus
COX	cyclooxygenase
DC	dendritic cell
EDA	extra domain A
EEL	outer elastic lamina
ELISA	enzyme-linked immunosorbent assay
GlcNAc	N-acetylglucosamine
HR	hazard ratio
HSP	heat shock protein
HSV	herpes simplex virus
HUVEC	human umbilical vein endothelial cells
ICAM	intercellular adhesion molecule
IEL	inner elastic lamina
IFN-β	interferon-β
IKK complex	IκB kinase complex
IL	interleukin
IMT	intima-media thickness
IRAK	IL-1R-associated protein kinase
LA	luminal area
LDL	low-density lipoprotein
LDLr-/-	low-density lipoprotein receptor-deficient
LPS	lipopolysaccharide
LRR	leucine-rich repeats
LTA	lipoteichoic acid
mAb	monoclonal antibody
MALP	macrophage-activating lipopeptide
MCP	monocyte chemoattractant protein
MDA-LDL	malondialdehyde LDL

mDAP	m-diaminopimelic acid
MDP	muramyl dipeptide
MI	myocardial infarction
MIP-1α	macrophage inflammatory protein-1α
MMP	matrix metalloproteinase
MurNAc	N-acetylmuramic acid
MyD88	myeloid differentiation factor 88
Nalp	NACHT, LRR and PYD domains
NAMLAA	N-acetyl muramyl-L-alanine amidase
NBS-LRR	nucleotide-binding site leucine-rich repeat
NEMO	NF-κB essential modulator
NF-κB	nuclear factor-κB
NOD	nucleotide-binding oligomerization domain
Ox-LDL	oxidized LDL
PAMP	pattern associated molecular patterns
PECAM	platelet endothelial cellular adhesion molecule
PG	peptidoglycan
PGLYRP2	peptidoglycan-recognition protein 2
PG-PS	peptidogycan-polysaccharides
PRR	pattern recognition receptor
PSGL-1	P-selectin glycoprotein ligand-1
PTA	percutaneous transluminal angioplasty
PTCA	percutaneous transluminal coronary angioplasty
SD	standard deviation
SEM	standard error of mean
SFA	saturated fatty acid
SMART	second manifestations of arterial disease
SMC	smooth muscle cell
SR	scavenger receptor
TA	teichoic acid
TAK	transforming growth factor-β-activated kinase
TIR	Toll interleukin-1 receptor
TIRAP/MAL	TIR-associated protein/MyD88 adaptor-like
TLR	Toll-like receptor
TNF	tumor necrosis factor
TRAF	tumor necrosis factor receptor-associated factor
TRAM	Toll receptor-associated molecule
TRIF	Toll receptor-associated activator of interferon
UFA	unsaturated fatty acid
VCAM	vascular cell adhesion molecule

CHAPTER 1

General introduction

Atherosclerosis

Nowadays, cardiovascular disease still is the leading cause of morbidity and mortality worldwide. Every year almost 20 million people experience cardiovascular events, such as myocardial infarction and stroke, and atherosclerosis is the primary cause of these events. Atherosclerosis is a multifactorial disease affecting mainly the large arteries. The name Atherosclerosis is derived from the two Greek words “athere” and “sclerosis”, which stand for “accumulation of lipid” and “thickening of the arterial intima”, respectively. Atherosclerosis silently starts in early childhood [1-3] and becomes clinically evident in later life [4]. Development of atherosclerotic disease is characterized by the migration and proliferation of vascular smooth muscle cells (SMC) and accumulation of lipids in the sub-endothelial layer. In addition to increased atherosclerotic plaque formation, constrictive arterial remodeling, i.e. shrinkage of the artery, leads to lumen reduction. In the early phase of atherosclerosis, compensatory expansive remodeling, i.e. enlargement, can prevent narrowing of the lumen. However, in the acute phase, atherosclerotic luminal narrowing can be accelerated by rupture of a vulnerable plaque. This results in abrupt occlusion of a coronary artery [5-7], which is the common cause of cardiovascular events or sudden cardiac death. The manifestations of this disease are usually not restricted to a single segment of the vascular bed. Patients with symptoms of cardiovascular disease often have signs of atherosclerosis at many sites of the arterial tree [8-13]. Therefore, after a first clinical manifestation of atherosclerotic disease, the risk of recurrence due to lesion progression at another site in the circulatory system is increased.

Atherosclerosis: an inflammatory disease

Ross postulated in 1973 an important theory that contributed to a better understanding of the pathogenesis of atherosclerosis. In his “response to injury”-hypothesis Ross suggested that atherosclerosis was the result of endothelial denudation [14]. He hypothesized that endothelial denudation caused migration of SMC into the intima, followed by proliferation of these cells, increased lipid deposition and extracellular matrix synthesis [14,15]. Over the years, this concept has been modified significantly. Nowadays it is appreciated that the immune system, activation of the endothelium and induction of inflammatory pathways jointly play important roles in the pathogenesis of atherosclerosis [16]. Inflammatory cells are a major component of atherosclerotic lesions and cells like monocyte-derived macrophages and T-lymphocytes are already present in early lesions [4,17,18], called fatty streaks. During lesion progression, the number of lipid droplets-containing macrophages (foam cells) even increases [4,16,19]. Furthermore, many inflammatory mediators, such as adhesion molecules [20-23], chemokines [24,25], cytokines [26-30] and proteases [31-34], are expressed in atherosclerotic lesions and their role in development and progression of atherosclerotic plaques is confirmed in mouse models [35-42]. Therefore, atherosclerosis is now considered an inflammatory disease.

In the past century, cohorts like the Framingham Heart Study have identified so-called traditional risk factors for cardiovascular disease. Both genetic and environmental factors, such as hypertension [43], diabetes mellitus, hypercholesterolemia [43],

smoking [43], high fat diet and stress [44], but also infectious microorganisms such as herpesviruses and *Chlamydia pneumoniae* [45,46] accelerate the progression of atherosclerosis into a clinically symptomatic stage [47,48]. These risk factors cause endothelial dysfunction and thereby the initiation of atherosclerotic lesion formation. Especially at atherosclerosis predilection sites, such as bifurcations, curvatures and branches, endothelial cells can get activated due to decreased shear stress and increased turbulence in the blood flow [49]. At these sites, adhesion molecules, responsible for rolling, adhesion and migration of leukocytes and T-lymphocytes, on the endothelial surface are upregulated [50]. Selectins and selectin ligands present on leukocytes and endothelial cells are required for capturing and rolling of leukocytes over the endothelial cells [51-53]. Chemoattractants and chemokines can activate leukocytes, resulting in increased integrin expression on the leukocyte surface. Binding of the activated integrins on the leukocyte to their counter ligands on the endothelial surface mediates firm adhesion to and subsequent spreading over the endothelial cells [54]. The leukocytes migrate to the subendothelial matrix and accelerate the inflammatory process. Atherosclerosis starts with the formation of a fatty streak [55], initially consisting of T-lymphocytes and monocytes that, once present in the arterial wall, are able to differentiate into macrophages, followed by ingestion of oxidized LDL particles and thereby changing in lipid-laden foam cells. Chemokines activate the T-lymphocytes, monocytes and monocyte-derived macrophages resulting in secretion of pro-inflammatory cytokines and subsequent recruitment of more monocytes and T-lymphocytes to the inflammatory site. Furthermore, already from the initiation of the atherosclerotic plaque development, SMC migrate from the media into the atherosclerotic plaque and proliferate. Slowly the fatty streak progresses into an advanced lesion. A fibrous cap is formed that walls off an atheromatous lipid core, containing a mixture of foam cells, lipid and debris, from the lumen. The apoptosis of foam cells, necrosis, increased proteolytic activity and more lipid accumulation within the lipid core eventually result in formation of a necrotic core. Besides leukocytes, platelets are also able to adhere to activated endothelial cells. When activated, these platelets release their granules, containing cytokines that may contribute further to the migration and proliferation of SMC and macrophages [56]. The macrophages accumulate as foam cells in the lipid core which is progressively growing. Furthermore, the activated macrophages release matrix metalloproteinases (MMPs) and other proteolytic enzymes which cause degradation of the matrix and as a result thinning of the fibrous cap covering the lipid core. Eventually, the fibrous cap might rupture and the thrombogenic material inside the necrotic core is exposed to the blood, leading to rapid thrombus formation.
Figure 1 illustrates the events involved in the development of atherosclerotic plaques and plaque rupture.

Immunity

As already mentioned, the immune system plays an important role in development of atherosclerosis. The immune system is a system of specialized cells and organs that protects the host against bacteria and viral infections. This defense against invading microbial pathogens is composed of two arms: the innate and the adaptive immune

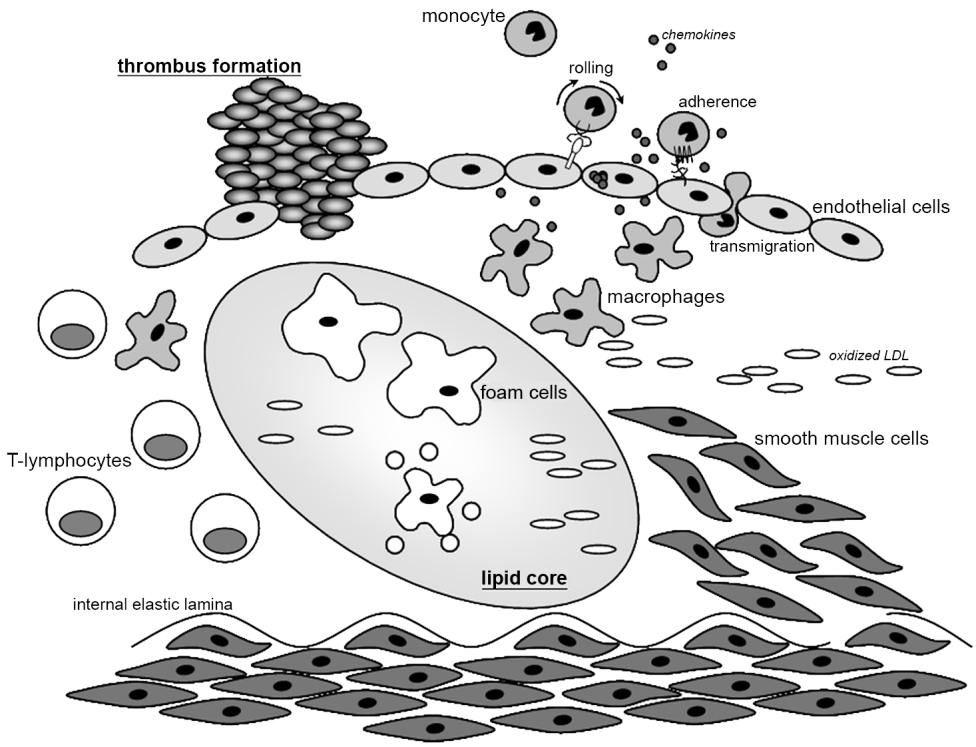

Figure 1. Atherosclerotic plaque formation and plaque rupture
Monocytes from the blood stream respond to chemoattractants and chemokines secreted by the damaged and activated endothelial cells. Selectins and selectin ligands trigger tethering and rolling of the monocytes over the endothelial cells. Firm adhesion is mediated by integrins on the monocyte surface and their counter receptors on the endothelial cells. Adhesion is followed by spreading and transendothelial migration. In the atherosclerotic plaque monocytes differentiate into macrophages, a process that is followed by uptake of oxidized LDL and formation of lipid-laden foam cells. Activated T-lymphocytes, monocytes and macrophages secrete pro-inflammatory cytokines, which recruit more monocytes and T-lymphocytes to the atherosclerotic plaque. Foam cells and lipids accumulate in an atheromatous lipid core. Proteolytic enzymes, secreted by the activated macrophages, degrade the matrix eventually leading to thinning of the fibrous cap and formation of a thrombus.

system. The early phase of the host-response to infection depends on innate immunity in which a variety of innate resistance mechanisms recognizes and responds to the presence of a pathogen and tissue damage. Activation of the innate immune system results in a fast response that does not change during life-time and will not result in long-lasting memory, so does not increase with repeated exposure to a given pathogen. In 1989, Charles Janeway proposed that infectious micro-organisms carry pathogen-associated molecular patterns (PAMPs) that can be detected by the immune system through a set of specialized receptors, the pattern recognition receptors (PRRs) [57]. This concept was confirmed by the discovery of human Toll-like receptors (TLRs) by Medzhitov *et al* in 1997 [58]. Nowadays, the TLRs are the largest family of innate immune receptors [59]. Several different types of phagocytic cells (neutrophils, monocytes, macrophages and dendritic cells (DC)) recognize PAMPs, but also cell types like vascular endothelial cells have been shown to participate in innate immunity.
Adaptive immunity (also known as acquired immunity) confers lifelong protective immunity to re-infection with the same pathogen. In the adaptive immune system

the responses to pathogens change and develop during life-time and activation of this system will result in long-lasting memory. The adaptive immune system is based on dedicated immune cells termed T-lymphocytes and B-lymphocytes. Previous research has demonstrated that TLRs are also important for optimal functioning of the acquired adaptive immune system.

Toll-like receptors

The Toll receptor was first discovered in the fruit fly *Drosophila melanogaster* [60]. In the last ten years, mammalian homologues of the Toll receptor have been identified, proteins that are now referred to as TLRs [58,61]. TLRs are transmembrane proteins that contain an extra-cellular ligand-recognition domain containing multiple leucine-rich repeats (LRR domain), a trans-membrane domain, and an intra-cellular Toll interleukin-1 (IL-1) receptor signaling domain (TIR domain) [62]. A functional immune system must have a mechanism for the recognition of pathogens while maintaining tolerance to self. The sensitivity and specificity of this recognition is defined, at least in part, by the ability of the TLRs to recognize specific microbial patterns. To date, eleven TLR family members (TLR1–11) have been identified in the human genome [63], and different TLRs appear to play crucial roles in the activation of the immune response to different PAMPs. A wide variety of agonists is known for many of the TLRs. Especially TLR2 and TLR4 recognize a broad range of surface molecular patterns. Upon ligand binding TLR2 forms a dimer with either TLR1 or TLR6. The heterodimer TLR2/1 detects tris-acylated lipopeptides [64], whereas the TLR2/6 heterodimer detects bis-acylated lipopeptides [65], peptidoglycan (PG) [66] and zymosan. TLR2 is also able to recognize lipoteichoic acid (LTA) [67,68], lipoarabinomannan [69], mycobacteria [70,71], necrotic cells [72], heat shock protein (HSP)60 [73] and many others. The TLR2/6 heterodimer can be recruited to macrophages, followed by phagocytosis and detection of intracellular PG [74]. TLR2 is predominantly expressed by monocytes, macrophages, DC and neutrophils [75,76], whereas TLR1 and TLR6 are expressed by many different cell types [75]. TLR3 recognizes double-stranded RNA [77], is an intracellular receptor and is almost exclusively present in DC. TLR4 has been identified as the signal transducer for lipopolysaccharide (LPS) which is present in the Gram-negative bacterial wall [78]. For LPS recognition TLR4 requires other small molecules, such as CD14, MD2 and LPS-binding protein as well [79,80]. TLR4 also recognizes taxol [81,82], HSP60 [73,83], extra domain A (EDA) of fibronectin [84], saturated fatty acids [85] and many others. Monocytes, macrophages, endothelial cells, DC and neutrophils express TLR4 [75]. TLR5 recognizes flagellin and therefore flagellated bacteria [86,87] and is expressed on monocytes, DC and the surface of intestinal epithelial cells [75,86]. TLR7 is the receptor for single-stranded RNA [88] but also recognizes several compounds that are structurally related to nucleic acids, such as imidozoquinoline [89]. TLR8 recognizes the imidazoquinoline resiquimod (R-848) in the mouse, but not in human [90]. Both receptors are expressed by DC. TLR9 is the receptor for non-methylated CpG DNA [91]. Both TLR8 and TLR9 can not be found on the cell surface but are expressed intracellularly. No ligand has been identified for the other TLRs so far. Interaction of TLRs with PAMPs on pathogens indicates the presence of infection and initiates signaling cascades leading to inflammatory and immune responses [92-94].

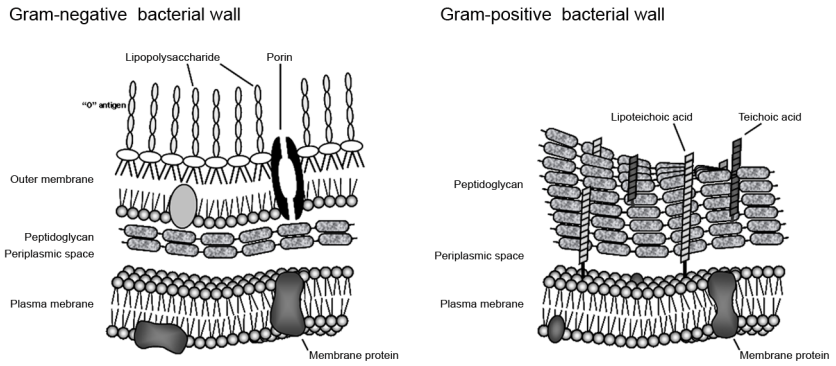

Figure 2. PG in Gram-negative and Gram-positive bacterial wall
PG is only a minor component of the Gram-negative bacterial wall, but a major cell wall component of Gram-positive bacteria. Furthermore, in the Gram-negative bacterial wall LPS is abundantly present and in the Gram-positive bacteria LTA and TA are integrated in the PG network. Modified from www.palaeos.com.

Peptidoglycan

PG is present in the cell walls of most bacteria; in Gram-positive bacteria PG consists of 10-70 layers accounting for 30-70% of the total bacterial cell wall mass (*Figure 2*) [95]. In contrast, in Gram-negative bacteria PG consists only of 1 or 2 layers and accounts thus for only 10% of the total cell wall [96]. In Gram-positive bacterial walls LTA and teichoic acid (TA) are dispersed throughout the PG network. These structures partially stay in the PG layers after digestion and purification and are known ligands able to activate the TLR2. Completely surrounding the cell, the presence of PG stabilizes the cell wall of the bacteria and thereby functions to withstand the high intracellular pressure exerted by the cytoplasm [97]. Therefore PG is essential for the survival of bacteria. PG is composed of alternating N-acetyl glucosamine (GlcNAc) and N-acetyl muramic acid (MurNAc) residues forming long sugar chains that are interlinked by peptide bridges (stem peptides) resulting in a large, complex macromolecule (*Figure 3*). The glycan chain is conserved in all bacteria and the peptide bridges can be attached to each other directly or indirectly via interpeptide bridges, which vary in length and amino acid sequence between Gram-positive and Gram-negative bacterial strains [98]. Some stem peptides are only attached to the glycan chain and do not cross-link with other stem peptides. PG is insoluble in its native form, but can be enzymatically cleaved into smaller soluble portions by specific PG-degrading enzymes [99].

Degradation of PG in human cells occurs by three different hydrolytic enzymes: lysozyme (N-acetylmuramidase), N-acetylglucosaminidase and N-acetylmuramyl-L-alanine amidase (NAMLAA) (*Figure 3*). Lysozyme is able to cleave PG sugar chains, producing monomeric muramyl peptides. N-acetylglucosaminidase is capable of hydrolyzing free N-acetylglucosamine groups from the non-reducing ends of the PG backbone, which results in the release of monomeric muropeptides. Both enzymes cleave the β-1-4 glycosidic bond between GlcNAc and MurNAc or MurNAc and GlcNAc, respectively [100,101]. NAMLAA, also known as peptidoglycan-recognition protein 2 (PGLYRP2) [102,103], hydrolyzes the lactamide bond between MurNAc and L-alanine at position 1 of the stem peptide [104]. Furthermore, other bacteriolytic enzymes,

such as endopeptidases, are able to degrade PG (for example by cleaving of PG at the interpeptide bridges). Bacterial PG is constantly being synthesized and degraded, processes during which PG components can be released. The sensitivity of PG for degradation by enzymatic activity is depending on the composition of the cell wall. When there is a high-degree of cross-linkage between the stem peptides, PG is more resistant to degradation. Furthermore, O-acetylated PG is more resistant to degradation than N-acetylated PG [105,106]. And finally, the presence of many TA and LTA molecules can partially inhibit degradation of PG. Thus, the extent of PG degradation depends on the structure of PG and on the presence of relevant bacteriolytic enzymes.
Surface expressed TLR2 is thought to mediate recognition of PG in the extracellular milieu. Recently, however, Travassos *et al* concluded that non-PG contaminants of PG preparations (lipoproteins and LTA) are recognized by TLR2 [107]. During PG isolation it is difficult to remove LTA and TA components completely. In contrast, Dziarski

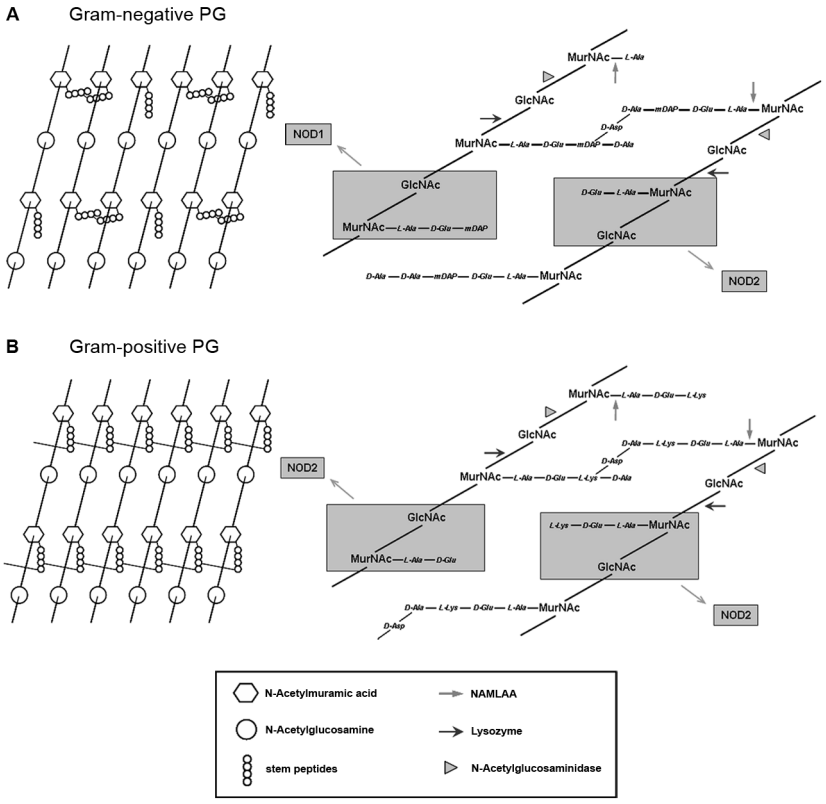

Figure 3. PG structure in Gram-negative and Gram-positive bacteria
The structure of PG differs between Gram-negative and Gram-positive bacteria. PG is composed of the two alternating sugar residues N-acetylmuramic acid (MurNAc) and N-acetylglucosamine (GlcNAc), which are connected via stem peptides (A and B left panel). Gram-negative bacteria contain a meso-diaminopimelic acid (mDAP) residue at position three of the stem peptide, whereas in most Gram-positive bacteria an L-lysine is present at that position (A and B right panel). The arrows indicate cleavage-sites by specific enzymes. Cleavage results in the release of PG fragments with different sizes and biological activities. Furthermore, the structural motifs of PG that can be recognized by intracellular nucleotide-binding oligomerization domain (NOD) receptors NOD1 and NOD2 are shown. NOD1 detects a PG structure from Gram-negative bacteria. NOD2 recognizes structures that are present in both Gram-negative and Gram-positive bacteria. Modified from the site of Community College of Baltimore County (www.cat.cc.md.us)

et al provided evidence that TLR2 is the true receptor for isolated *Staphylococcus aureus* PG [108]. Besides recognition in the extracellular milieu, bacteria can also be phagocytosed by antigen-presenting cells (APC). Degradation and subsequent release of PG components takes place inside the phagosomes. Furthermore, PG fragments can also be released during bacterial replication and infection. Released PG motifs are recognized by intracellular innate antigen receptors, as discussed below. Recognition of PG by the innate immune receptors is shown in *Figure 4*.

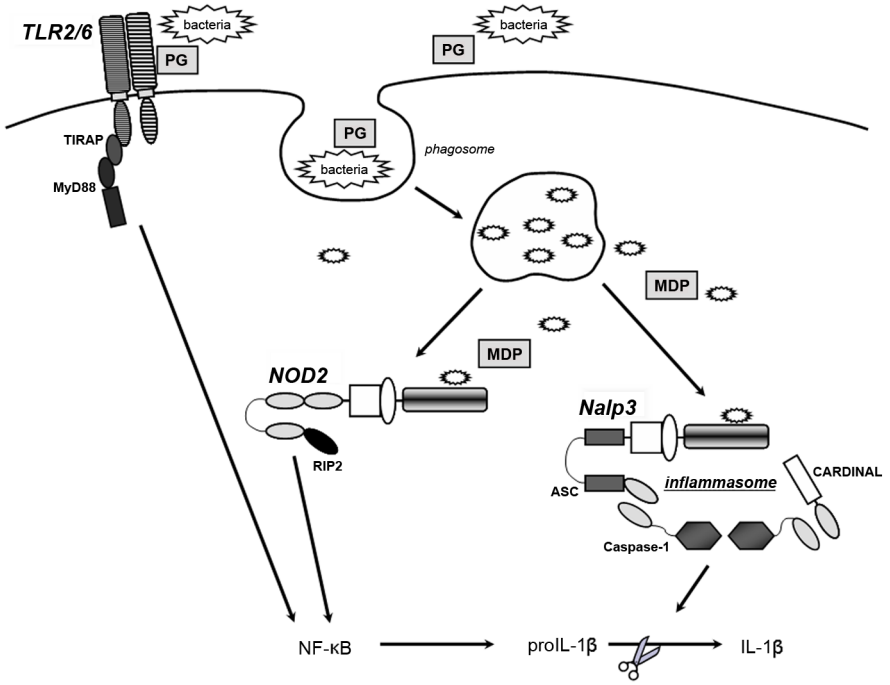

Figure 4. PG recognition by innate immune receptors
PG is present in the cell wall of Gram-positive and Gram-negative bacteria. Membrane-bound TLR2/6 heterodimers recognize extracellular PG, recruit MyD88 adaptor molecules with the intracellular TIR domain and activate NF-κB. When bacteria are phagocytosed by antigen-presenting cells, PG is degraded into specific PG fragments. The minimal motif that is required for NOD2 recognition is muramyl dipeptide (MDP), which is present in both Gram-positive and Gram-negative PG. Upon stimulation RIP2 is recruited and the homophilic CARD-CARD interaction activates NF-κB followed by synthesis of proIL-1β. MDP can also be recognized by the Nalp3 inflammasome. Nalp3 binds to CARDINAL and ASC and activates Caspase-1, which in turn cleaves proIL-1β thereby generating the pro-inflammatory IL-1β. Modified from Martinon and Tschopp, 2005 [134].

NOD1 and NOD2 receptors

Nucleotide-binding oligomerization domain (NOD) proteins belong to the nucleotide-binding site leucine-rich repeat (NBS-LRR) family (also called CATERPILLER [CARD, transcription enhancer, R- (purine-) binding, pyrin, lots of LRRs] family). Mammals have two closely related NOD family members -NOD1 and NOD2-, containing 1 or 2 amino-terminal caspase-recruitment domains (CARDs), respectively. Both NOD1 (CARD4) and NOD2 (CARD15) are intracellular PRRs containing three functional domains: a C-terminal leucine-rich repeat (LRR) domain serving as ligand-recognition domain, a centrally located nucleotide-binding domain, and an N-terminal protein/protein interaction domain (CARD) involved in signaling [109]. CARD and nucleotide-

binding site (NBS) domains are crucial for the activation of NF-κB. The C-terminal LRR domain might play a negative regulatory role in the activation of NF-κB [110] and is involved in the recognition of PG [111,112]. NODs are general sensors for both Gram-positive and Gram-negative bacteria, but it appears that specific fragments of the PG structure are responsible for its pro-inflammatory properties. Interestingly, NOD1 and NOD2 are distinct and non-overlapping in their sensing specificity for PG fragments (*Figure 3*). NOD1 specifically detects gram-negative specific, meso-diaminopimelic acid containing (mDAP type) PG, composed of the following muropeptide: GlcNAc-MurNAc-L-ala-D-Glu-mDAP (GM-Tri_{DAP}) [113,114]. Even without the glycan backbone NOD1 is able to recognize PG via γ-D-Glu-mDAP, the minimal motif necessary for PG recognition by NOD1 [113,115]. However, γ-D-Glu-mDAP is not a naturally occurring bacterial product, whereas TriDAP is present in most bacteria. In contrast to NOD1, NOD2 recognizes PG from both Gram-positive and Gram-negative bacteria. The minimal motif necessary for NOD2 engagement is muramyl dipeptide (MDP) [112,116], but just as γ-D-Glu-mDAP, MDP can not be formed naturally. Furthermore, recognition of PG by NOD2 depends on the presence of MurNAc and therefore NOD2 is also able to recognize the naturally occurring GlcNAc-MurNAc-L-ala-D-Glu (GM-DP), which is present in both Gram-positive and Gram-negative bacteria. Upon stimulation with PG fragments, NOD proteins have been shown to regulate NF-κB activation [110,117,118] and to induce apoptosis [119]. This stimulation probably occurs through the serine/threonine kinase RIP2 (also known as RICK or CARDIAK), thereby inducing the expression of pro-inflammatory mediators [120] and the induction of inflammation and atherosclerosis [121].

NOD1 is ubiquitously expressed in a variety of adult tissues [110,117], as opposed to NOD2 which has been found mainly in macrophages, DC, granulocytes and Paneth and epithelial cells of the intestine [118,122-124].

Interestingly, mutations in NOD2 have been shown to be associated with Crohn's disease (CD) and Blau syndrome, both inflammatory diseases. A frame-shift mutation and two single nucleotide polymorphisms in the NOD2 gene were found to be associated with CD [125,126] and point mutations in the nucleotide-binding domain of NOD2 have been shown to cause Blau syndrome [127]. For most of the mutations, inactivation of NF-κB after stimulation with MDP is defective, which results in decreased production of pro-inflammatory cytokines. Hence, the putative involvement of these receptors is x-fold: 1) they recognize microbial antigens, 2) they recognize self-antigens that are released during tissue damage, 3) they are involved in production of inflammatory compounds and 4) they regulate cellular apoptosis.

In this thesis we have used PG derived from *S. aureus* as described previously [99], which does not contain the motif required for NOD1 ligation but does contain the motif necessary for NOD2 ligation.

Nalp3 / Cryopyrin

A third class of innate receptors that are able to detect microbial agents are the Nalps. These intracellular receptors are, like NOD1 and NOD2, NBS-LRR proteins. Nalp (NACHT, LRR and PYD domains) proteins contain a C-terminal LRR domain, a centrally located nucleotide-binding domain or NACHT (neuronal apoptosis inhibitor protein, CIITA, HET-E and TP1) and at the N-terminus, the protein-protein interaction domain PYD (pyrin domain). Already 14 Nalps have been identified in the human genome [128]. Because all Nalps contain an LRR domain, it is possible that Nalps are activated in a similar way as NODs through the LRR-dependent sensing of a specific molecular motif. Until now, only Nalp1, 3 and 5 have been studied extensively. Recently, Martinon *et al* demonstrated that, similar to NOD2, MDP is the minimal structure that allows activation of the Nalp3 inflammasome [129], see *Figure 4*. Nalp3 is also known as PYPAF1/Cryopyrin/CIAS1. It binds PYCARD/ASC and activates caspase-1 and NF-κB [130]. Caspase-1 is able to cleave pro-IL-1β thereby generating the mature pro-inflammatory IL-1β. Expression of Nalp3 is restricted to immune cells. Mutations in the NACHT domain of Nalp3 have been associated with hereditary fever syndromes and chronic inflammatory diseases [131-133]. Furthermore, Blau syndrome is caused by mutations in the nucleotide-binding domain/NACHT domain of NOD2 that correspond to mutation R260W in Nalp3.

Outline of the thesis

The aim of this thesis is to assess whether bacterial PG is involved in atherosclerotic plaque initiation and progression. ***Chapter 2*** reviews the determinants that are known to play an important role in atherosclerotic plaque development, notably inflammation, infection and the immune system. Furthermore, this chapter focuses on exogenous and endogenous ligands that are able to stimulate TLRs and the role of these ligands and both the TLRs and intracellular NOD receptors in the atherosclerotic process. In ***Chapter 3*** systemic antibody levels against PG in serum of both patients with and without severe atherosclerotic disease are determined. Furthermore, possible relations between the immune response against PG and the extent of atherosclerotic disease are examined. In addition, in ***Chapter 4*** this first clinical study is extended by postulating that the systemic antibody levels against PG might be prognostic determinants for the occurrence of cardiovascular interventions after coronary angioplasty. PG is a well-known TLR2/NOD ligand, but the role of TLR2 in vascular occlusive disease is still not well-defined. ***Chapter 5*** assesses the hypothesis that TLR2 is involvement in development of atherosclerotic occlusive disease. The effect of TLR2 activation on production and secretion of pro-inflammatory cytokines and chemokines by vascular cells has been studied as well as the induction of TLR2 stimulation on intimal and atherosclerotic plaque formation in *in vivo* mouse models. In human atherosclerotic lesions PG is mainly present in macrophage-rich atheromatous lesions. Macrophages are derived from blood-borne monocytes, and monocyte adhesion to activated endothelial cells is an important first event in the inflammatory response. ***Chapter 6*** determines whether stimulation of primary human monocytes with TLR/NOD ligands promotes adhesion molecule expression, adhesion to endothelial cells under flow conditions and their subsequent migration. To elucidate whether TLR/NOD ligation is involved in lesion progression and development, we determined the effect of PG and $Pam_3Cys\text{-}SK_4$ on plaque characteristics in advanced lesions. In ***Chapter 7*** we determine whether local stimulation with PG promotes plaque development and stability after placement of non-constrictive polyethylene cuffs around the femoral arteries of atherosclerosis-susceptible ApoE*3Leiden transgenic mice. PG is a ligand that can be recognized by intracellular NOD1 and NOD2 receptors after processing into small motifs.
Chapter 8 assesses the hypothesis that NOD1 and NOD2 protein is expressed in human atherosclerotic plaques (Athero-Express). Furthermore, we determined whether NOD1 and NOD2 expression is related to plaque characteristics and MMP activity. ***Chapter 9*** summarizes and discusses the results of the studies that have been described in the preceding chapters.

REFERENCES

1. Stary HC. Macrophage foam cells in the coronary artery intima of human infants. Ann N Y Acad Sci 1985;454:5-8.
2. Stary HC. Evolution and progression of atherosclerotic lesions in coronary arteries of children and young adults. Arteriosclerosis 1989;9:I19-I32.
3. Stary HC, Blankenhorn DH, Chandler AB, Glagov S, Insull W, Jr., Richardson M, et al. A definition of the intima of human arteries and of its atherosclerosis-prone regions. A report from the Committee on Vascular Lesions of the Council on Arteriosclerosis, American Heart Association. Circulation 1992;85:391-405.
4. Stary HC, Chandler AB, Dinsmore RE, Fuster V, Glagov S, Insull W, Jr., et al. A definition of advanced types of atherosclerotic lesions and a histological classification of atherosclerosis. A report from the Committee on Vascular Lesions of the Council on Arteriosclerosis, American Heart Association. Circulation 1995;92:1355-74.
5. Davies MJ. Acute coronary thrombosis--the role of plaque disruption and its initiation and prevention. Eur Heart J 1995;16 Suppl L:3-7.
6. Libby P. Molecular bases of the acute coronary syndromes. Circulation 1995;91:2844-50.
7. Lee RT, Libby P. The unstable atheroma. Arterioscler Thromb Vasc Biol 1997;17:1859-67.
8. Burke AP, Farb A, Malcom GT, Liang YH, Smialek J, Virmani R. Coronary risk factors and plaque morphology in men with coronary disease who died suddenly. N Engl J Med 1997;336:1276-82.
9. Mann J, Davies MJ. Mechanisms of progression in native coronary artery disease: role of healed plaque disruption. Heart 1999;82:265-8.
10. Goldstein JA, Demetriou D, Grines CL, Pica M, Shoukfeh M, O'Neill WW. Multiple complex coronary plaques in patients with acute myocardial infarction. N Engl J Med 2000;343:915-22.
11. Nissen SE. Who is at risk for atherosclerotic disease? Lessons from intravascular ultrasound. Am J Med 2002;112 Suppl 8A:27S-33S.
12. Rioufol G, Finet G, Ginon I, Andre-Fouet X, Rossi R, Vialle E, et al. Multiple atherosclerotic plaque rupture in acute coronary syndrome: a three-vessel intravascular ultrasound study. Circulation 2002;106:804-8.
13. Buffon A, Biasucci LM, Liuzzo G, D'Onofrio G, Crea F, Maseri A. Widespread coronary inflammation in unstable angina. N Engl J Med 2002;347:5-12.
14. Ross R, Glomset JA. Atherosclerosis and the arterial smooth muscle cell: Proliferation of smooth muscle is a key event in the genesis of the lesions of atherosclerosis. Science 1973;180:1332-9.
15. Ross R, Glomset J, Harker L. Response to injury and atherogenesis. Am J Pathol 1977;86:675-84.
16. Ross R. Atherosclerosis--an inflammatory disease. N Engl J Med 1999;340:115-26.
17. Emeson EE, Robertson AL, Jr. T lymphocytes in aortic and coronary intimas. Their potential role in atherogenesis. Am J Pathol 1988;130:369-76.
18. Stary HC, Chandler AB, Glagov S, Guyton JR, Insull W, Jr., Rosenfeld ME, et al. A definition of initial, fatty streak, and intermediate lesions of atherosclerosis. A report from the Committee on Vascular Lesions of the Council on Arteriosclerosis, American Heart Association. Arterioscler Thromb 1994;14:840-56.
19. van der Wal AC, Becker AE, van der Loos CM, Das PK. Site of intimal rupture or erosion of thrombosed coronary atherosclerotic plaques is characterized by an inflammatory process irrespective of the dominant plaque morphology. Circulation 1994;89:36-44.
20. Poston RN, Haskard DO, Coucher JR, Gall NP, Johnson-Tidey RR. Expression of intercellular adhesion molecule-1 in atherosclerotic plaques. Am J Pathol 1992;140:665-73.
21. Richardson M, Hadcock SJ, DeReske M, Cybulsky MI. Increased expression *in vivo* of VCAM-1 and E-selectin by the aortic endothelium of normolipemic and hyperlipemic diabetic rabbits. Arterioscler Thromb 1994;14:760-9.
22. Tenaglia AN, Buda AJ, Wilkins RG, Barron MK, Jeffords PR, Vo K, et al. Levels of expression of P-selectin, E-selectin, and intercellular adhesion molecule-1 in coronary atherectomy specimens from patients with stable and unstable angina pectoris. Am J Cardiol 1997;79:742-7.
23. Nakashima Y, Raines EW, Plump AS, Breslow JL, Ross R. Upregulation of VCAM-1 and ICAM-1 at atherosclerosis-prone sites on the endothelium in the ApoE-deficient mouse. Arterioscler Thromb Vasc Biol 1998;18:842-51.

24. Yla-Herttuala S, Lipton BA, Rosenfeld ME, Sarkioja T, Yoshimura T, Leonard EJ, et al. Expression of monocyte chemoattractant protein 1 in macrophage-rich areas of human and rabbit atherosclerotic lesions. Proc Natl Acad Sci U S A 1991;88:5252-6.

25. Boisvert WA, Santiago R, Curtiss LK, Terkeltaub RA. A leukocyte homologue of the IL-8 receptor CXCR-2 mediates the accumulation of macrophages in atherosclerotic lesions of LDL receptor-deficient mice. J Clin Invest 1998;101:353-63.

26. Moyer CF, Sajuthi D, Tulli H, Williams JK. Synthesis of IL-1 alpha and IL-1 beta by arterial cells in atherosclerosis. Am J Pathol 1991;138:951-60.

27. Rosenfeld ME, Yla-Herttuala S, Lipton BA, Ord VA, Witztum JL, Steinberg D. Macrophage colony-stimulating factor mRNA and protein in atherosclerotic lesions of rabbits and humans. Am J Pathol 1992;140:291-300.

28. Tipping PG, Hancock WW. Production of tumor necrosis factor and interleukin-1 by macrophages from human atheromatous plaques. Am J Pathol 1993;142:1721-8.

29. Uyemura K, Demer LL, Castle SC, Jullien D, Berliner JA, Gately MK, et al. Cross-regulatory roles of interleukin (IL)-12 and IL-10 in atherosclerosis. J Clin Invest 1996;97:2130-8.

30. Sukovich DA, Kauser K, Shirley FD, DelVecchio V, Halks-Miller M, Rubanyi GM. Expression of interleukin-6 in atherosclerotic lesions of male ApoE-knockout mice: inhibition by 17beta-estradiol. Arterioscler Thromb Vasc Biol 1998;18:1498-505.

31. Galis ZS, Sukhova GK, Lark MW, Libby P. Increased expression of matrix metalloproteinases and matrix degrading activity in vulnerable regions of human atherosclerotic plaques. J Clin Invest 1994;94:2493-503.

32. Galis ZS, Muszynski M, Sukhova GK, Simon-Morrissey E, Libby P. Enhanced expression of vascular matrix metalloproteinases induced *in vitro* by cytokines and in regions of human atherosclerotic lesions. Ann N Y Acad Sci 1995;748:501-7.

33. Galis ZS, Sukhova GK, Kranzhofer R, Clark S, Libby P. Macrophage foam cells from experimental atheroma constitutively produce matrix-degrading proteinases. Proc Natl Acad Sci U S A 1995;92:402-6.

34. Nikkari ST, O'Brien KD, Ferguson M, Hatsukami T, Welgus HG, Alpers CE, et al. Interstitial collagenase (MMP-1) expression in human carotid atherosclerosis. Circulation 1995;92:1393-8.

35. Dong ZM, Chapman SM, Brown AA, Frenette PS, Hynes RO, Wagner DD. The combined role of P- and E-selectins in atherosclerosis. J Clin Invest 1998;102:145-52.

36. Rajavashisth T, Qiao JH, Tripathi S, Tripathi J, Mishra N, Hua M, et al. Heterozygous osteopetrotic (op) mutation reduces atherosclerosis in LDL receptor- deficient mice. J Clin Invest 1998;101:2702-10.

37. Aiello RJ, Bourassa PA, Lindsey S, Weng W, Natoli E, Rollins BJ, et al. Monocyte chemoattractant protein-1 accelerates atherosclerosis in apolipoprotein E-deficient mice. Arterioscler Thromb Vasc Biol 1999;19:1518-25.

38. Dawson TC, Kuziel WA, Osahar TA, Maeda N. Absence of CC chemokine receptor-2 reduces atherosclerosis in apolipoprotein E-deficient mice. Atherosclerosis 1999;143:205-11.

39. Huber SA, Sakkinen P, Conze D, Hardin N, Tracy R. Interleukin-6 exacerbates early atherosclerosis in mice. Arterioscler Thromb Vasc Biol 1999;19:2364-7.

40. Mallat Z, Besnard S, Duriez M, Deleuze V, Emmanuel F, Bureau MF, et al. Protective role of interleukin-10 in atherosclerosis. Circ Res 1999;85:e17-e24.

41. Rouis M, Adamy C, Duverger N, Lesnik P, Horellou P, Moreau M, et al. Adenovirus-mediated overexpression of tissue inhibitor of metalloproteinase-1 reduces atherosclerotic lesions in apolipoprotein E-deficient mice. Circulation 1999;100:533-40.

42. Collins RG, Velji R, Guevara NV, Hicks MJ, Chan L, Beaudet AL. P-Selectin or intercellular adhesion molecule (ICAM)-1 deficiency substantially protects against atherosclerosis in apolipoprotein E-deficient mice. J Exp Med 2000;191:189-94.

43. Glasser SP, Selwyn AP, Ganz P. Atherosclerosis: risk factors and the vascular endothelium. Am Heart J 1996;131:379-84.

44. Kritchevsky D. Diet and atherosclerosis. Am Heart J 1999;138:S426-S430.

45. Jackson LA, Campbell LA, Schmidt RA, Kuo CC, Cappuccio AL, Lee MJ, et al. Specificity of detection of *Chlamydia pneumoniae* in cardiovascular atheroma: evaluation of the innocent bystander hypothesis. Am J Pathol 1997;150:1785-90.

46. Libby P, Egan D, Skarlatos S. Roles of infectious agents in atherosclerosis and restenosis: an assessment of the evidence and need for future research. Circulation 1997;96:4095-103.

47. Berliner JA, Navab M, Fogelman AM, Frank JS, Demer LL, Edwards PA, et al. Atherosclerosis: basic mechanisms. Oxidation, inflammation, and genetics. Circulation 1995;91:2488-96.

48. Berenson GS, Srinivasan SR, Bao W, Newman WP, III, Tracy RE, Wattigney WA. Association between multiple cardiovascular risk factors and atherosclerosis in children and young adults. The Bogalusa Heart Study. N Engl J Med 1998;338:1650-6.

49. Malek AM, Alper SL, Izumo S. Hemodynamic shear stress and its role in atherosclerosis. JAMA 1999;282:2035-42.

50. Urbich C, Walter DH, Zeiher AM, Dimmeler S. Laminar shear stress upregulates integrin expression: role in endothelial cell adhesion and apoptosis. Circ Res 2000;87:683-9.

51. Abbassi O, Lane CL, Krater S, Kishimoto TK, Anderson DC, McIntire LV, et al. Canine neutrophil margination mediated by lectin adhesion molecule-1 *in vitro*. J Immunol 1991;147:2107-15.

52. Lawrence MB, Springer TA. Neutrophils roll on E-selectin. J Immunol 1993;151:6338-46.

53. Mayadas TN, Johnson RC, Rayburn H, Hynes RO, Wagner DD. Leukocyte rolling and extravasation are severely compromised in P selectin-deficient mice. Cell 1993;74:541-54.

54. Hynes RO. Integrins: versatility, modulation, and signaling in cell adhesion. Cell 1992;69:11-25.

55. Strong JP, Malcom GT, McMahan CA, Tracy RE, Newman WP, III, Herderick EE, et al. Prevalence and extent of atherosclerosis in adolescents and young adults: implications for prevention from the Pathobiological Determinants of Atherosclerosis in Youth Study. JAMA 1999;281:727-35.

56. Bombeli T, Schwartz BR, Harlan JM. Adhesion of activated platelets to endothelial cells: evidence for a GPIIbIIIa-dependent bridging mechanism and novel roles for endothelial intercellular adhesion molecule 1 (ICAM-1), alphavbeta3 integrin, and GPIbalpha. J Exp Med 1998;187:329-39.

57. Janeway CA, Jr. Approaching the asymptote? Evolution and revolution in immunology. Cold Spring Harb Symp Quant Biol 1989;54 Pt 1:1-13.

58. Medzhitov R, Preston-Hurlburt P, Janeway CA, Jr. A human homologue of the Drosophila Toll protein signals activation of adaptive immunity. Nature 1997;388:394-7.

59. Aderem A, Ulevitch RJ. Toll-like receptors in the induction of the innate immune response. Nature 2000;406:782-7.

60. Hashimoto C, Hudson KL, Anderson KV. The Toll gene of Drosophila, required for dorsal-ventral embryonic polarity, appears to encode a transmembrane protein. Cell 1988;52:269-79.

61. Rock FL, Hardiman G, Timans JC, Kastelein RA, Bazan JF. A family of human receptors structurally related to Drosophila Toll. Proc Natl Acad Sci U S A 1998;95:588-93.

62. O'Neill L. The Toll/interleukin-1 receptor domain: a molecular switch for inflammation and host defence. Biochem Soc Trans 2000;28:557-63.

63. Abreu MT, Arditi M. Innate immunity and toll-like receptors: clinical implications of basic science research. J Pediatr 2004;144:421-9.

64. Takeuchi O, Sato S, Horiuchi T, Hoshino K, Takeda K, Dong Z, et al. Cutting edge: role of Toll-like receptor 1 in mediating immune response to microbial lipoproteins. J Immunol 2002;169:10-4.

65. Takeuchi O, Kawai T, Muhlradt PF, Morr M, Radolf JD, Zychlinsky A, et al. Discrimination of bacterial lipoproteins by Toll-like receptor 6. Int Immunol 2001;13:933-40.

66. Ozinsky A, Underhill DM, Fontenot JD, Hajjar AM, Smith KD, Wilson CB, et al. The repertoire for pattern recognition of pathogens by the innate immune system is defined by cooperation between toll-like receptors. Proc Natl Acad Sci U S A 2000;97:13766-71.

67. Schwandner R, Dziarski R, Wesche H, Rothe M, Kirschning CJ. Peptidoglycan- and lipoteichoic acid-induced cell activation is mediated by toll-like receptor 2. J Biol Chem 1999;274:17406-9.

68. Lehner MD, Morath S, Michelsen KS, Schumann RR, Hartung T. Induction of cross-tolerance by lipopolysaccharide and highly purified lipoteichoic acid via different Toll-like receptors independent of paracrine mediators. J Immunol 2001;166:5161-7.

69. Means TK, Lien E, Yoshimura A, Wang S, Golenbock DT, Fenton MJ. The CD14 ligands lipoarabinomannan and lipopolysaccharide differ in their requirement for Toll-like receptors. J Immunol 1999;163:6748-55.

70. Lien E, Sellati TJ, Yoshimura A, Flo TH, Rawadi G, Finberg RW, et al. Toll-like receptor 2 functions as a pattern recognition receptor for diverse bacterial products. J Biol Chem 1999;274:33419-25.

71. Underhill DM, Ozinsky A, Smith KD, Aderem A. Toll-like receptor-2 mediates mycobacteria-induced proinflammatory signaling in macrophages. Proc Natl Acad Sci U S A 1999;96:14459-63.

72. Li M, Carpio DF, Zheng Y, Bruzzo P, Singh V, Ouaaz F, et al. An essential role of the NF-kappa B/Toll-like receptor pathway in induction of inflammatory and tissue-repair gene expression by necrotic cells. J Immunol 2001;166:7128-35.

73. Vabulas RM, Ahmad-Nejad P, Da Costa C, Miethke T, Kirschning CJ, Hacker H, et al. Endocytosed HSP60s use toll-like receptor 2 (TLR2) and TLR4 to activate the toll/interleukin-1 receptor signaling pathway in innate immune cells. J Biol Chem 2001;276:31332-9.

74. Underhill DM, Ozinsky A, Hajjar AM, Stevens A, Wilson CB, Bassetti M, et al. The Toll-like receptor 2 is recruited to macrophage phagosomes and discriminates between pathogens. Nature 1999;401:811-5.

75. Muzio M, Bosisio D, Polentarutti N, D'amico G, Stoppacciaro A, Mancinelli R, et al. Differential expression and regulation of toll-like receptors (TLR) in human leukocytes: selective expression of TLR3 in dendritic cells. J Immunol 2000;164:5998-6004.

76. Flo TH, Halaas O, Torp S, Ryan L, Lien E, Dybdahl B, et al. Differential expression of Toll-like receptor 2 in human cells. J Leukoc Biol 2001;69:474-81.

77. Alexopoulou L, Holt AC, Medzhitov R, Flavell RA. Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. Nature 2001;413:732-8.

78. Takeuchi O, Hoshino K, Kawai T, Sanjo H, Takada H, Ogawa T, et al. Differential roles of TLR2 and TLR4 in recognition of gram-negative and gram-positive bacterial cell wall components. Immunity 1999;11:443-51.

79. Shimazu R, Akashi S, Ogata H, Nagai Y, Fukudome K, Miyake K, et al. MD-2, a molecule that confers lipopolysaccharide responsiveness on Toll-like receptor 4. J Exp Med 1999;189:1777-82.

80. Gangloff M, Weber AN, Gay NJ. Conserved mechanisms of signal transduction by Toll and Toll-like receptors. J Endotoxin Res 2005;11:294-8.

81. Kawasaki K, Akashi S, Shimazu R, Yoshida T, Miyake K, Nishijima M. Mouse toll-like receptor 4.MD-2 complex mediates lipopolysaccharide-mimetic signal transduction by Taxol. J Biol Chem 2000;275:2251-4.

82. Kawasaki K, Akashi S, Shimazu R, Yoshida T, Miyake K, Nishijima M. Involvement of TLR4/MD-2 complex in species-specific lipopolysaccharide-mimetic signal transduction by Taxol. J Endotoxin Res 2001;7:232-6.

83. Ohashi K, Burkart V, Flohe S, Kolb H. Cutting edge: heat shock protein 60 is a putative endogenous ligand of the toll-like receptor-4 complex. J Immunol 2000;164:558-61.

84. Okamura Y, Watari M, Jerud ES, Young DW, Ishizaka ST, Rose J, et al. The extra domain A of fibronectin activates Toll-like receptor 4. J Biol Chem 2001;276:10229-33.

85. Lee JY, Sohn KH, Rhee SH, Hwang D. Saturated fatty acids, but not unsaturated fatty acids, induce the expression of cyclooxygenase-2 mediated through Toll-like receptor 4. J Biol Chem 2001;276:16683-9.

86. Gewirtz AT, Navas TA, Lyons S, Godowski PJ, Madara JL. Cutting edge: bacterial flagellin activates basolaterally expressed TLR5 to induce epithelial proinflammatory gene expression. J Immunol 2001;167:1882-5.

87. Hayashi F, Smith KD, Ozinsky A, Hawn TR, Yi EC, Goodlett DR, et al. The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5. Nature 2001;410:1099-103.

88. Lund JM, Alexopoulou L, Sato A, Karow M, Adams NC, Gale NW, et al. Recognition of single-stranded RNA viruses by Toll-like receptor 7. Proc Natl Acad Sci U S A 2004;101:5598-603.

89. Hemmi H, Kaisho T, Takeuchi O, Sato S, Sanjo H, Hoshino K, et al. Small anti-viral compounds activate immune cells via the TLR7 MyD88-dependent signaling pathway. Nat Immunol 2002;3:196-200.

90. Jurk M, Heil F, Vollmer J, Schetter C, Krieg AM, Wagner H, et al. Human TLR7 or TLR8 independently confer responsiveness to the antiviral compound R-848. Nat Immunol 2002;3:499.

91. Hemmi H, Takeuchi O, Kawai T, Kaisho T, Sato S, Sanjo H, et al. A Toll-like receptor recognizes bacterial DNA. Nature 2000;408:740-5.

92. Takeda K, Kaisho T, Akira S. Toll-like receptors. Annu Rev Immunol 2003;21:335-76.

93. Akira S. Toll-like receptor signaling. J Biol Chem 2003;278:38105-8.

94. Takeda K, Akira S. TLR signaling pathways. Semin Immunol 2004;16:3-9.

95. Snowden MA, Perkins HR. Peptidoglycan cross-linking in *Staphylococcus aureus*. An apparent random polymerisation process. Eur J Biochem 1990;191:373-7.

96. Labischinski H, Goodell EW, Goodell A, Hochberg ML. Direct proof of a "more-than-single-layered" peptidoglycan architecture of Escherichia coli W7: a neutron small-angle scattering study. J Bacteriol 1991;173:751-6.

97. Holtje JV. Growth of the stress-bearing and shape-maintaining murein sacculus of Escherichia coli. Microbiol Mol Biol Rev 1998;62:181-203.

98. Schleifer KH, Kandler O. Peptidoglycan types of bacterial cell walls and their taxonomic implications. Bacteriol Rev 1972;36:407-77.

99. Rosenthal RS, Dziarski R. Isolation of peptidoglycan and soluble peptidoglycan fragments. Methods Enzymol 1994;235:253-85.

100. Jolles P, Jolles J. What's new in lysozyme research? Always a model system, today as yesterday. Mol Cell Biochem 1984;63:165-89.

101. Striker R, Kline ME, Haak RA, Rest RF, Rosenthal RS. Degradation of gonococcal peptidoglycan by granule extract from human neutrophils: demonstration of N-acetylglucosaminidase activity that utilizes peptidoglycan substrates. Infect Immun 1987;55:2579-84.

102. Gelius E, Persson C, Karlsson J, Steiner H. A mammalian peptidoglycan recognition protein with N-acetylmuramoyl-L-alanine amidase activity. Biochem Biophys Res Commun 2003;306:988-94.

103. Zhang Y, van der FL, Voerman JS, Melief MJ, Laman JD, Wang M, et al. Identification of serum N-acetylmuramoyl-l-alanine amidase as liver peptidoglycan recognition protein 2. Biochim Biophys Acta 2005;1752:34-46.

104. Hoijer MA, Melief MJ, Calafat J, Roos D, van den Beemd RW, van Dongen JJ, et al. Expression and intracellular localization of the human N-acetylmuramyl-L-alanine amidase, a bacterial cell wall-degrading enzyme. Blood 1997;90:1246-54.

105. Rosenthal RS, Folkening WJ, Miller DR, Swim SC. Resistance of O-acetylated gonococcal peptidoglycan to human peptidoglycan-degrading enzymes. Infect Immun 1983;40:903-11.

106. Clarke AJ, Dupont C. O-acetylated peptidoglycan: its occurrence, pathobiological significance, and biosynthesis. Can J Microbiol 1992;38:85-91.

107. Travassos LH, Girardin SE, Philpott DJ, Blanot D, Nahori MA, Werts C, et al. Toll-like receptor 2-dependent bacterial sensing does not occur via peptidoglycan recognition. EMBO Rep 2004;5:1000-6.

108. Dziarski R, Gupta D. *Staphylococcus aureus* peptidoglycan is a toll-like receptor 2 activator: a reevaluation. Infect Immun 2005;73:5212-6.

109. Girardin SE, Sansonetti PJ, Philpott DJ. Intracellular vs extracellular recognition of pathogens--common concepts in mammals and flies. Trends Microbiol 2002;10:193-9.

110. Inohara N, Koseki T, del Peso L, Hu Y, Yee C, Chen S, et al. Nod1, an Apaf-1-like activator of caspase-9 and nuclear factor-kappaB. J Biol Chem 1999;274:14560-7.

111. Inohara N, Ogura Y, Chen FF, Muto A, Nunez G. Human Nod1 confers responsiveness to bacterial lipopolysaccharides. J Biol Chem 2001;276:2551-4.

112. Girardin SE, Boneca IG, Viala J, Chamaillard M, Labigne A, Thomas G, et al. Nod2 is a general sensor of peptidoglycan through muramyl dipeptide (MDP) detection. J Biol Chem 2003;278:8869-72.

113. Chamaillard M, Hashimoto M, Horie Y, Masumoto J, Qiu S, Saab L, et al. An essential role for NOD1 in host recognition of bacterial peptidoglycan containing diaminopimelic acid. Nat Immunol 2003;4:702-7.

114. Girardin SE, Boneca IG, Carneiro LA, Antignac A, Jehanno M, Viala J, et al. Nod1 detects a unique muropeptide from gram-negative bacterial peptidoglycan. Science 2003;300:1584-7.

115. Girardin SE, Travassos LH, Herve M, Blanot D, Boneca IG, Philpott DJ, et al. Peptidoglycan molecular requirements allowing detection by Nod1 and Nod2. J Biol Chem 2003;278:41702-8.

116. Inohara N, Ogura Y, Fontalba A, Gutierrez O, Pons F, Crespo J, et al. Host recognition of bacterial muramyl dipeptide mediated through NOD2. Implications for Crohn's disease. J Biol Chem 2003;278:5509-12.

117. Bertin J, Nir WJ, Fischer CM, Tayber OV, Errada PR, Grant JR, et al. Human CARD4 protein is a novel CED-4/Apaf-1 cell death family member that activates NF-kappaB. J Biol Chem 1999;274:12955-8.

118. Ogura Y, Inohara N, Benito A, Chen FF, Yamaoka S, Nunez G. Nod2, a Nod1/Apaf-1 family member that is restricted to monocytes and activates NF-kappaB. J Biol Chem 2001;276:4812-8.

119. Inohara N, Nunez G. NODs: intracellular proteins involved in inflammation and apoptosis. Nat Rev Immunol 2003;3:371-82.

120. Inohara N, Koseki T, Lin J, del Peso L, Lucas PC, Chen FF, et al. An induced proximity model for NF-kappa B activation in the Nod1/RICK and RIP signaling pathways. J Biol Chem 2000;275:27823-31.

121. Collins T, Cybulsky MI. NF-kappaB: pivotal mediator or innocent bystander in atherogenesis? J Clin Invest 2001;107:255-64.

122. Gutierrez O, Pipaon C, Inohara N, Fontalba A, Ogura Y, Prosper F, et al. Induction of Nod2 in myelomonocytic and intestinal epithelial cells via nuclear factor-kappa B activation. J Biol Chem 2002;277:41701-5.

123. Ogura Y, Lala S, Xin W, Smith E, Dowds TA, Chen FF, et al. Expression of NOD2 in Paneth cells: a possible link to Crohn's ileitis. Gut 2003;52:1591-7.

124. Lala S, Ogura Y, Osborne C, Hor SY, Bromfield A, Davies S, et al. Crohn's disease and the NOD2 gene: a role for paneth cells. Gastroenterology 2003;125:47-57.

125. Hugot JP, Chamaillard M, Zouali H, Lesage S, Cezard JP, Belaiche J, et al. Association of NOD2 leucine-rich repeat variants with susceptibility to Crohn's disease. Nature 2001;411:599-603.

126. Ogura Y, Bonen DK, Inohara N, Nicolae DL, Chen FF, Ramos R, et al. A frameshift mutation in NOD2 associated with susceptibility to Crohn's disease. Nature 2001;411:603-6.

127. Miceli-Richard C, Lesage S, Rybojad M, Prieur AM, Manouvrier-Hanu S, Hafner R, et al. CARD15 mutations in Blau syndrome. Nat Genet 2001;29:19-20.

128. Tschopp J, Martinon F, Burns K. NALPs: a novel protein family involved in inflammation. Nat Rev Mol Cell Biol 2003;4:95-104.

129. Martinon F, Agostini L, Meylan E, Tschopp J. Identification of bacterial muramyl dipeptide as activator of the NALP3/cryopyrin inflammasome. Curr Biol 2004;14:1929-34.

130. Manji GA, Wang L, Geddes BJ, Brown M, Merriam S, Al Garawi A, et al. PYPAF1, a PYRIN-containing Apaf1-like protein that assembles with ASC and regulates activation of NF-kappa B. J Biol Chem 2002;277:11570-5.

131. Hoffman HM, Mueller JL, Broide DH, Wanderer AA, Kolodner RD. Mutation of a new gene encoding a putative pyrin-like protein causes familial cold autoinflammatory syndrome and Muckle-Wells syndrome. Nat Genet 2001;29:301-5.

132. Aganna E, Martinon F, Hawkins PN, Ross JB, Swan DC, Booth DR, et al. Association of mutations in the NALP3/CIAS1/PYPAF1 gene with a broad phenotype including recurrent fever, cold sensitivity, sensorineural deafness, and AA amyloidosis. Arthritis Rheum 2002;46:2445-52.

133. Feldmann J, Prieur AM, Quartier P, Berquin P, Certain S, Cortis E, et al. Chronic infantile neurological cutaneous and articular syndrome is caused by mutations in CIAS1, a gene highly expressed in polymorphonuclear cells and chondrocytes. Am J Hum Genet 2002;71:198-203.

134. Martinon F, Tschopp J. NLRs join TLRs as innate sensors of pathogens. Trends Immunol 2005;26:447-54.

CHAPTER 2

Exogenous and endogenous ligands triggering the innate immune system in atherosclerotic disease

Manon M Oude Nijhuis[1,2], J Karlijn van Keulen[1,2],
Gerard Pasterkamp[1], Paul Quax[3], Dominique PV de Kleijn[1,2]

1. Experimental Cardiology Laboratory, University Medical Center Utrecht, Utrecht
2. Interuniversity Cardiology Institute of the Netherlands (ICIN), Utrecht
3. TNO-Quality of Life, Gaubius Laboratory, Leiden, the Netherlands

ACCEPTED FOR PUBLICATION

ABSTRACT

Innate immunity is the first line of defense against invading micro-organisms. The family of Toll-like receptors (TLRs) recognizes pathogen-associated molecular patterns (PAMPs) that are carried by the invading micro-organisms. Infectious pathogens have been implicated to play an important role in atherosclerosis. Nowadays, evidence is accumulating that TLRs play an important role in the initiation and progression of atherosclerosis too. A lot is known about the exogenous ligands that are able to activate the TLRs, but it is also known that endogenous ligands have the capacity to activate TLRs when exogenous ligands are absent. Studies on knockout mice, epidemiological studies and even human polymorphism studies confirmed the important role of TLRs in development and progression of atherosclerotic disease. Studies with antagonists against TLR ligands and vaccination studies demonstrated that TLR signaling might be a potential target for intervention in the initiation and progression of atherosclerosis.

Atherosclerosis is a chronic inflammatory disease of the vascular wall. Besides inflammation, metabolism and haemodynamics play a key role in the initiation and progression of atherosclerotic disease [1]. Disease onset can be triggered by risk factors like hypertension, diabetes mellitus, or high plasma concentrations of low-density lipoprotein (LDL) cholesterol. Fatty streaks are the precursors to atherosclerotic lesions and develop already during early childhood [2]. Susceptibility to plaque formation is the greatest in vascular regions where haemodynamic strain is changed, such as curvatures. At these low-shear rate sites, the endothelial cells become activated and permeability, adhesion molecule expression on the endothelial surface and chemokine production increases.

Modified lipoproteins and inflammation in atherosclerosis

Accumulation and modification of lipoproteins in the arterial intima initiate the development of atherosclerosis. The innate immune system responds to this accumulation, as shown by *in vitro* cell culture experiments, and thereby participates in the atherosclerotic process. Modified lipoproteins are capable of inducing cytokine and chemokine expression, which are mediators of innate immunity [3,4]. Proteoglycan and protein-bound lipoprotein particles can undergo modifications, such as oxidation of the lipid or protein moieties, non-enzymatic glycation, and several forms of enzymatic modifications [5-7]. The secretion of cytokines and chemokines, in turn, will cause expression of adhesion molecules on the surface of endothelial cells. Sequentially, leukocytes, monocytes, lymphocytes, and platelets are able to adhere to the endothelial cells and migrate between them to enter the intimal layer of the vessel wall. Leukocyte/monocyte adhesion to activated endothelial cells is a multistep process [8,9]. Molecules that play an important role in leukocyte-endothelial adhesion include L-selectin (CD62L), E-selectin (CD62E), and P-selectin (CD62P), expressed by leukocytes, endothelial cells and platelets, respectively [10-13]. Furthermore, intercellular adhesion molecule-1 (ICAM-1/CD54) and vascular adhesion molecule-1 (VCAM-1/CD106) expressed by the activated endothelium play an important role in firm adhesion of the leukocytes to the endothelial cells [14,15]. Once firmly adhered to the endothelial cells, leukocytes migrate into the underlying intima in response to chemoattractant stimuli [16]. If the monocytes become activated, and remain in the intimal layer, they mature into macrophages, take up oxidized lipids to become foam cells, and release a variety of inflammatory mediators. At this point, the fatty streak becomes an advanced, complicated lesion with a fibrous cap overlying a necrotic and calcified core [17,18]. Eventually, atheromatous lesions might rupture, resulting in thrombus formation that is able to block the arterial lumen, thereby causing myocardial infarction (MI) or stroke [1,17,18].

Immunity

Both inflammation and the immune system play an important role in the development and perhaps even destabilization of the atherosclerotic plaque. The immune system can be divided in innate and adaptive immunity. Innate immunity, also known as natural immunity, is the first line of defense against invading micro-organisms and is not very specific to the threatening situation but provides a rapid response against

the invading pathogens. Micro-organisms and pathogens carry pathogen-associated molecular patterns (PAMPs) that are recognized by pattern recognition receptors, including scavenger receptors (SRs) and Toll-like receptors (TLRs). Immune competent cells, such as monocytes, macrophages, dendritic cells (DC), and leukocytes recognize PAMPs, but also cell types like vascular endothelial cells have been shown to participate in innate immunity. Adaptive immunity, also known as acquired immunity, responds not as quickly as innate immune responses, but is highly specific and does result in long-lasting immunologic memory. In this adaptive immune response T-lymphocytes and B-lymphocytes play an important role. Furthermore, the uptake of modified lipoproteins might result in presentation of processed ligands to specific T-lymphocytes, therefore linking innate and adaptive immunity [19].

Evidence is accumulating that not only exogenous ligands are able to activate TLRs but that they can also be stimulated by endogenous ligands that are mostly produced during stress and cell damage. In this review we will focus on the role TLRs and their exogenous and endogenous ligands play in innate immunity and in the development of atherosclerotic disease. Since not only inflammation but also infection has been described to play an important role in development of cardiovascular disease, we will also discuss the role of infectious pathogens in atherosclerotic disease development.

Toll-like receptors

The Toll receptor was first discovered in the fruit fly *Drosophila melanogaster* [20]. In recent years, mammalian homologues of the Toll receptor have been identified, proteins that are now referred to as TLRs [21,22]. Both are transmembrane proteins that contain an extra-cellular ligand-recognition domain containing multiple leucine-rich repeats (LRR domain), a trans-membrane domain, and intra-cellular Toll interleukin-1 (IL-1) receptor signaling domain (TIR domain). TLRs play an important role in both our innate and acquired immune systems. They are involved in responses to infection and in development of sepsis and inflammatory bowel disease [23].

All TLRs share homology in their cytoplasmic TIR domains [21,24] and to date, eleven TLRs have been identified in mammals [25]. TLRs can be divided in 5 subfamilies, based on their genomic structure and their primary amino acid sequence; the TLR2, TLR3, TLR4, TLR5, and TLR9 subfamilies [24]. The TLR2 subfamily is composed of TLR1, TLR2, TLR6, and TLR10. TLR1 and TLR6 form heterodimers with TLR2. The homology among these receptors is high, more than 69% similarity in overall amino acid sequence, especially in the transmembrane and cytoplasmic domain [26]. The TIR domains of both receptors share over 90% identity. All TLR2 family members have the same genomic structure; all of the coding sequences are located in one exon. TLR3 is composed of five exons, but the protein is encoded by exons 2 through 5. The TLR4 gene has four exons and, in addition, the TLR5 gene consists of five exons. Both are encoded by only one or two exons. The TLR9 subfamily is composed of TLR7, TLR8, and TLR9. These family members are encoded by two exons [27,28]. TLR7 and TLR8 show almost 73% similarity in their amino acid sequences and more than 42% identity.

TLRs recognize PAMPs on the surface of pathogens such as Gram-positive and Gram-negative bacteria, fungi and viruses. TLR2 and TLR4, in particular, recognize

a broad range of surface molecular patterns. From the TLR2 it is known that upon ligand binding a dimer is formed with either TLR1 or TLR6. The heterodimer TLR2/1 detects tris-acylated lipopeptides [29], whereas the TLR2/6 heterodimer detects bis-acylated lipopeptides [30], peptidoglycan (PG) [31] and zymosan. The TLR2 is also able to recognize lipoteichoic acid (LTA) and lipoarabinomannan [32]. The TLR4 has been identified as the signal transducer for lipopolysaccharide (LPS) which is present in the Gram-negative bacterial wall [33]. TLR4 also recognizes taxol [34], saturated fatty [35] acids and many others. TLR3 recognizes double-stranded RNA [36], TLR5 recognizes flagellin [37], and TLR7 is the receptor for single-stranded RNA [38], but also recognizes several compounds that are structurally related to nucleic acids such as imidazoquinoline [39]. TLR9 is the receptor for non-methylated CpG DNA [40]. TLR8 recognizes the imidazoquinoline resiquimod (R-848) in the mouse, but not in human [41]. No ligand has been detected for the other TLRs so far. TLR3, TLR8 and TLR9 are intracellular TLRs, the others are transmembrane receptors. Interaction of TLRs with PAMPs on pathogens indicates the presence of infection and initiates signaling cascades leading to inflammatory and immune responses [24,42,43].

Toll-like receptor signaling

After binding to a ligand intracellular signaling adaptors are recruited to the TLR. Binding of these adaptors to the TIR domain of the TLR is the first step of intracellular signaling [42,44,45]. The intracellular TLR signaling pathway is depicted in *Figure 1*. To date, four adaptor proteins, MyD88 (myeloid differentiation factor 88), TIRAP/MAL (TIR-associated protein/MyD88 adaptor-like), TRIF (Toll Receptor-associated activator of Interferon), and TRAM (Toll receptor-associated molecule) have been shown to be able to bind the TIR domain of TLRs. MyD88 is utilized by all the TLRs, except TLR3 as shown in MyD88 knockout mice where the TLR3 ligand double-stranded RNA is able to induce Nuclear Factor-κB (NF-κB) activation. On binding to their ligands, TLRs recruit MyD88 to the receptor complex, which is then joined by IL-1R-associated protein kinase 1 (IRAK-1), IRAK-4 and tumor necrosis factor receptor–associated factor 6 (TRAF6). IRAK-1 and TRAF6 then dissociate from this complex and associate with another complex composed of transforming growth factor-β–activated kinase (TAK-1) and TAK-1 binding proteins 1 and 2. TAK-1 is activated, which in turn activates the IκB kinase (IKK) complex. The kinase activity of this complex is modulated by its IKKγ subunit, the transcription factor NF-κB essential modulator (NEMO). IKK-mediated phosphorylation of IκB leads to its ubiquitination and degradation, thereby unmasking the nuclear localization domain of NF-κB. After its translocation into the nucleus, NF-κB activates multiple pro-inflammatory genes, including tumor necrosis factor (TNF), IL-1 and IL-6. The molecular mechanisms underlying regulation at practically all these steps are not well-understood and are under intense investigation. TIRAP/MAL was identified to be involved in the MyD88-dependent pathways of TLR2/1, TLR2/6, and TLR4 but not other TLRs. The MyD88-independent adaptor molecule TRIF is essential for the TLR3 and TLR4 signaling, leading to the production of interferon-β (IFN-β) and the activation and maturation of DC. Furthermore, TRAM has been shown to be involved specifically in TLR4-, but not TLR3-mediated, MyD88-independent IFN-β production. So, most TLRs activate the MyD88-dependent pathway, resulting

in transcription of NF-κB, but some TLRs use different adaptor molecules. TLR4 activation results in recruitment of all four adaptor molecules to the receptor complex, whereas TLR3 activation is entirely dependent on TRIF.

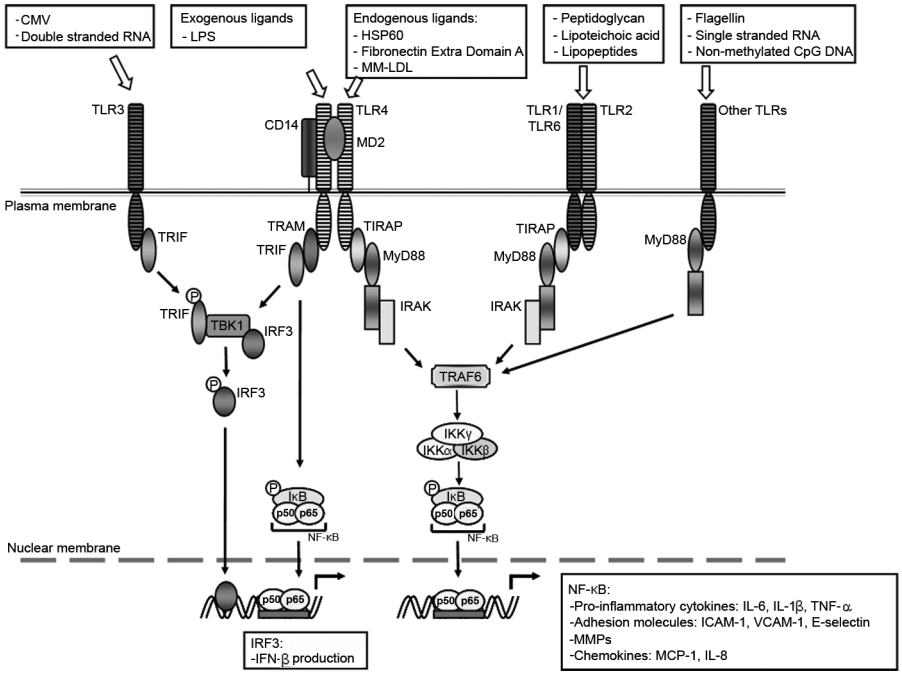

Figure 1. TLR ligation and intracellular signaling pathways
Schematic representation of proteins involved in the signaling pathway after TLR ligation. TLRs can be activated by either exogenous or endogenous ligands. Upon ligand binding TLRs recruit intracellular signaling adaptors to their receptor-signaling domain. Four adaptor molecules have been shown to bind the TIR domain of TLRs, resulting in activation and nuclear translocation of NF-κB. NF-κB activates pro-inflammatory cytokines, like IL-6, IL-1β and TNF-α, increases expression of adhesion molecules such as ICAM-1, VCAM-1 and E-selectin, and increases chemokine release (MCP-1 and IL-8). Modified from Takeda and Akira, 2004 [43].

Exogenous ligands involved in atherosclerotic disease

The role of TLRs in innate immunity is well described, especially the role that LPS plays in inflammation and atherosclerotic plaque development. LPS, also known as endotoxin, is a glycolipid that is present in the outer wall of Gram-negative bacteria. Even in healthy individuals LPS can be detected in the plasma. Several observations suggest that this exogenous TLR4 ligand could be pro-atherogenic. Presence of LPS in inflammatory cells, such as monocytes and macrophages, and endothelial cells causes upregulation of adhesion molecule expression, production of cytokines and superoxide, loss of endothelial monolayer integrity and barrier function, and apoptosis [46] thereby confirming that LPS is able to cause vascular inflammation and endothelial dysfunction. PG is present in the cell wall of most bacteria; in large amounts it can be found in Gram-positive bacteria and only in small amounts in Gram-negative bacteria. Stimulation of the TLR2 by PG results in production and secretion of pro-inflammatory cytokines, like IL-1, IL-6 and TNF-α, by monocytes and macrophages. Zeuke *et al* investigated whether TLR stimulation on human coronary

artery endothelial cells could increase cytokine and adhesion molecule expression on the surface. Stimulation of the TLR4 by LPS results in enhanced secretion of IL-6, IL-8, and monocyte chemoattractant protein-1 (MCP-1) and upregulation of ICAM-1, VCAM, and ELAM-1 [47]. Furthermore, stimulation of TLR2 by LTA and AraLam and TLR4 stimulation by *Escherichia coli*-LPS, purified *E. coli*-LPS and *Neisseria meningitidis*-LPS on human blood monocytes results in high expression of mRNA and increased protein synthesis of MCP-1 and MIP-1α [48]. Genetically altered mice studies showed that a decrease in expression of MCP-1 will result in decreased lesion formation in atherosclerosis-susceptible hypercholesterolemic mice [49]. MCP-1 attracts leukocytes bearing the chemokine receptor CCR-2, principally mononuclear phagocytes, and plays an important role in leukocyte spreading on the endothelial surface and leukocyte migration [50].

In addition, *in vivo* after cuff placement local activation of TLR4 by LPS augments neointima formation, whereas in mice with a defective TLR4, LPS application results in a smaller neointima than in the wild type strain [51]. Furthermore, outward arterial remodeling in the TLR4 defective mice is significantly less as compared to the wild type mice both after cuff placement around the femoral artery and LPS application (outward arterial remodeling with neointima formation) or carotid ligation (outward arterial remodeling without neointima formation), suggesting that TLR4 plays an important role in outward arterial remodeling [52]. Outward arterial remodeling also occurs in the absence of the exogenous TLR4 ligand LPS. Since an upregulation of endogenous ligands, such as heat shock protein 60 (HSP60) and Extra Domain A (EDA) of fibronectin, can be found in the arterial wall, TLR4-involvement in the absence of exogenous ligands might be dependent on stimulation by these endogenous ligands. Injections of LPS in rabbits on hypercholesterolemic diets and in Apolipoprotein E-deficient (ApoE-/-) mice increased the rapid development of atherosclerotic disease [53,54]. Michelsen *et al* demonstrated that TLR4 knockout mice crossed onto the ApoE background show significantly reduced atherosclerotic lesion development [55]. Furthermore, in hyperlipidemic mice lacking expression of MyD88, early atherosclerosis is significantly reduced [56]. In those mice macrophage recruitment to the arterial wall is decreased resulting in reduced chemokine levels. In contrast, there are also data suggesting that pathogens do not play a role in atherogenesis. Wright *et al* showed previously that in germ free mice atherosclerosis is not different from mice with ambient microbial challenge. These results seem to disagree with a possible role for TLRs in atherogenesis [57]. However, Wright and colleagues did not quantify the amount of plaque present in the arteries and did not look at plaque phenotype but only looked at plasma cholesterol and triglyceride levels and extent of free cholesterol or cholesteryl ester accumulation in the aorta.

In human atherosclerotic plaques, elevated mRNA and protein levels of TLR1, TLR2 and TLR4 have been found [58,59]. In those atherosclerotic plaques, expression of TLR2 and TLR4 has been described in macrophages and endothelial cells. TLR2 and TLR4 expression by endothelial cells in normal arteries is low, but is significantly increased in endothelial cells lining atherosclerotic lesions [58]. Xu *et al* showed that TLR4 is

expressed in lipid-rich, macrophage-infiltrated atherosclerotic lesions of mice and humans [59]. They also found that OxLDL induces upregulation of TLR4 expression in human monocyte-derived macrophages. And although Edfeldt *et al* demonstrated that in both the macrophages and endothelial cells TLR2 and TLR4 colocalizes with NF-κB, this does not prove that in that particular situation stimulation of the TLR causes NF-κB activation. Therefore, it would be important to identify TLR ligands in atherosclerotic lesions.

Bacterial PG has been detected in atherosclerotic plaques that reveal an inflammatory, unstable phenotype [60]. In those plaques, the presence of PG has been associated with the presence of macrophages. In a recent study, Travassos *et al* concluded that non-PG contaminants of PG preparations (lipoproteins and LTA) are responsible for recognition by TLR2 and that PG detection is more likely to occur through nucleotide-binding oligomerization domain (NOD)2 (CARD15), an intracellular protein [61]. NOD2 is a general sensor for both Gram-positive and Gram-negative bacteria, through the recognition of muramyl dipeptide, the minimal motif found in all PGs. In contrast to NOD2, NOD1 (CARD4) specifically recognizes DAP-type PG, which is present in all Gram-negative bacteria. Since Dziarsky *et al*, in response to the study by Travassos, demonstrated that *Staphylococcus aureus* PG can be recognized by TLR2, the issue about which receptors recognize PG is still a matter of debate.

As in many diseases, gene polymorphisms play an important role in the atherosclerotic process as well. The TLR4 polymorphism Asp299Gly is a single nucleotide polymorphism in the TLR4 gene, resulting in an amino acid substitution in the extracellular domain of the receptor. Due to decreased ligand signaling, this polymorphism declines the capacity of the innate immune system to elicit an inflammatory reaction [62]. In a retrospective study, 810 persons were screened for the presence (55 persons) or absence of this Asp299Gly polymorphism [63]. During 5-year follow-up, the persons with the polymorphism had a lower risk of developing carotid atherosclerosis as compared to individuals with wild type TLR4 (odds ratio 0.54; 95% confidence interval 0.32-0.98). Furthermore, the individuals with the Asp299Gly TLR4 allele revealed less common carotid intima-media thickness. In a case-control study, among 216 controls and 183 patients with acute coronary syndromes, the presence of the Asp299Gly allele was associated with a decreased risk of acute coronary events (odds ratio 0.41; 95% confidence interval 0.18-0.95) [64]. The results of both studies suggest that the presence of the TLR4 polymorphism protects against the progression of atherosclerosis. In contrast, Edfeldt *et al* found that among 852 cases and 1054 controls the Asp299Gly genotype was associated with an increased risk of MI in men (odds ratio 1.4; 95% confidence interval 1.0-1.9) [65]. Other groups did not find significant associations between Asp299Gly and atherosclerosis. Zee *et al* reported no association with risk of atherothrombosis, MI or stroke [66]. In a study among 293 patients with familial hypercholesterolemia the Asp299Gly polymorphism has no impact on the progression of atherosclerosis [67]. In accordance, Yang *et al* found no significant difference between the genotype frequencies and the number of coronary arteries with >50% stenosis [68].

Endogenous ligands

As already mentioned, antigen-presenting cells, like macrophages and DC, express TLRs, but also human endothelial cells have been shown to express TLR4 and low levels of TLR2. Human adventitial fibroblasts express a functional TLR4 as well. All these cells are able to produce a variety of cytokines after TLR stimulation. In the human atherosclerotic plaque, expression of TLR2 and TLR4 has been described in macrophages and the endothelial cells. But the question that remains is; which ligands are able to activate TLRs in the arterial wall?

A lot is known about the exogenous ligands that can activate the TLRs, but it is also known that endogenous ligands are able to activate TLRs when exogenous ligands are absent. These endogenous ligands for TLR4 are mostly produced during stress or cell damage. The best-described endogenous ligands for TLR4 are HSP60 [69], EDA [70], and to a lesser extend also hyaluronan [71], and biglycan [72]. The ligands HSP60, EDA and hyaluronan have previously been associated with arthritic [73] and oncological disease [74], and biglycan is found in septic lungs [75] and fibrogenesis [76] which have, like in atherosclerotic disease, local inflammation and matrix turn-over in common. Since no exogenous ligands are necessary for development of murine atherosclerosis [57], the endogenous TLR4 ligands may play a potential role in the development and progression of atherosclerosis.

EDA levels are elevated in plasma of ApoE-/- mice and EDA mRNA levels are increased during, TLR4-dependent, outward remodeling. Furthermore, in ApoE-/- mice lacking EDA, atherosclerotic lesion areas are reduced, lesions are thinner and lipid content of foam cells is lowered compared to lesions in animals that do express EDA fibronectin [77]. Heat-shock proteins are highly conserved molecules that participate in protein folding and assembly, and are crucial for correct transportation of proteins through the cell. Heat-shock protein synthesis is dramatically increased during cellular stress. The LPS-hyporesponsive C3H/HeJ mice strain, possessing a nonfunctional TLR4 owing to a point mutation in the TLR4 gene, is resistant to human HSP60-induced macrophage activation, which suggests a role for TLR4 in cellular activation by human HSP60 [69].

EDA is an extra domain of fibronectin incorporated by alternative splicing of fibronectin mRNA, which occurs during cell injury. EDA is able to induce the production of interleukins [78]. Okamura *et al* showed that EDA is capable of activating NF-κB via TLR4 [70]. Similar to LPS and HSP60, the response to EDA is blunted in C3H/HeJ cells. Hyaluronan is one of the major glycosaminoglycans of the extracellular matrix. During inflammation, hyaluronan is rapidly degraded and degradation products are able to activate DC via TLR4 [71]. Biglycan belongs to the family of small, leucine-rich repeat proteoglycans and interacts with fibrillar collagens, thereby modulating the organisation of the extracellular matrix. The leucine-rich repeats of biglycan protein facilitate protein-protein interactions. In addition, biglycan is able to bind SRs and the endocytic mannose receptor. Schaeffer *et al* demonstrated that biglycan interacts with TLR4 and to a lesser extent with TLR2 in macrophages [72] and is able to increase MyD88-dependent activation of NF-κB, resulting in expression of TNF-α by macrophages, followed by recruitment of more macrophages that are able to produce biglycan. In turn this biglycan is able to stimulate TLR2 and TLR4 resulting in a feed-

forward cycle. Biglycan has been shown to be present in murine atherosclerotic plaques [79] and human coronary lesions [80]. In addition, biglycan in human atherosclerotic plaques co-localizes with ApoE and ApoA-I which are characteristics of atherosclerotic disease [81]. It should be recognized, however, that recent publications have revealed that the endogenous TLR ligands often suffer from LPS contaminations.
The aforementioned endogenous ligands for TLR4 may be expressed when cells undergo pathological cell death or are damaged and may thereby subsequently result in inflammation and an immune response [69-71]. As endogenous ligands can also trigger TLR4, it is possible that this receptor plays a role in atherosclerotic lesion development in the absence of pathogens. Following vascular injury or functional arterial remodeling, expression of pro-inflammatory proteins may be essential for vascular repair. To understand their potential role in atherogenesis, it remains to be determined how expression and release of endogenous TLR-ligands is regulated.

Infectious pathogens and atherosclerosis

A number of infectious agents have been proposed as a potential cause of atherosclerotic disease since the late 1800s and early 1900s. Agents that have been implicated in atherosclerosis are cytomegalovirus (CMV), herpes simplex virus (HSV), *Helicobacter pylori* and *Chlamydia pneumoniae* [82,83]. Exposure to these organisms occurs early in life. However, literature shows a lot of conflicting data regarding the association between these infectious pathogens and atherosclerotic disease or cardiovascular events. And therefore it is difficult to draw conclusions regarding their role in disease pathogenesis.
CMV is a member of the herpes virus group, which includes HSV 1&2 and Epstein-Barr virus, and is a virus that infects most people world-wide. This virus is able to remain dormant within the body over a long period. CMV has been associated with an increased risk in atherosclerotic disease and a prior CMV infection has been linked to increased risk of restenosis after percutaneous coronary angioplasty [84]. There are, however, conflicting data about antibody responses against CMV and development of atherosclerosis. In a population-based cohort study Sorlie *et al* showed that high antibody levels against CMV correlate with an increased risk of coronary artery disease (CAD) during a 5-year follow-up [85], whereas Ridker *et al* could not find any association between antibodies against CMV or HSV and MI or stroke after 12-year follow-up in a prospective, nested case-control study [86].
H. pylori is a spiral-shaped Gram-negative bacterium that is known to cause infection of the stomach. Ameriso *et al* found that *H. pylori* is present in carotid atherosclerotic lesions [87] and Pieniazek showed that *H. pylori* infection increases the risk of CAD [88]. In contrast, Tsai *et al* demonstrated that *H. pylori* seropositivity is not associated with several coronary risk factors in either cases or controls [89]. A meta-analysis of 18 epidemiological studies involving over 10000 patients failed to demonstrate a significant association between *H. pylori* infection and CAD [90]. Furthermore, Menge *et al* stated in a review that data regarding the association between *H. pylori* infection and CAD are inconclusive and need further investigation [91].
In addition, several studies have shown that infectious burden (number of microbial infections) is associated with the extent of atherosclerotic disease and risk for

cardiovascular death [92,93]. With respect to a potential role for infectious agents in the development of atherosclerotic disease *C. pneumoniae* has been the best-described pathogen. *C. pneumoniae* is an intracellular Gram-negative bacterium and *C. pneumoniae* infection is ubiquitous and common in young adults, but has the highest incidence in the elderly. It is transmitted by respiratory aerosol transmission and causes 5-10% of all community-acquired pneumonia or bronchitis. The presence of *C. pneumoniae* in atherosclerotic lesions has been detected by PCR, immunocytochemical staining, immunofluorescence, and in situ hybridization. Gibbs *et al* reviewed 17 studies on the detection of *C. pneumoniae* and found that it is present in 303 of 597 (50.8%) atherosclerotic lesions versus 5 of 131 (3.8%) specimen without atherosclerosis [94]. Of 18 seroepidemiological studies performed before 1997, most of them show a positive association between *C. pneumoniae* antibodies and coronary disease, as reviewed by Danesh *et al* [82]. Most of these studies are cross-sectional or case-control studies. Danesh also performed a meta-analysis of 15 prospective studies published through May 2000 [95]. Again they found a positive correlation between *C. pneumoniae* infection and atherosclerotic disease, but the odds ratio found in this study was a lot lower than found in their first study (1.15 compared with 2.0). This was confirmed by Bloemenkamp *et al* who showed in a meta-analysis that the odds ratio for cross-sectional or case-control studies is higher than that of prospective studies [96]. These more recent data suggest that a strong causal relationship, as postulated by the first studies investigating the correlation between *C. pneumoniae* and atherosclerosis, is not very likely. Therefore it is still not known whether *C. pneumoniae* is able to induce the atherosclerotic process, might play a role in progression of the disease, or just simply is an innocent bystander that does not contribute to atherosclerotic development. The use of antibiotics effective against *C. pneumoniae* could be an important treatment option. Some small studies showed beneficial effects of anti-chlamydial antibiotics on outcomes of MI or angina pectoris [97]. Unfortunately, these studies are often too small and the treatments too short to show convincing evidence. The WIZARD study is a large, randomized trial that includes patients with previous MI and elevated *C. pneumoniae* titers. In this study a 3-month course of azithromycin shows no beneficial effects on secondary cardiac events after a median follow-up of 14 months [98]. Another large trial, Azithromycin and Coronary Events Study (ACES), showed similar negative results [99,100]. This is supported by a study by Gieffers *et al* showing that *C. pneumoniae* are able to survive within monocytes treated with high-dose, but short duration, azithromycin [101]. Andraws *et al* performed a meta-analysis on the effects of antibiotic therapy on the outcome of patients with CAD [102]. They found that there is no overall benefit of anti-chlamydial antibiotic therapy in reducing mortality or cardiovascular events in patients with CAD. The failure to improve clinical outcome by treatment with anti-chlamydial antibiotics does not exclude *C. pneumoniae* as a potential etiology of acute coronary events, but suggests that a single course of treatment might be ineffective over a lifetime of a patient or that *C. pneumoniae* are resistant to treatment.

Targeting the innate immune system as a treatment of atherosclerosis

Excess stimulation of host cells by bacterial LPS causes the pathogenesis of Gram-negative septic shock. This stimulation leads to expression and release of pro-inflammatory marker genes, which in turn can initiate events leading to systemic toxicity. However, cells that are part of the innate immune system respond to initial exposure of LPS to defend the host from a Gram-negative infection. It has been reported that TLR4-derived signaling pathways are differentially modulated by fatty acids [103]. Epidemiological and biochemical studies have shown that different types of dietary fatty acids can modify the risks of many chronic diseases, such as atherosclerotic diseases, cancer and inflammatory diseases. Unfortunately, the molecular and cellular mechanisms by which dietary fatty acids exert such effects are not well understood. As already mentioned, TLR4 is the LPS receptor and it has been demonstrated that LPS responsiveness can be determined by activation of NF-κB and expression of cyclooxygenase 2 (COX-2). It has been demonstrated that saturated fatty acids (SFAs), but not unsaturated fatty acids (UFAs), induce NF-κB activation and expression of COX-2 and other inflammatory markers in macrophages.

To prevent LPS toxicity, some research groups have investigated the effects of using a receptor antagonist to block activation of cells. Lipid A is the unique fatty-acylated diphosphorylated diglucosamine portion of LPS that is a common element of LPS from most pathogenic bacteria and is mainly responsible for the toxic effects of LPS [104]. Therefore, antagonism of the interaction of lipid A with target cells is an attractive target for the treatment of sepsis, septic shock or other indications. Addition of the first lipid A analogue, E5531, to blood *in vitro* and *in vivo*, inhibited LPS but the activity of the analogue decreased as a function of time, due to interaction of E5531 with plasma lipoprotiens [105]. Mullarkey *et al* described the activity of E5564, a second-generation LPS antagonist derived from the structure of *R. sphaeroides* [106]. Compared with E5531, E5564 is structurally and synthetically less complex, and has even superior activity and pharmacological characteristics. *In vivo* studies showed that administration of low doses E5564, also known as eritoran, into human volunteers and animals results in extremely active eritoran when co-administered with LPS, but its activity decreases shortly after ending the infusions [107]. Still, eritoran is inactivated more slowly than E5531, suggesting that eritoran may be a more pharmacological effective antagonist of LPS [106]. In a study performed by Rossignol *et al* healthy volunteers were injected with high doses of eritoran. The majority of eritoran bound to high-density lipoproteins and for up to 72 hours after injection, the *ex vivo* response of blood to LPS was inhibited by ≥85% [108]. *In vitro* analyses revealed that eritoran is inactivated by high-density lipoproteins, but not by low-density, very low-density lipoproteins, or albumin. Eritoran has also been used in a phase II clinical trial. Patients with severe sepsis were randomized to receive a high dose eritoran, a low dose eritoran, or placebo. Twenty-eight-day all-cause mortality in the high dose group was reduced compared to placebo.

Vaccination against atherosclerosis

Atherosclerosis is an inflammatory disease in which the immune system plays an important role too. The role for immunity and therefore immune cells in atherosclerosis

suggests important similarities to autoimmune disorders, such as arthritis, lupus, and multiple sclerosis [109]. A lot of research has been done on the effect of vaccination on development and progression of autoimmune disorders and it has been shown that autoimmune diseases can be prevented by pretreatment with vaccines [110]. So, the next important question is whether vaccination might prevent atherosclerosis as well.

As already discussed autoantigens like HSP60 and OxLDL play an important role in atherosclerosis. Furthermore, autoantibodies against these autoantigens have been shown to be associated with atherosclerosis. Therefore, *in vivo* experiments were performed to investigate the effect of active immunization of naïve animals with these antibodies. The idea is that immunization will result in a specific immune response that is able to attenuate atherosclerosis. Several studies showed the effect of immunization with malondialdehyde (MDA)-LDL and OxLDL on atherogenesis. George *et al* demonstrated that immunization of ApoE-/- mice with MDA-LDL for 12 weeks at 2-week intervals results in high titers of anti-MDA-LDL antibodies and atherosclerotic lesion size at the aortic sinus is significantly lower as compared with littermates that are immunized with PBS [111]. Immunization of hypercholesterolemic rabbits with homologous LDL at the start of a cholesterol-rich diet and at 3 weeks results in increased levels of antibodies against LDL at 16 weeks and significantly reduced atherosclerotic lesions in the proximal aorta [112]. In addition, Palinski *et al* immunized LDL receptor-deficient rabbits with homologous MDA-LDL, which generates high-titers of autoantibodies and results in reduction in the extent of atherosclerotic lesions in the aortic tree after 6.5 months [113]. All these studies suggest that an immune response to modified LDL protects against the development of atherosclerosis.

In contrast, there are also conflicting results. Increased levels of antibodies against OxLDL predicted progression of carotid atherosclerosis in a case-control study among 60 Finnish men [114] and predicted MI occurrence and mortality in a prospective nested case-control study among 50-year-old men that were followed for 20 years [115].

It has been postulated that immunization with OxLDL results in production of two different antibodies; pathogenic antibodies, that are able to promote atherosclerosis, and autoantibodies, which have a protective role in atherosclerosis. These antibodies can be cleared as autoantibody-antigen complex instead of accumulating in the arterial wall. Shaw *et al* were able to isolate monoclonal antibodies to epitopes of MDA-LDL and OxLDL that could inhibit the uptake of OxLDL by macrophages and antibodies that could bind apoptotic cells and inhibit their phagocytosis by macrophages [116].

DC are able to induce innate and adaptive immune responses. Binding to pathogens triggers maturation of DC. This is followed by secretion of cytokines and chemokines by the DC that recruit innate cells such as macrophages and neutrophils to the site of infection. The dendritic cells migrate to draining lymph nodes, where they activate and expand antigen-specific T-lymphocytes, cells that are part of the adaptive immune system. Activated T-lymphocytes migrate to the site of inflammation, clear the infection and give rise to memory. CD4+ T-lymphocytes can be divided in Th1 and Th2 cells that counterbalance each other. Th1 cells induce macrophage activation,

promote inflammation by secreting IFN-γ and Th1 differentiation can be promoted by IL-12, IL-23 and IL-27 [117]. Th2 cells suppress inflammation and reduce activity of macrophages via secretion of anti-inflammatory cytokines such as IL-4, IL-5, IL-10 and IL-13 [117]. TLR ligands generally induce antigen-presenting cells to produce IL-12 and therefore favor Th1-type adaptive immune responses. DC that are activated with imiquimod and R848 (both TLR7 ligands) switch from an immature to a mature phenotype and induce CD4+ T-lymphocyte differentiation into Th1 cells mediated by IL-12 and IFN-α [118]. However, MyD88-deficient mice fail to generate Th1-type immune responses to *Mycobacterium tuberculosis*, which can activate TLRs such as TLR2 and TLR4, whereas Th2 responses are completely preserved [119]. Re *et al* demonstrated that TLR4 stimulation by LPS induces the production of IL-12 p70 and interferon gamma-inducible protein (IP)-10, which both are associated to Th1 responses [120]. In contrast, TLR2 stimulation by PG fails to induce the production of IL-12 p70 and IP-10 but does result, however, in the release of IL-12 inhibitory p40 homodimer, producing conditions that are predicted to favor Th2 development. Lauw *et al* performed a study in which healthy volunteers received an intravenous injection of LPS [121]. After 3-6 hours whole-blood production of Th1 cytokines IFN-γ and IL-2 is significantly reduced while production of Th2 cytokines IL-4 and IL-5 is slightly increased. So, LPS exposure influences lymphocyte cytokine production, resulting in a shift from a Th1 toward a Th2 cytokine response. Some studies found that LPS has no effect on Th2 responses [122] and since that discovery it appeared that the dose and timing of LPS exposure are critical determinants. When LPS is used at a low dose, it causes allergen-specific Th2 responses and increases levels of IL-4, IL-5 and IL-13, whereas high dose LPS induces a Th1 response [123].

Emeson showed that deletion of CD4+ and CD8+ T-lymphocytes reduces fatty streak formation in C57BL/6 mice [124]. Furthermore, as shown by Zhou *et al* atherosclerosis is enhanced after transfer of CD4+ T-lymphocytes from atherosclerotic ApoE-/- mice into apoE-/- × SCID-/- immunodeficient mice, indicating a pro-atherogenic role of T-lymphocytes [125]. The transplanted cells produce high levels of IFN-γ, suggesting a Th1-related pro-atherogenic effect. Some studies suggest that Th2-driven immune responses may be athero-protective. In mouse models a switch towards Th2-driven immunity is associated with production of protective OxLDL antibodies [126]. In ApoE-/- mice antibodies against MDA-LDL are associated with reduced atherosclerotic lesion size [111,112].

So, although consistence exists on the fact that Th1 cells in general are pro-atherogenic and Th2 cells anti-atherogenic, the effect of TLR ligands on the balance between Th1 and Th2 is less clear. Some TLR ligands and in certain doses cause athero-protective Th2 responses, whereas exposure to other ligands might cause Th1 responses inducing atherosclerotic development.

Summary

Infectious pathogens have been implicated to play an important role in atherosclerosis. However, literature shows a lot of conflicting data regarding the association between these infectious pathogens and atherosclerotic disease or cardiovascular events. Data of studies performed before 1997 show positive correlations between *C. pneumoniae* infection and coronary disease, whereas studies performed at later time points suggest that a causal relationship between *C. pneumoniae* and atherosclerosis, is not very likely.

Besides infection, exogenous and endogenous ligands of the TLRs, and therefore the signaling pathway elicited via TLRs, play an important role in innate immunity and atherosclerosis. Already 11 TLRs have been found and, in addition, for most of the TLRs the ligands have been identified. Some TLRs need to form dimers before a signaling cascade can be initiated. These activated intracellular signaling cascades may result in protection of the host. Extensive investigations showed that a number of endogenous molecules, such as EDA of Fibronectin and HSP60, may be important activators of the immune system resulting in induction of pro-inflammatory cytokine production by monocytes and macrophages. These endogenous molecules are able to mediate these effects via TLR stimulation comparable to TLR stimulation by LPS and bacterial lipoproteins. Some studies however reported that the endogenous ligands are a result of contaminated pathogens. Therefore, the role of these endogenous ligands has to be studied more intensively in mice.

Manipulation of the effects of infectious agents or of the TLR signaling pathways might be important as therapy for human disease, but still there are considerable risks associated with such manipulations. TLR agonists might be used as vaccines and antagonists of these ligands might improve prognosis of septic patients. Unfortunately, the other side of the coin might be that these agonists are able to increase atherosclerotic development. Since it has been found that TLR polymorphisms protect against the progression of atherosclerotic disease, in the future the genotype of patients will be an important factor in determining the best therapy for the patient. Future studies will continue examining the mechanisms of disease development and eventually might lead to the discovery of new targets to intervene in the development of atherosclerosis.

REFERENCES

1. Malek AM, Alper SL, Izumo S. Hemodynamic shear stress and its role in atherosclerosis. JAMA 1999;282:2035-42.
2. Strong JP, Malcom GT, McMahan CA, Tracy RE, Newman WP, III, Herderick EE, et al. Prevalence and extent of atherosclerosis in adolescents and young adults: implications for prevention from the Pathobiological Determinants of Atherosclerosis in Youth Study. JAMA 1999;281:727-35.
3. Hansson GK. Immune mechanisms in atherosclerosis. Arterioscler Thromb Vasc Biol 2001;21:1876-90.
4. Hansson GK, Libby P, Schonbeck U, Yan ZQ. Innate and adaptive immunity in the pathogenesis of atherosclerosis. Circ Res 2002;91:281-91.
5. Steinberg D. Low density lipoprotein oxidation and its pathobiological significance. J Biol Chem 1997;272:20963-6.
6. Camejo G, Hurt-Camejo E, Wiklund O, Bondjers G. Association of apo B lipoproteins with arterial proteoglycans: pathological significance and molecular basis. Atherosclerosis 1998;139:205-22.
7. Williams KJ, Tabas I. The response-to-retention hypothesis of atherogenesis reinforced. Curr Opin Lipidol 1998;9:471-4.
8. Beekhuizen H, van Furth R. Monocyte adherence to human vascular endothelium. J Leukoc Biol 1993;54:363-78.
9. Jang Y, Lincoff AM, Plow EF, Topol EJ. Cell adhesion molecules in coronary artery disease. J Am Coll Cardiol 1994;24:1591-601.
10. McEver RP. Selectins. Curr Opin Immunol 1994;6:75-84.
11. Moore KL, Patel KD, Bruehl RE, Li F, Johnson DA, Lichenstein HS, et al. P-selectin glycoprotein ligand-1 mediates rolling of human neutrophils on P-selectin. J Cell Biol 1995;128:661-71.
12. Asa D, Raycroft L, Ma L, Aeed PA, Kaytes PS, Elhammer AP, et al. The P-selectin glycoprotein ligand functions as a common human leukocyte ligand for P- and E-selectins. J Biol Chem 1995;270:11662-70.
13. Dong ZM, Chapman SM, Brown AA, Frenette PS, Hynes RO, Wagner DD. The combined role of P- and E-selectins in atherosclerosis. J Clin Invest 1998;102:145-52.
14. Chuluyan HE, Issekutz AC. VLA-4 integrin can mediate CD11/CD18-independent transendothelial migration of human monocytes. J Clin Invest 1993;92:2768-77.
15. Springer TA. Traffic signals for lymphocyte recirculation and leukocyte emigration: the multistep paradigm. Cell 1994;76:301-14.
16. Muller WA, Randolph GJ. Migration of leukocytes across endothelium and beyond: molecules involved in the transmigration and fate of monocytes. J Leukoc Biol 1999;66:698-704.
17. Ross R. Atherosclerosis--an inflammatory disease. N Engl J Med 1999;340:115-26.
18. Lusis AJ. Atherosclerosis. Nature 2000;407:233-41.
19. Nicoletti A, Caligiuri G, Tornberg I, Kodama T, Stemme S, Hansson GK. The macrophage scavenger receptor type A directs modified proteins to antigen presentation. Eur J Immunol 1999;29:512-21.
20. Hashimoto C, Hudson KL, Anderson KV. The Toll gene of Drosophila, required for dorsal-ventral embryonic polarity, appears to encode a transmembrane protein. Cell 1988;52:269-79.
21. Medzhitov R, Preston-Hurlburt P, Janeway CA, Jr. A human homologue of the Drosophila Toll protein signals activation of adaptive immunity. Nature 1997;388:394-7.
22. Rock FL, Hardiman G, Timans JC, Kastelein RA, Bazan JF. A family of human receptors structurally related to Drosophila Toll. Proc Natl Acad Sci U S A 1998;95:588-93.
23. Abreu MT, Fukata M, Arditi M. TLR signaling in the gut in health and disease. J Immunol 2005;174:4453-60.
24. Takeda K, Kaisho T, Akira S. Toll-like receptors. Annu Rev Immunol 2003;21:335-76.
25. Abreu MT, Arditi M. Innate immunity and toll-like receptors: clinical implications of basic science research. J Pediatr 2004;144:421-9.
26. Takeuchi O, Kawai T, Sanjo H, Copeland NG, Gilbert DJ, Jenkins NA, et al. TLR6: A novel member of an expanding toll-like receptor family. Gene 1999;231:59-65.
27. Chuang TH, Ulevitch RJ. Cloning and characterization of a sub-family of human toll-like receptors: hTLR7, hTLR8 and hTLR9. Eur Cytokine Netw 2000;11:372-8.

28. Du X, Poltorak A, Wei Y, Beutler B. Three novel mammalian toll-like receptors: gene structure, expression, and evolution. Eur Cytokine Netw 2000;11:362-71.

29. Takeuchi O, Sato S, Horiuchi T, Hoshino K, Takeda K, Dong Z, et al. Cutting edge: role of Toll-like receptor 1 in mediating immune response to microbial lipoproteins. J Immunol 2002;169:10-4.

30. Takeuchi O, Kawai T, Muhlradt PF, Morr M, Radolf JD, Zychlinsky A, et al. Discrimination of bacterial lipoproteins by Toll-like receptor 6. Int Immunol 2001;13:933-40.

31. Ozinsky A, Underhill DM, Fontenot JD, Hajjar AM, Smith KD, Wilson CB, et al. The repertoire for pattern recognition of pathogens by the innate immune system is defined by cooperation between toll-like receptors. Proc Natl Acad Sci U S A 2000;97:13766-71.

32. Means TK, Lien E, Yoshimura A, Wang S, Golenbock DT, Fenton MJ. The CD14 ligands lipoarabinomannan and lipopolysaccharide differ in their requirement for Toll-like receptors. J Immunol 1999;163:6748-55.

33. Takeuchi O, Hoshino K, Kawai T, Sanjo H, Takada H, Ogawa T, et al. Differential roles of TLR2 and TLR4 in recognition of gram-negative and gram-positive bacterial cell wall components. Immunity 1999;11:443-51.

34. Kawasaki K, Akashi S, Shimazu R, Yoshida T, Miyake K, Nishijima M. Involvement of TLR4/MD-2 complex in species-specific lipopolysaccharide-mimetic signal transduction by Taxol. J Endotoxin Res 2001;7:232-6.

35. Lee JY, Sohn KH, Rhee SH, Hwang D. Saturated fatty acids, but not unsaturated fatty acids, induce the expression of cyclooxygenase-2 mediated through Toll-like receptor 4. J Biol Chem 2001;276:16683-9.

36. Alexopoulou L, Holt AC, Medzhitov R, Flavell RA. Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. Nature 2001;413:732-8.

37. Hayashi F, Smith KD, Ozinsky A, Hawn TR, Yi EC, Goodlett DR, et al. The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5. Nature 2001;410:1099-103.

38. Lund JM, Alexopoulou L, Sato A, Karow M, Adams NC, Gale NW, et al. Recognition of single-stranded RNA viruses by Toll-like receptor 7. Proc Natl Acad Sci U S A 2004;101:5598-603.

39. Hemmi H, Kaisho T, Takeuchi O, Sato S, Sanjo H, Hoshino K, et al. Small anti-viral compounds activate immune cells via the TLR7 MyD88-dependent signaling pathway. Nat Immunol 2002;3:196-200.

40. Hemmi H, Takeuchi O, Kawai T, Kaisho T, Sato S, Sanjo H, et al. A Toll-like receptor recognizes bacterial DNA. Nature 2000;408:740-5.

41. Jurk M, Heil F, Vollmer J, Schetter C, Krieg AM, Wagner H, et al. Human TLR7 or TLR8 independently confer responsiveness to the antiviral compound R-848. Nat Immunol 2002;3:499.

42. Akira S. Toll-like receptor signaling. J Biol Chem 2003;278:38105-8.

43. Takeda K, Akira S. TLR signaling pathways. Semin Immunol 2004;16:3-9.

44. Akira S, Takeda K. Functions of toll-like receptors: lessons from KO mice. C R Biol 2004;327:581-9.

45. Palsson-McDermott EM, O'Neill LA. Signal transduction by the lipopolysaccharide receptor, Toll-like receptor-4. Immunology 2004;113:153-62.

46. Bannerman DD, Goldblum SE. Direct effects of endotoxin on the endothelium: barrier function and injury. Lab Invest 1999;79:1181-99.

47. Zeuke S, Ulmer AJ, Kusumoto S, Katus HA, Heine H. TLR4-mediated inflammatory activation of human coronary artery endothelial cells by LPS. Cardiovasc Res 2002;56:126-34.

48. Moller AS, Ovstebo R, Westvik AB, Joo GB, Haug KB, Kierulf P. Effects of bacterial cell wall components (PAMPs) on the expression of monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein-1alpha (MIP-1alpha) and the chemokine receptor CCR2 by purified human blood monocytes. J Endotoxin Res 2003;9:349-60.

49. Gu L, Okada Y, Clinton SK, Gerard C, Sukhova GK, Libby P, et al. Absence of monocyte chemoattractant protein-1 reduces atherosclerosis in low density lipoprotein receptor-deficient mice. Mol Cell 1998;2:275-81.

50. Boring L, Gosling J, Cleary M, Charo IF. Decreased lesion formation in CCR2-/- mice reveals a role for chemokines in the initiation of atherosclerosis. Nature 1998;394:894-7.

51. Vink A, Schoneveld AH, van der Meer JJ, van Middelaar BJ, Sluijter JP, Smeets MB, et al. *In vivo* evidence for a role of toll-like receptor 4 in the development of intimal lesions. Circulation 2002;106:1985-90.

52. Hollestelle SC, De Vries MR, Van Keulen JK, Schoneveld AH, Vink A, Strijder CF, et al. Toll-like receptor 4 is involved in outward arterial remodeling. Circulation 2004;109:393-8.

53. Lehr HA, Sagban TA, Ihling C, Zahringer U, Hungerer KD, Blumrich M, et al. Immunopathogenesis of atherosclerosis: endotoxin accelerates atherosclerosis in rabbits on hypercholesterolemic diet. Circulation 2001;104:914-20.

54. Ostos MA, Recalde D, Zakin MM, Scott-Algara D. Implication of natural killer T cells in atherosclerosis development during a LPS-induced chronic inflammation. FEBS Lett 2002;519:23-9.

55. Michelsen KS, Wong MH, Shah PK, Zhang W, Yano J, Doherty TM, et al. Lack of Toll-like receptor 4 or myeloid differentiation factor 88 reduces atherosclerosis and alters plaque phenotype in mice deficient in apolipoprotein E. Proc Natl Acad Sci U S A 2004;101:10679-84.

56. Bjorkbacka H, Kunjathoor VV, Moore KJ, Koehn S, Ordija CM, Lee MA, et al. Reduced atherosclerosis in MyD88-null mice links elevated serum cholesterol levels to activation of innate immunity signaling pathways. Nat Med 2004;10:416-21.

57. Wright SD, Burton C, Hernandez M, Hassing H, Montenegro J, Mundt S, et al. Infectious agents are not necessary for murine atherogenesis. J Exp Med 2000;191:1437-42.

58. Edfeldt K, Swedenborg J, Hansson GK, Yan ZQ. Expression of toll-like receptors in human atherosclerotic lesions: a possible pathway for plaque activation. Circulation 2002;105:1158-61.

59. Xu XH, Shah PK, Faure E, Equils O, Thomas L, Fishbein MC, et al. Toll-like receptor-4 is expressed by macrophages in murine and human lipid-rich atherosclerotic plaques and upregulated by oxidized LDL. Circulation 2001;104:3103-8.

60. Laman JD, Schoneveld AH, Moll FL, van Meurs M, Pasterkamp G. Significance of peptidoglycan, a proinflammatory bacterial antigen in atherosclerotic arteries and its association with vulnerable plaques. Am J Cardiol 2002;90:119-23.

61. Travassos LH, Girardin SE, Philpott DJ, Blanot D, Nahori MA, Werts C, et al. Toll-like receptor 2-dependent bacterial sensing does not occur via peptidoglycan recognition. EMBO Rep 2004;5:1000-6.

62. Arbour NC, Lorenz E, Schutte BC, Zabner J, Kline JN, Jones M, et al. TLR4 mutations are associated with endotoxin hyporesponsiveness in humans. Nat Genet 2000;25:187-91.

63. Kiechl S, Lorenz E, Reindl M, Wiedermann CJ, Oberhollenzer F, Bonora E, et al. Toll-like receptor 4 polymorphisms and atherogenesis. N Engl J Med 2002;347:185-92.

64. Ameziane N, Beillat T, Verpillat P, Chollet-Martin S, Aumont MC, Seknadji P, et al. Association of the Toll-like receptor 4 gene Asp299Gly polymorphism with acute coronary events. Arterioscler Thromb Vasc Biol 2003;23:e61-e64.

65. Edfeldt K, Bennet AM, Eriksson P, Frostegard J, Wiman B, Hamsten A, et al. Association of hypo-responsive toll-like receptor 4 variants with risk of myocardial infarction. Eur Heart J 2004;25:1447-53.

66. Zee RY, Hegener HH, Gould J, Ridker PM. Toll-like receptor 4 Asp299Gly gene polymorphism and risk of atherothrombosis. Stroke 2005;36:154-7.

67. Netea MG, Hijmans A, van Wissen S, Smilde TJ, Trip MD, Kullberg BJ, et al. Toll-like receptor-4 Asp299Gly polymorphism does not influence progression of atherosclerosis in patients with familial hypercholesterolaemia. Eur J Clin Invest 2004;34:94-9.

68. Yang IA, Holloway JW, Ye S. TLR4 Asp299Gly polymorphism is not associated with coronary artery stenosis. Atherosclerosis 2003;170:187-90.

69. Ohashi K, Burkart V, Flohe S, Kolb H. Cutting edge: heat shock protein 60 is a putative endogenous ligand of the toll-like receptor-4 complex. J Immunol 2000;164:558-61.

70. Okamura Y, Watari M, Jerud ES, Young DW, Ishizaka ST, Rose J, et al. The extra domain A of fibronectin activates Toll-like receptor 4. J Biol Chem 2001;276:10229-33.

71. Termeer C, Benedix F, Sleeman J, Fieber C, Voith U, Ahrens T, et al. Oligosaccharides of Hyaluronan activate dendritic cells via toll-like receptor 4. J Exp Med 2002;195:99-111.

72. Schaefer L, Babelova A, Kiss E, Hausser HJ, Baliova M, Krzyzankova M, et al. The matrix component biglycan is proinflammatory and signals through Toll-like receptors 4 and 2 in macrophages. J Clin Invest 2005;115:2223-33.

73. Shiozawa K, Hino K, Shiozawa S. Alternatively spliced EDA-containing fibronectin in synovial fluid as a predictor of rheumatoid joint destruction. Rheumatology (Oxford) 2001;40:739-42.

74. Adamia S, Maxwell CA, Pilarski LM. Hyaluronan and hyaluronan synthases: potential therapeutic targets in cancer. Curr Drug Targets Cardiovasc Haematol Disord 2005;5:3-14.

75. Venkatesan N, Ebihara T, Roughley PJ, Ludwig MS. Alterations in large and small proteoglycans in bleomycin-induced pulmonary fibrosis in rats. Am J Respir Crit Care Med 2000;161:2066-73.

76. Sime PJ, Sarnstrand B, Xing Z, Graham F, Fisher L, Gauldie J. Adenovirus-mediated gene transfer of the proteoglycan biglycan induces fibroblastic responses in the lung. Chest 1997;111:137S.

77. Tan MH, Sun Z, Opitz SL, Schmidt TE, Peters JH, George EL. Deletion of the alternatively spliced fibronectin EIIIA domain in mice reduces atherosclerosis. Blood 2004;104:11-8.

78. Saito S, Yamaji N, Yasunaga K, Saito T, Matsumoto S, Katoh M, et al. The fibronectin extra domain A activates matrix metalloproteinase gene expression by an interleukin-1-dependent mechanism. J Biol Chem 1999;274:30756-63.

79. Kunjathoor VV, Chiu DS, O'Brien KD, LeBoeuf RC. Accumulation of biglycan and perlecan, but not versican, in lesions of murine models of atherosclerosis. Arterioscler Thromb Vasc Biol 2002;22:462-8.

80. Riessen R, Isner JM, Blessing E, Loushin C, Nikol S, Wight TN. Regional differences in the distribution of the proteoglycans biglycan and decorin in the extracellular matrix of atherosclerotic and restenotic human coronary arteries. Am J Pathol 1994;144:962-74.

81. O'Brien KD, Olin KL, Alpers CE, Chiu W, Ferguson M, Hudkins K, et al. Comparison of apolipoprotein and proteoglycan deposits in human coronary atherosclerotic plaques: colocalization of biglycan with apolipoproteins. Circulation 1998;98:519-27.

82. Danesh J, Collins R, Peto R. Chronic infections and coronary heart disease: is there a link? Lancet 1997;350:430-6.

83. Ridker PM. Inflammation, infection, and cardiovascular risk: how good is the clinical evidence? Circulation 1998;97:1671-4.

84. Zhou YF, Leon MB, Waclawiw MA, Popma JJ, Yu ZX, Finkel T, et al. Association between prior cytomegalovirus infection and the risk of restenosis after coronary atherectomy. N Engl J Med 1996;335:624-30.

85. Sorlie PD, Nieto FJ, Adam E, Folsom AR, Shahar E, Massing M. A prospective study of cytomegalovirus, herpes simplex virus 1, and coronary heart disease: the atherosclerosis risk in communities (ARIC) study. Arch Intern Med 2000;160:2027-32.

86. Ridker PM, Hennekens CH, Stampfer MJ, Wang F. Prospective study of herpes simplex virus, cytomegalovirus, and the risk of future myocardial infarction and stroke. Circulation 1998;98:2796-9.

87. Ameriso SF, Fridman EA, Leiguarda RC, Sevlever GE. Detection of *Helicobacter pylori* in human carotid atherosclerotic plaques. Stroke 2001;32:385-91.

88. Pieniazek P, Karczewska E, Duda A, Tracz W, Pasowicz M, Konturek SJ. Association of *Helicobacter pylori* infection with coronary heart disease. J Physiol Pharmacol 1999;50:743-51.

89. Tsai CJ, Huang TY. Relation of *Helicobacter pylori* infection and angiographically demonstrated coronary artery disease. Dig Dis Sci 2000;45:1227-32.

90. Danesh J, Peto R. Risk factors for coronary heart disease and infection with *Helicobacter pylori*: meta-analysis of 18 studies. BMJ 1998;316:1130-2.

91. Menge H, Lang A, Brosius B, Hopert R, Lollgen H. [*Helicobacter pylori* and coronary heart diseases--hypotheses and facts]. Z Gastroenterol 2000;38:315-23.

92. Espinola-Klein C, Rupprecht HJ, Blankenberg S, Bickel C, Kopp H, Rippin G, et al. Impact of infectious burden on extent and long-term prognosis of atherosclerosis. Circulation 2002;105:15-21.

93. Rupprecht HJ, Blankenberg S, Bickel C, Rippin G, Hafner G, Prellwitz W, et al. Impact of viral and bacterial infectious burden on long-term prognosis in patients with coronary artery disease. Circulation 2001;104:25-31.

94. Gibbs RG, Carey N, Davies AH. *Chlamydia pneumoniae* and vascular disease. Br J Surg 1998;85:1191-7.

95. Danesh J, Whincup P, Walker M, Lennon L, Thomson A, Appleby P, et al. *Chlamydia pneumoniae* IgG titres and coronary heart disease: prospective study and meta-analysis. BMJ 2000;321:208-13.

96. Bloemenkamp DG, Mali WP, Visseren FL, van der GY. Meta-analysis of sero-epidemiologic studies of the relation between *Chlamydia pneumoniae* and atherosclerosis: does study design influence results? Am Heart J 2003;145:409-17.

97. Grayston JT. Antibiotic treatment of atherosclerotic cardiovascular disease. Circulation 2003;107:1228-30.

98. O'Connor CM, Dunne MW, Pfeffer MA, Muhlestein JB, Yao L, Gupta S, et al. Azithromycin for the secondary prevention of coronary heart disease events: the WIZARD study: a randomized controlled trial. JAMA 2003;290:1459-66.

99. Jackson LA. Description and status of the azithromycin and coronary events study (ACES). J Infect Dis 2000;181 Suppl 3:S579-S581.

100. Pokrovskaia EV. [Final confirmation of inefficiency of antibiotics in secondary prevention of coronary heart disease. Results of PROVE IT (gatifloxacin trial) and ACES]. Kardiologiia 2004;44:82-3.

101. Gieffers J, Fullgraf H, Jahn J, Klinger M, Dalhoff K, Katus HA, et al. *Chlamydia pneumoniae* infection in circulating human monocytes is refractory to antibiotic treatment. Circulation 2001;103:351-6.

102. Andraws R, Berger JS, Brown DL. Effects of antibiotic therapy on outcomes of patients with coronary artery disease: a meta-analysis of randomized controlled trials. JAMA 2005;293:2641-7.

103. Rhee SH, Hwang D. Murine TOLL-like receptor 4 confers lipopolysaccharide responsiveness as determined by activation of NF kappa B and expression of the inducible cyclooxygenase. J Biol Chem 2000;275:34035-40.

104. Takada H, Kotani S. Structural requirements of lipid A for endotoxicity and other biological activities. Crit Rev Microbiol 1989;16:477-523.

105. Rose JR, Mullarkey MA, Christ WJ, Hawkins LD, Lynn M, Kishi Y, et al. Consequences of interaction of a lipophilic endotoxin antagonist with plasma lipoproteins. Antimicrob Agents Chemother 2000;44:504-10.

106. Mullarkey M, Rose JR, Bristol J, Kawata T, Kimura A, Kobayashi S, et al. Inhibition of endotoxin response by e5564, a novel Toll-like receptor 4-directed endotoxin antagonist. J Pharmacol Exp Ther 2003;304:1093-102.

107. Wong YN, Rossignol D, Rose JR, Kao R, Carter A, Lynn M. Safety, pharmacokinetics, and pharmacodynamics of E5564, a lipid A antagonist, during an ascending single-dose clinical study. J Clin Pharmacol 2003;43:735-42.

108. Rossignol DP, Wasan KM, Choo E, Yau E, Wong N, Rose J, et al. Safety, pharmacokinetics, pharmacodynamics, and plasma lipoprotein distribution of eritoran (E5564) during continuous intravenous infusion into healthy volunteers. Antimicrob Agents Chemother 2004;48:3233-40.

109. Wick G, Knoflach M, Xu Q. Autoimmune and inflammatory mechanisms in atherosclerosis. Annu Rev Immunol 2004;22:361-403.

110. von Herrath MG. Vaccination to prevent type 1 diabetes. Expert Rev Vaccines 2002;1:25-8.

111. George J, Afek A, Gilburd B, Levkovitz H, Shaish A, Goldberg I, et al. Hyperimmunization of apo-E-deficient mice with homologous malondialdehyde low-density lipoprotein suppresses early atherogenesis. Atherosclerosis 1998;138:147-52.

112. Ameli S, Hultgardh-Nilsson A, Regnstrom J, Calara F, Yano J, Cercek B, et al. Effect of immunization with homologous LDL and oxidized LDL on early atherosclerosis in hypercholesterolemic rabbits. Arterioscler Thromb Vasc Biol 1996;16:1074-9.

113. Palinski W, Miller E, Witztum JL. Immunization of low density lipoprotein (LDL) receptor-deficient rabbits with homologous malondialdehyde-modified LDL reduces atherogenesis. Proc Natl Acad Sci U S A 1995;92:821-5.

114. Salonen JT, Yla-Herttuala S, Yamamoto R, Butler S, Korpela H, Salonen R, et al. Autoantibody against oxidised LDL and progression of carotid atherosclerosis. Lancet 1992;339:883-7.

115. Wu R, Nityanand S, Berglund L, Lithell H, Holm G, Lefvert AK. Antibodies against cardiolipin and oxidatively modified LDL in 50-year-old men predict myocardial infarction. Arterioscler Thromb Vasc Biol 1997;17:3159-63.

116. Shaw PX, Horkko S, Tsimikas S, Chang MK, Palinski W, Silverman GJ, et al. Human-derived anti-oxidized LDL autoantibody blocks uptake of oxidized LDL by macrophages and localizes to atherosclerotic lesions *in vivo*. Arterioscler Thromb Vasc Biol 2001;21:1333-9.

117. Robinson DS, O'Garra A. Further checkpoints in Th1 development. Immunity 2002;16:755-8.

118. Ito T, Amakawa R, Kaisho T, Hemmi H, Tajima K, Uehira K, et al. Interferon-alpha and interleukin-12 are induced differentially by Toll-like receptor 7 ligands in human blood dendritic cell subsets. J Exp Med 2002;195:1507-12.

119. Su SB, Silver PB, Grajewski RS, Agarwal RK, Tang J, Chan CC, et al. Essential role of the MyD88 pathway, but nonessential roles of TLRs 2, 4, and 9, in the adjuvant effect promoting Th1-mediated autoimmunity. J Immunol 2005;175:6303-10.

120. Re F, Strominger JL. Toll-like receptor 2 (TLR2) and TLR4 differentially activate human dendritic cells. J Biol Chem 2001;276:37692-9.

121. Lauw FN, ten Hove T, Dekkers PE, de Jonge E, van Deventer SJ, van Der PT. Reduced Th1, but not Th2, cytokine production by lymphocytes after *in vivo* exposure of healthy subjects to endotoxin. Infect Immun 2000;68:1014-8.

122. Cochran JR, Khan AM, Elidemir O, Xue H, Cua B, Fullmer J, et al. Influence of lipopolysaccharide exposure on airway function and allergic responses in developing mice. Pediatr Pulmonol 2002;34:267-77.

123. Eisenbarth SC, Piggott DA, Huleatt JW, Visintin I, Herrick CA, Bottomly K. Lipopolysaccharide-enhanced, toll-like receptor 4-dependent T helper cell type 2 responses to inhaled antigen. J Exp Med 2002;196:1645-51.

124. Emeson EE, Shen ML, Bell CG, Qureshi A. Inhibition of atherosclerosis in CD4 T-cell-ablated and nude (nu/nu) C57BL/6 hyperlipidemic mice. Am J Pathol 1996;149:675-85.

125. Zhou X, Nicoletti A, Elhage R, Hansson GK. Transfer of CD4(+) T cells aggravates atherosclerosis in immunodeficient apolipoprotein E knockout mice. Circulation 2000;102:2919-22.

126. Zhou X, Paulsson G, Stemme S, Hansson GK. Hypercholesterolemia is associated with a T helper (Th) 1/Th2 switch of the autoimmune response in atherosclerotic apo E-knockout mice. J Clin Invest 1998;101:1717-25.

CHAPTER 3

IgM antibody level against pro-inflammatory bacterial peptidoglycan is inversely correlated with extent of atherosclerotic disease

Manon M Oude Nijhuis[1,2], Yolanda van der Graaf [3], Marie-José Melief [4], Arjan H Schoneveld[1,2], Dominique PV de Kleijn[1,2], Jon D Laman[4], Gerard Pasterkamp[1]

1. Experimental Cardiology Laboratory, University Medical Center Utrecht, Utrecht
2. Interuniversity Cardiology Institute of the Netherlands (ICIN), Utrecht
3. Julius Center, University Medical Center Utrecht, Utrecht
4. Department of Immunology and MS Center ErasMS, Erasmus MC, Rotterdam

Atherosclerosis, 2004; 173(2): 245-251

ABSTRACT

Objective: Atherosclerosis may lead to acute clinical events by rupture of a vulnerable atherosclerotic plaque. Previously, we demonstrated that peptidoglycan (PG), a major cell wall component of Gram-positive bacteria that induces production of pro-inflammatory cytokines through Toll-like receptor 2 (TLR2) or nucleotide-binding oligomerization domain (NOD) receptors, is prevalent in atherosclerotic lesions with histological features associated with plaque vulnerability. We hypothesized that in atherosclerotic patients antibody levels against PG may differ compared with matched controls.

Methods and results: ELISA was performed to measure immunoglobulin levels against PG in sera of 80 atherosclerotic patients versus 77 control patients with an increased cardiovascular risk, frequency-matched for age, sex and risk factors for atherosclerotic disease. In all patients and controls intima-media thickness was assessed using an array transducer. Significantly lower levels of IgM directed against PG were found in atherosclerotic patients compared with the controls without clinically manifest disease (p=0.02). The IgM levels against PG decreased with increasing mean common carotid intima-media thickness (p=0.006).

Conclusions: These results show that patients suffering from atherosclerotic disease have decreased IgM antibody levels against PG. The data suggest that an antibody response against PG could have a protective effect against the development or activity of atherosclerotic disease.

INTRODUCTION

Atherosclerosis is a chronic inflammatory disease [1]. During the inflammatory response mediators such as chemokines, cytokines and growth factors are released. These mediators are able to recruit inflammatory cells such as T-lymphocytes and macrophages to the site of injury [2].
Peptidoglycan (PG) may promote chronic inflammation at non-mucosal sites [3]. This antigen is present in the cell walls of most bacteria and is the major constituent (30-70%) of Gram-positive cell walls. PG surrounds the cell and gives strength to withstand the turgor pressure exerted by the cytoplasm. During gram-positive infections, PG can activate granulocytes [4] and upregulate the expression of adhesion molecules on endothelial cells [5]. PG can induce polyclonal antibody formation in peripheral blood mononuclear cells of healthy donors dependent on the presence of T-lymphocytes and monocytes [6]. Furthermore, PG is able to induce production of pro-inflammatory cytokines such as interleukin (IL)-1, IL-6 and tumor necrosis factor (TNF)-α by monocytes/macrophages *in vitro* through engagement of Toll-like receptor 2 (TLR2) [7,8] or nucleotide-binding oligomerization domain (NOD) receptors [9,10]. These macrophages are crucial in lipid metabolism and the development of atherosclerotic lesions [11]. Recently, we demonstrated that PG is present in atherosclerotic plaques with histological features of a vulnerable plaque phenotype [12]. Furthermore, PG may play a role in the pathogenesis of rheumatoid arthritis, which also is a chronic inflammatory disease [13,14]. In view of the postulated functional roles of antibodies against self antigens in atherosclerosis, we hypothesized that levels of antibodies directed against PG may be different in atherosclerotic patients as compared to matched controls. In the present study, we determined systemic antibody levels against PG in patients with severe atherosclerotic disease and compared these levels to those in a control group without clinically manifest disease. Furthermore, we examined a possible relation between the immune response against PG and the extent of atherosclerotic disease using duplex wall thickness measurements of the carotid artery.

METHODS

Patients
All patients were participants of the Second Manifestations of ARTerial disease (SMART) study; an ongoing, single-center, prospective cohort study of approximately 3500 patients referred to the University Medical Center Utrecht for the first time because of atherosclerotic vascular disease or treatment of atherosclerotic risk factors [15]. The study was approved by the ethics committee of the University Medical Center Utrecht, and written informed consent was obtained from all participants.
Eighty atherosclerotic patients that fulfilled all of the following three criteria were selected: 1- a history of clinically manifest atherosclerotic disease, 2- atherosclerotic disease at the time of hospital-entry AND 3- one cardiovascular risk factor (hypertension, diabetes mellitus or hyperlipidemia). Patients were additionally screened for the presence of clinically silent atherosclerotic lesions in the carotid and femoral arteries

and aorta using three approaches. First, atherosclerotic luminal narrowing in the femoral artery was evaluated by measuring the ratio of the systolic blood pressure measured at the ankle to the systolic blood pressure measured in both arms (ankle-brachial pressure index, ABPI). When patients had a lowered ABPI (≤0.9) they were classified having silent atherosclerosis. Second, stenosis of the common and internal carotid arteries was measured bilaterally with Doppler-assisted duplex scanning. When carotid stenosis exceeded 70%, patients were diagnosed having clinically silent atherosclerosis. Third, ultrasonography of the abdomen was performed to measure the anteroposterior juxtarenal diameter and the distal anteroposterior diameter of the aorta. When distal anteroposterior diameter ≥3 cm or ≥1.5 times anteroposterior juxtarenal diameter, patients were diagnosed having clinically silent atherosclerosis. Seventy-seven controls from the SMART study were frequency-matched for sex, age and one of three major risk factors (hypertension, diabetes mellitus and hyperlipidemia). These controls did neither reveal clinically evident nor silent atherosclerosis.

Isolation of PG from human intestinal flora

PG was prepared from faeces of a healthy subject [16]. In short, homogenized faeces was filtrated and centrifuged for 45 minutes at 5,000 g. Four volumes of 96% ethanol were added and after 2 h the precipitate was centrifuged for 15 min at 5,000 g. After dissolving in Milli-Q the suspension was centrifuged for 1 h at 100,000 g. Size exclusion chromatography was done on the clear supernatant using dilutions of 15-60 mg/30 ml. Collected fractions containing carbohydrates but no proteins were pooled, dialyzed and lyophilized. About 50 mg PG could be retrieved from 100 g of faeces. This represents a full representation of the multitude of bacterial species occurring in the normal intestinal flora. Since the PG structure varies from one bacterial species to another, PG derived from the full flora provides a broad range of PG structures.

Enzyme-linked immunosorbent-assay (ELISA) for anti-PG immunoglobulin level

The direct ELISA with faeces PG coated to microtiter wells to measure anti-PG antibody level has been developed and validated previously [14,17]. As a reference for calculating antibody levels against PG, a pooled standard serum (≈500 bloodbank donors) selected for high anti-PG reactivity was used. In microtiter plates 50 µl 10 µg/ml PG diluted in PBS was coated overnight at 50˚C. 100 µl serum diluted in PBS/Tween 0.2% was added and incubated for 1 h at 37˚C. Dilutions of 1:400, 1:1600 and 1:200 were used for IgM, IgG and IgA measurements, respectively. Peroxidase conjugated rabbit anti-human IgM, IgG or IgA (Jackson Immunoresearch, Inc., Westgroove, PA) diluted in PBS/Tween 0.2% was used as secondary antibody during 1 h at 37˚C. The development of the colorimetric assay took place in the dark for 10-20 min after the addition of 100 µl of substrate with *ortho*-phenylene diamine/H_2O_2. The reaction was stopped by adding 50 µl 4 M H_2SO_4. Optical density was measured at 492 nm. On each plate the standard serum was included and the background absorption of the conjugate was tested in duplicate. Levels of antibodies were expressed as a ratio calculated as follows:

ratio = $OD_{sample} - OD_{background} / OD_{standard\ serum} - OD_{background}$

Total immunoglobulin determination

To examine whether total immunoglobulin concentrations were in the normal range total IgM and IgG concentrations of all patients were measured using immunonephelometry according to routine and validated clinical chemistry protocols.

Risk score

Previously, a risk score based on data of pre-existing disease and risk factors available in the SMART cohort (SMART risk score) was developed and validated by comparing the SMART risk score to the Framingham risk score and the EPOZ risk score [18]. The three risk scores strongly correlated with each other. Single points were given for male gender, age, cardiovascular risk factors, and history and presence of cardiovascular disease, to a maximum of 24 points (Table 1). If more than two risk indicators were missing, the risk score was classified as missing (n=9 patients). If two or fewer risk indicators were missing, the missing indicator was given 0 points (n=1 patient).

Intima-media thickness measurement

In all patients intima-media thickness (IMT) was measured in the left and right common carotid arteries as a measure for the extent of atherosclerosis. Arteries were examined in anterolateral, posterolateral and mediolateral direction using an ATL Ultramark 9 machine (Advanced Technology Laboratories, Bethel, WA) equipped with a 10 MHz linear array transducer. The intima-media surface of the selected area was calculated on-line using built-in software of the ultrasound system. The mean IMT of the six measurements in each patient was calculated. Investigators who performed the ELISAs were blinded for the outcome of IMT measurements.

Statistical analysis

A Mann-Whitney test was used to compare the ratios of IgM, IgG and IgA measured in the PG-ELISA of the atherosclerotic patients and the controls versus standard serum. All patients and controls were divided into quartiles on the basis of IMT or SMART risk score. Differences in IgM levels against PG between the quartiles were measured with one-way ANOVA and Tukey post hoc multiple comparisons. All values are presented as mean ± standard error of mean (SEM). A p-value of <0.05 was considered significant.

Indicator	Points
Demographic characteristics	
Male gender	1
Age ≥ 30	1
Age ≥ 40	1
Age ≥ 50	1
Age ≥ 60	1
Age ≥ 70	1
Risk factors	
Body mass index ≥ 30	1
Smoking, current or past	1
Hyperlipidemia *	1
Medication for indication hyperlipidemia	1
Hyperglycemia †	1
Medication for indication hyperglycemia	1
Hypertension ‡	1
Medication for indication hypertension	1
History of vascular disease	
Angina pectoris	1
Myocardial infarction	1
Transient ischemic attack or stroke	1
Carotid endarterectomy	1
Intervention on leg arteries §	1
Aortic aneurysmectomy	1
Existing vascular disease	
Peripheral arterial disease ||	1
Internal carotid artery stenosis ≥ 50% ¶	1
Abdominal aortic aneurysm #	1
Renal failure **	1

Table 1. SMART risk score based on pre-existing vascular disease and risk factors
* *Total cholesterol ≥ 6.5 mmol/l, triglycerides ≥ 2.3 mmol/l, or HDL-cholesterol ≤ 1.0 mmol/l*
† *Fasting serum glucose ≥ 7.0 mmol/l or non-fasting serum glucose ≥ 11.1 mmol/l*
‡ *Systolic pressure ≥ 160 mmHg or diastolic pressure ≥ 95 mmHg*
§ *Every vascular intervention on the aorta, iliacal, femoral and crural arteries*
|| *Resting ABPI ≤ 0.90 or post exercise ABPI decreasing ≥ 20% in at least one leg*
¶ *Peak systolic velocity >150 cm/s corresponding with diameter reduction ≥ 50% on at least one leg*
\# *Distal anteroposterior diameter ≥ 3 cm or ≥ 1.5 times the anteroposterior juxtarenal diameter*
** *Serum creatinine > 120 µmol/l or microalbuminuria > 20 mg/mmol*

RESULTS

Systemic antibody level against PG in atherosclerotic patients

The study population consisted of 80 atherosclerotic patients (40 males, 40 females; mean age 60, range 31-79) and 77 sex-, age and risk factor-matched controls without clinically manifest disease (38 males, 39 females; mean age 58, range 32-76). Forty, 34 and 6 atherosclerotic patients entered the hospital with (a)symptomatic carotid stenosis, peripheral arterial disease and abdominal aortic aneurysm, respectively. Furthermore, 5, 7 and 68 atherosclerotic patients suffered from hypertension, diabetes mellitus and hyperlipidemia, respectively. In Table 2, the baseline characteristics of the study population are presented. The ELISA results show that atherosclerotic patients have significantly lower IgM antibody levels against PG compared to control patients (0.527 ± 0.037 versus 0.630 ± 0.039, p=0.02). No differences were observed in IgG and IgA antibody levels against PG in atherosclerotic patients compared to control patients (0.400 ± 0.023 versus 0.348 ± 0.023, p=0.1 for IgG and 0.853 ± 0.074 versus 0.932 ± 0.079, p=0.4 for IgA, respectively). Total IgM and IgG immunoglobulin concentrations

of the control patients were not significantly different from atherosclerotic patients (1.26 ± 0.07 g/l versus 1.09 ± 0.07 g/l, p=0.1 and 9.93 ± 0.34 g/l versus 10.13 ± 0.28 g/l, p=0.6, respectively). Furthermore, no relation between IgM, IgG and IgA antibody levels against PG and age was observed (p=0.07, p=0.9 and p=0.2, respectively). In the same patient cohort at another time point, measurement of IgM antibody levels against PG could be reproduced (0.711 ± 0.055 versus 0.860 ± 0.057, atherosclerotic patients versus control patients, p=0.03). For repeat experiments both primary optical density readings as well as ratio of patient over reference signals were ranked for all patients. No differences between the experiments were observed in the rankings, demonstrating that the experiments were comparable and the data reproducible.

Characteristics of the study population	Controls	Atherosclerotic patients
Total number	77	80
Age (years)	58	60
Women (%)	49	50
Body mass index (kg/m^2)	26	26
Smoking (%)	29	50
Mean common carotid intima-media thickness (mm)	0.78	1.04
SMART risk score	6.77	9.96

Table 2. Characteristics of the study population

Antibody titers against PG in relation to IMT and SMART risk score

Figure 1 presents the association between common carotid IMT and IgM antibody level against PG in all patients and controls. The cut-off values of the IMT run from 0.05 cm to 0.06 cm in quartile 1, from 0.07 cm to 0.09 cm in quartile 2, from 0.10 cm to 0.12 cm in quartile 3 and from 0.13 cm to 0.15 cm in quartile 4. The IgM level against PG decreased with increasing mean IMT (p=0.006); the p for trend is 0.008.

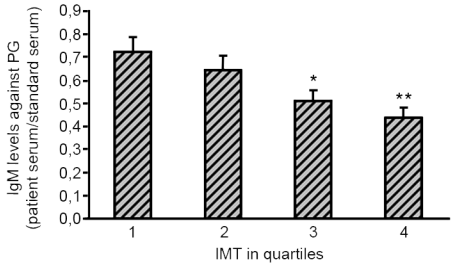

Figure 1. IgM antibody level against peptidoglycan in relation to common carotid intima-media thickness
*The IgM antibody level against PG decreased with increasing IMT (R^2=0.0537, p=0.006, p for trend is 0.008). The decrease was significant between the first and third and first and fourth quartile. *p=0.02 and **p=0.004*

The decrease was significant between the first and third and between the first and fourth quartile (p=0.02 and p=0.004, respectively). After normalization of the specific IgM response against PG to the total IgM response, a decrease could still be observed with increasing IMT (p=0.009). During the repeat measurements these data could be reproduced. The IgM antibody level against PG decreased with increasing mean IMT

(p=0.001). Between the first and third and between the first and fourth quartile, this decrease was again significant (p=0.01 and p=0.009, respectively).

Figure 2 demonstrates the relationship between IgM antibody levels against PG and the SMART risk score in all patients and controls. The IgM antibody level against PG decreased with increasing SMART risk score (p=0.003); the p for trend is < 0.001. The significant decrease in IgM antibody levels against PG was due to significant differences between the first and second, first and third and between the first and fourth quartiles (p=0.003, p=0.003 and p<0.001, respectively). Again after normalization of the specific IgM response against PG to the total IgM response, the IgM response decreased with increasing SMART risk score (p=0.02).

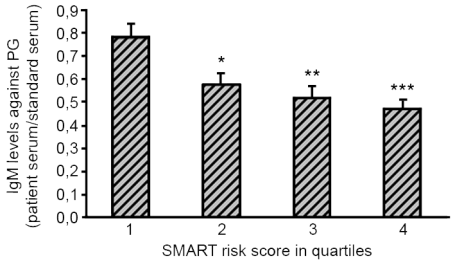

Figure 2. IgM antibody level against peptidoglycan in relation to SMART risk score
*The SMART risk score showed an inverse relation with IgM antibody levels against PG (R^2=0.0834, p=0.003, p for trend is <0.001). Differences were significant between the first and second, first and third and first and fourth quartiles *p=0.003, **p=0.003 and ***p<0.001*

DISCUSSION

Previously we have demonstrated that the bacterial cell wall component PG is present in atherosclerotic plaques [12]. Since individual systemic immunoglobulin isotypes are involved in the process of immune exclusion, i.e. prevention of redistribution of microbial antigens from the mucosa to other sites in the body [19], we hypothesized that atherosclerotic patients may differ from matched controls in their levels of antibodies directed against PG. We indeed observed that atherosclerotic patients had a significantly lower systemic IgM antibody level against PG than control patients closely matched for risk factors, but comparable IgG and IgA. In addition, the level of IgM antibodies against PG negatively correlated with common carotid IMT and a risk score for atherosclerotic disease, suggesting a functional relationship. The results show that there is no statistically significant difference in total IgM concentration. There was however a trend towards a decrease in total IgM in atherosclerotic patients as well as a trend toward a decrease in IgM levels against PG with age. Since these trends might partly explain the lowered IgM PG titers we also normalized the specific IgM to the total IgM. After normalization a decrease in IgM antibody levels could still be observed with increasing common carotid IMT and SMART risk scores.
There are three obvious interpretations of these findings: First, the decrease in antibody levels to PG in atherosclerotic patients might be due to a generalized depression of antibody levels. However, as mentioned earlier, even after correction for total IgM the

difference in IgM levels against PG among groups was still evident. Second, decreased IgM antibody levels against PG might be explained by decreased exposure to the antigen. This possibility is hard to address, but seems unlikely with respect to the ubiquitous nature of PG, being present at high level in all Gram-positive bacteria and low level in Gram-negative bacteria to which we are continuously exposed at all mucosa, including the healthy intestinal and skin flora. The unaltered levels of IgG and IgA specific for PG also argue against this option. Third, the decreased IgM levels may reflect an increased consumption of anti-PG IgM antibodies, caused by binding to intact bacteria or cell wall fragments with subsequent immune complex formation and enhanced removal.

To our knowledge, this is the first study that has examined the relationship between the presence of immunoglobulins against PG and atherosclerosis. Other studies also demonstrated significantly lower titers of IgM against oxidized LDL (OxLDL), a key autoantigen with neo-epitopes, in patients with a history of atherosclerosis, as well as negative associations between levels of IgM OxLDL antibodies and IMT [20,21]. Another study indicated that low IgM and IgG antibody levels against OxLDL may be associated with the presence of major risk factors for developing atherosclerotic disease [22]. These results support the concept that a lowered antibody response to pro-inflammatory antigens like PG or OxLDL may be causally related with the activity of atherosclerotic disease.

In a few other studies, immunization with OxLDL was found to be correlated with inhibition of the progression of atherosclerosis. In mice and rabbits, it has been demonstrated that immunization with OxLDL leads to increased immunoglobulin levels and immunization with both OxLDL and HSP-65 inhibits progression of atherosclerosis [23,24]. Furthermore, Caligiuri *et al* suggest that protection from atherosclerosis is associated with specific antibody responses to OxLDL, developing in the spleen [25]. This is further supported by Witztum and coworkers who reported that IgM antibodies reactive to phosphorylcholine are deposited in atherosclerotic lesions, can recognize OxLDL and block its uptake by macrophages [26]. These results suggest that an immune response against (auto)antigens has a protective effect against the development of atherosclerotic lesions. In this study we showed the opposite, a lowered immune response to the antigen PG is associated with more pronounced atherosclerotic disease. While OxLDL and HSP65 are auto-antigens, PG is the first exogenous antigen that shows a negative correlation between specific IgM and atherosclerotic activity. However, the role of the immune system in the pathogenesis of atherosclerosis is complex [27]. Therefore, we can not conclude that all increased antibody responses are protective, while decreased antibody responses are pro-atherogenic.

In atherogenesis, the earliest changes are observed in the subendothelial matrix where fatty streaks develop with the invasion of monocytes, T-lymphocytes and smooth muscle cells. The fatty streak progresses into an advanced lesion with necrotic core formation that is covered by a fibrous cap. Eventually a plaque can rupture, leading to unstable coronary syndromes or myocardial infarction (MI). Previously, we studied PG expression in advanced lesions [12] and demonstrated that PG is present in lesions with an inflammatory and vulnerable plaque phenotype. In this study we found that in atherosclerotic patients the IgM antibody levels against PG are significantly lower

compared to the controls, suggesting that the presence of PG is associated with a decreased IgM antibody titer against PG. It is unknown whether the results of the present study can be applied not only to advanced lesion formation but also to the early stages of atherosclerotic disease. It is highly likely that any control population will also hide early stage atherosclerotic lesions so this answer will probably be given by post mortem studies in which non-atherosclerotic segments need to be investigated for the presence of PG.

Other research groups showed previously that PG may play a role in the pathogenesis of rheumatoid arthritis, which also is a chronic inflammatory disease [13,14]. In these studies rheumatoid arthritis patients had significantly lower systemic IgG antibody levels against PG compared to healthy controls.

Previous studies suggest that an association exists between chronic infection with *Chlamydia pneumoniae* and manifestations of coronary heart disease (CHD) [28,29]. *C. pneumoniae* has been found within atherosclerotic tissues and high-titer IgG antibodies against *C. pneumoniae* are associated with an increased risk of CHD death and MI [30]. Furthermore, few studies have suggested there is a strong correlation between *Helicobacter pylori* infection and adverse cardiovascular events or systemic inflammation markers [31,32]. Most attention has been paid to the potential role of Gram-negative bacteria, like *C. pneumoniae*, in atherogenesis and the occurrence of adverse events. The present observation suggests that the role of Gram-positive bacteria and their most recognized ligand, PG, needs to be taken into account when the role of bacterial load in atherogenesis is discussed.

In summary, in atherosclerotic patients systemic IgM antibodies against the pro-inflammatory antigen PG are significantly lower compared to controls with increased cardiovascular risk but without clinically manifest disease, perhaps due to preferential downregulation of the IgM antibody response. This result supports the idea that immune modulation may affect the progression of atherosclerotic disease. Further studies are required to assess which mechanism underlies lower IgM antibody levels, and whether IgM directed against PG is beneficial for example by reducing its access to plaques.

This is a cross-sectional study. Therefore, this study cannot exclude the possibility that the decrease in IgM antibody levels against PG is a consequence of the atherosclerotic process itself. To show that the levels of IgM antibody titers against PG are preceding the atherosclerotic process and thereby contribute to atherosclerosis, prospective studies need to be performed.

ACKNOWLEDGEMENTS

This study was supported by the Dutch Heart Foundation (grant 2001-077). The SMART study is supported by a grant of the University Medical Center Utrecht. The authors gratefully thank the Department of Medical Immunology, University Medical Center Utrecht for technical assistance during total immunoglobulin determinations.

Appendix

Smart studygroup

The SMART Study Group consists of:

A. Algra, MD, FAHA, Julius Center for Health Sciences and Primary Care, and Rudolf Magnus Institute for Neurosciences, Department of Neurology

P.A.F.M. Doevendans, MD, PhD, Department of Cardiology

B.C. Eikelboom, MD, PhD, Department of Vascular Surgery

Y. van der Graaf, MD, PhD, Julius Center for Health Sciences and Primary Care

D.E. Grobbee, MD, PhD, Julius Center for Health Sciences and Primary Care

L.J. Kappelle, MD, PhD, Department of Neurology

H.A. Koomans, MD, PhD, Department of Nephrology

W.P.Th.M. Mali, MD, PhD, Department of Radiology

F.L. Moll, MD, PhD, Department of Vascular Surgery

G.E.H.M. Rutten, MD, PhD, Julius Center for Health Sciences and Primary Care

F.L.J. Visseren, MD, PhD, Department of Vascular Medicine

REFERENCES

1. Ross R. Atherosclerosis--an inflammatory disease. N Engl J Med 1999;340:115-26.
2. Glass CK, Witztum JL. Atherosclerosis. the road ahead. Cell 2001;104:503-16.
3. Schrijver IA, Melief MJ, Eulderink F, Hazenberg MP, Laman JD. Bacterial peptidoglycan polysaccharides in sterile human spleen induce proinflammatory cytokine production by human blood cells. J Infect Dis 1999;179:1459-68.
4. Martinez-Martinez L, Timmerman CP, Fleer A, Verhoef J. Chemiluminescence of human polymorphonuclear leucocytes after stimulation with whole cells and cell-wall components of Staphylococcus epidermidis. J Med Microbiol 1993;39:196-203.
5. Dobrina A, Nardon E, Vecile E, Cinco M, Patriarca P. Leptospira icterohemorrhagiae and leptospire peptidolgycans induce endothelial cell adhesiveness for polymorphonuclear leukocytes. Infect Immun 1995;63:2995-9.
6. Rasanen L, Lehto M, Jokinen I, Leinikki P. Polyclonal antibody formation of human lymphocytes to bacterial components. Immunology 1986;58:577-81.
7. Wang JE, Jørgensen PF, Almlöf M, Thiemermann C, Foster SJ, Aasen AO, et al. Peptidoglycan and lipoteichoic acid from *Staphylococcus aureus* induce tumor necrosis factor alpha, interleukin 6 (IL-6), and IL-10 production in both T cells and monocytes in a human whole blood model. Infect Immun 2000;68:3965-70.
8. Yoshimura A, Lien E, Ingalls RR, Tuomanen E, Dziarski R, Golenbock D. Cutting edge: recognition of Gram-positive bacterial cell wall components by the innate immune system occurs via Toll-like receptor 2. J Immunol 1999;163:1-5.
9. Girardin SE, Boneca IG, Carneiro LA, Antignac A, Jehanno M, Viala J, et al. Nod1 detects a unique muropeptide from gram-negative bacterial peptidoglycan. Science 2003;300:1584-7.
10. Girardin SE, Boneca IG, Viala J, Chamaillard M, Labigne A, Thomas G, et al. Nod2 is a general sensor of peptidoglycan through muramyl dipeptide (MDP) detection. J Biol Chem 2003;278:8869-72.
11. Kaul D. Molecular link between cholesterol, cytokines and atherosclerosis. Mol Cell Biochem 2001;219:65-71.
12. Laman JD, Schoneveld AH, Moll FL, van Meurs M, Pasterkamp G. Significance of peptidoglycan, a proinflammatory bacterial antigen in atherosclerotic arteries and its association with vulnerable plaques. Am J Cardiol 2002;90:119-23.
13. Schrijver IA, Melief MJ, Tak PP, Hazenberg MP, Laman JD. Antigen-presenting cells containing bacterial peptidoglycan in synovial tissues of rheumatoid arthritis patients coexpress costimulatory molecules and cytokines. Arthritis Rheum 2000;43:2160-8.
14. Schrijver IA, De Man YA, Melief MJ, Van Laar JM, Markusse HM, Klasen IS, et al. Reduced systemic IgG levels against peptidoglycan in rheumatoid arthritis (RA) patients. Clin Exp Immunol 2001;123:140-6.
15. Simons PC, Algra A, van de Laak MF, Grobbee DE, van der Graaf Y. Second manifestations of ARTerial disease (SMART) study: rationale and design. Eur J Epidemiol 1999;15:773-81.
16. Hazenberg MP, Pennock-Schroder AM, Wensinck F, Van de Merwe JP. Effect of a soluble bacterial carbohydrate fraction on the viscosity of intestinal contents in healthy subjects and patients with Crohn's disease. Eur J Clin Invest 1989;19:61-4.
17. Hazenberg MP, de Visser H, Bras MJ, Prins ME, Van de Merwe JP. Serum antibodies to peptidoglycan-polysaccharide complexes from the anaerobic intestinal flora in patients with Crohn's disease. Digestion 1990;47:172-80.
18. Simons PC, Algra A, Bots ML, Grobbee DE, van der Graaf Y. Common carotid intima-media thickness and arterial stiffness: indicators of cardiovascular risk in high-risk patients. The SMART Study (Second Manifestations of ARTerial disease). Circulation 1999;100:951-7.
19. Brandtzaeg P. The role of humoral mucosal immunity in the induction and maintenance of chronic airway infections. Am J Respir Crit Care Med 1995;151:2081-6.
20. Hulthe J, Wikstrand J, Lidell A, Wendelhag I, Hansson GK, Wiklund O. Antibody titers against oxidized LDL are not elevated in patients with familial hypercholesterolemia. Arterioscler Thromb Vasc Biol 1998;18:1203-11.
21. Hulthe J, Bokemark L, Fagerberg B. Antibodies to oxidized LDL in relation to intima-media thickness in carotid and femoral arteries in 58-year-old subjectively clinically healthy men. Arterioscler Thromb Vasc Biol 2001;21:101-7.

22. Wu R, de Faire U, Lemne C, Witztum JL, Frostegard J. Autoantibodies to OxLDL are decreased in individuals with borderline hypertension. Hypertension 1999;33:53-9.

23. Ameli S, Hultgardh-Nilsson A, Regnstrom J, Calara F, Yano J, Cercek B, et al. Effect of immunization with homologous LDL and oxidized LDL on early atherosclerosis in hypercholesterolemic rabbits. Arterioscler Thromb Vasc Biol 1996;16:1074-9.

24. Maron R, Sukhova G, Faria AM, Hoffmann E, Mach F, Libby P, et al. Mucosal administration of heat shock protein-65 decreases atherosclerosis and inflammation in aortic arch of low-density lipoprotein receptor-deficient mice. Circulation 2002;106:1708-15.

25. Caligiuri G, Nicoletti A, Poirier B, Hansson GK. Protective immunity against atherosclerosis carried by B cells of hypercholesterolemic mice. J Clin Invest 2002;109:745-53.

26. Shaw PX, Horkko S, Chang MK, Curtiss LK, Palinski W, Silverman GJ, et al. Natural antibodies with the T15 idiotype may act in atherosclerosis, apoptotic clearance, and protective immunity. J Clin Invest 2000;105:1731-40.

27. Virella G, Lopes-Virella MF. Humoral immunity and atherosclerosis. Nat Med 2003;9:243-4.

28. Schumacher A, Seljeflot I, Lerkerod AB, Sommervoll L, Otterstad JE, Arnesen H. Positive *Chlamydia pneumoniae* serology is associated with elevated levels of tumor necrosis factor alpha in patients with coronary heart disease. Atherosclerosis 2002;164:153-60.

29. Schumacher A, Seljeflot I, Lerkerod AB, Sommervoll L, Otterstad JE, Arnesen H. Does infection with *Chlamydia pneumoniae* and/or *Helicobacter pylori* increase the expression of endothelial cell adhesion molecules in humans? Clin Microbiol Infect 2002;8:654-61.

30. Siscovick DS, Schwartz SM, Corey L, Grayston JT, Ashley R, Wang SP, et al. *Chlamydia pneumoniae*, herpes simplex virus type 1, and cytomegalovirus and incident myocardial infarction and coronary heart disease death in older adults : the Cardiovascular Health Study. Circulation 2000;102:2335-40.

31. De Backer J, Mak R, De Bacquer D, Van Renterghem L, Verbraekel E, Kornitzer M, et al. Parameters of inflammation and infection in a community based case-control study of coronary heart disease. Atherosclerosis 2002;160:457-63.

32. Kowalski M, Rees W, Konturek PC, Grove R, Scheffold T, Meixner H, et al. Detection of *Helicobacter pylori* specific DNA in human atheromatous coronary arteries and its association to prior myocardial infarction and unstable angina. Dig Liver Dis 2002;34:398-402.

CHAPTER 4

Assessment of the prognostic value of immunoglobulin levels against bacterial peptidoglycan in patients undergoing coronary angioplasty

Manon M Oude Nijhuis[1,2], Yolanda van der Graaf [3], Jeroen Koerselman[3], Jon D Laman[4], Pieter R Stella[5], Dominique PV de Kleijn[1,2], Diederick E Grobbee[3], Gerard Pasterkamp[1]

1. Experimental Cardiology Laboratory, University Medical Center Utrecht, Utrecht
2. Interuniversity Cardiology Institute of the Netherlands (ICIN), Utrecht
3. Julius Center, University Medical Center Utrecht, Utrecht
4. Department of Immunology and MS Center ErasMS, Erasmus MC, Rotterdam
5. Department of Cardiology, University Medical Center Utrecht, Utrecht

SUBMITTED FOR PUBLICATION

ABSTRACT

Objective: Peptidoglyan (PG) is a membrane compound of bacteria that is known to induce production of pro-inflammatory cytokines through ligation of innate immune receptors. Previously, we demonstrated that in atherosclerotic patients systemic IgM antibody levels against PG are significantly lower compared to control patients and that common carotid intima-media thickness is inversely related with anti-PG IgM levels. Therefore, in the present study, we examined systemic antibody levels against PG as a prognostic determinant of cardiovascular outcome in patients undergoing coronary angioplasty.

Methods and results: A prospective case-cohort study was conducted in 655 patients, admitted for elective coronary angioplasty. Cases (N=136) were patients with a first cardiovascular event during a median follow-up time of 2.6 years (range 0.4-4.6). A total of 101 patients served as controls. ELISA was performed on serum collected at time of coronary angioplasty to measure immunoglobulin levels against PG. The risk of a first cardiovascular event during follow-up was not associated with IgM, IgG and IgA levels against PG, both unadjusted and adjusted for gender, age, diabetes, hypertension and hyperlipidemia.

Conclusion: In patients undergoing coronary angioplasty, systemic IgM, IgG and IgA antibody levels against pro-inflammatory PG are not prognostic determinants of cardiovascular outcome.

INTRODUCTION

Atherosclerosis is a vascular disease that is characterized by local inflammatory cells and increased systemic markers for inflammation [1]. Atherosclerosis starts with damage to the endothelial lining of the blood vessels that attracts monocytes to the site of injury [2]. The monocytes migrate into the arterial wall and differentiate into large lipid-laden macrophages under the action of cytokines and chemokines [3]. A self-sustaining reaction is initiated when the resident macrophages, T-cells and mast cells release cytokines and chemokines that recruit and activate more inflammatory cells [4].
Biomarkers for the occurrence of adverse cardiovascular events have been studied extensively, including antibodies against bacterial antigens like *Chlamydia pneumoniae* [5]. *C. pneumoniae* has the capacity to initiate and propagate inflammation in ways that contribute to atherosclerosis.
Previously, we found that the antigen peptidoglycan (PG) is prevalent in atherosclerotic lesions with an inflammatory unstable phenotype [6]. The presence of PG was mainly observed in macrophage-rich regions. In addition, we demonstrated in atherosclerotic patients that systemic IgM antibody levels against PG are significantly lower compared to control patients and that common carotid intima-media thickness is inversely related with anti-PG IgM levels [7].
PG is present in the cell walls of most bacteria. It is the major constituent of Gram-positive bacteria (30-70% of the cell wall mass) and is also present in Gram-negative bacterial walls but to a smaller extent (only 10% of the cell wall) [8]. PG surrounds the cell, providing strength to withstand the turgor pressure exerted by the cytoplasm and is able to induce production of pro-inflammatory cytokines such as interleukin (IL)-1, IL-6 and tumor necrosis factor (TNF)-α by monocytes/macrophages *in vitro* [9,10] through engagement of Toll-like receptor 2 (TLR2) [11], nucleotide-binding oligomerization domain (NOD) receptors [12] or Nalp3/cryopirin [13].
Our previous results raised the question whether a deficient immunological response to PG is functionally or indirectly related to atherosclerotic disease. In support of this notion, in an *in vivo* mouse-model we found that TLR2 ligation is capable of inducing neointima formation [14]. Therefore, in the present study we examined systemic antibody levels against PG as a prognostic determinant of cardiovascular outcome after coronary angioplasty.

MATERIAL AND METHODS

The study was approved by the Institutional Review Board of the University Medical Center Utrecht. Written informed consent was obtained from all patients. The procedures followed were in accordance with the institutional guidelines and the investigation conforms to the principles outlined in the Declaration of Helsinki.

Patients
A prospective case-cohort study was performed within the “Second Manifestations

of ARTerial disease (SMART) study". The latter study is an ongoing prospective cohort study conducted at the University Medical Center Utrecht [15]. At enrolment, medical history is recorded with a standardized questionnaire, and height, weight, and blood pressure are measured. Blood and urine samples are taken. Information on occurrence of new fatal or non-fatal cardiovascular disease and cardiovascular interventions during follow-up is obtained by contacting the patients every six months with a questionnaire. Follow-up for this study ended March 1, 2003.

For the purpose of the present study, we used a case-cohort design. Cases and non-cases originated from a study performed by Koerselman *et al* in which the presence and extent of coronary collaterals as long-term prognostic determinant of cardiovascular outcome was examined [16]. The study population consisted of 655 patients, who were admitted for elective percutaneous transluminal coronary angioplasty (PTCA) and took part in SMART between January 1, 1998 and July 8, 2002. For the control group, a 20% random sample of 131 of the 655 patients (20%) was selected. Cases consisted of all patients in whom a cardiovascular event occurred during follow-up, in total 152 patients, 25 of whom had also been selected in the random sample. The baseline angiographic data for 258 patients who underwent PTCA were retrieved (131 + 152 - 25 = 258). From 237 patients blood samples were available. Therefore, in this study data are shown for 136 cases and 101 non-cases.

Cardiovascular outcome

In the present study, we determined whether specific antibody levels against PG are predictive for adverse cardiovascular outcome in atherosclerotic patients undergoing coronary angioplasty. Therefore, we defined adverse cardiovascular outcome as a composite of cardiovascular death, non-fatal myocardial infarction (MI), non-fatal stroke, or any cardiovascular intervention. The adverse outcome of interest was defined as the first cardiovascular event occurring during follow-up. In case potential outcomes of interest occurred, additional information was collected from either the patients' specialist or general practitioner.

Cardiovascular death was defined as fatal cerebral infarction, fatal MI, sudden death, or fatal rupture of an abdominal aortic aneurysm. Non-fatal MI was defined by at least two out of the three following characteristics present: 1) ischemic chest pain of ≥ 30 min duration, 2) an increase in MB-fraction of creatine kinase to more than twice the upper level of normal and 3) characteristic changes on the electrocardiogram consistent with the diagnosis. Non-fatal stroke was defined as focal brain injury persisting for more than 24 hours, combined with an increase in handicap of at least one point on the Rankin Scale. A cardiovascular intervention was defined as any coronary artery bypass grafting (CABG), PTCA, carotid endarteriectomy, or revascularization (surgical or with percutaneous transluminal angioplasty [PTA]) of the aorta or one of its branches, or of the iliac, femoral, or crural arteries [15]. All events were reviewed by three members of an independent Clinical Event Committee for final diagnosis and classification, and coded as described previously. If an event was classified differently, consensus was obtained.

Isolation of PG from human intestinal flora

PG from faeces of a healthy subject was prepared as described previously [17]. In short, faeces was homogenized, filtrated and centrifuged for 45 min at 5000 x g. Four volumes of 96% ethanol were added to one volume of supernatant. After 2 h the precipitate was centrifuged for 15 min at 5000 x g. The pellet was dissolved in Milli-Q, the suspension was centrifuged for 1 h at 100000 x g and the supernatant was collected. Size exclusion chromatography was done on the clear supernatant using dilutions of 15-60 mg/30 ml. Collected fractions containing carbohydrates but no proteins were pooled, dialyzed and lyophilized. From 100 g of faeces about 50 mg PG could be retrieved by this procedure. Preparation of PG from faeces using this method gives a representation of the multitude of bacterial species occurring in the normal intestinal flora. Since the PG structure varies from one bacterial species to another, PG derived from the full flora provides a broad range of PG structures.

Enzyme-linked immunosorbent-assay (ELISA) to measure anti-PG level

To measure anti-PG antibody levels in serum an ELISA was performed. This direct ELISA has been developed and validated previously [18]. In microtiter plates 50 µl, 10 µg/ml PG diluted in PBS was coated overnight at 50°C. One hundred µl serum diluted in PBS/Tween 0.2% was added to the wells and incubated for 1 h at 37°C. Dilutions of 1:400, 1:1600 and 1:200 were used for IgM, IgG and IgA measurements, respectively. As detecting antibody peroxidase conjugated rabbit anti-human IgM, IgG or IgA (Jackson Immunoresearch, Inc., Westgroove, PA) diluted in PBS/Tween 0.2% was used during 1 h at 37°C. After washing three times, development of the colorimetric assay took place in the dark for 10-20 min after the addition of 100 µl of substrate with *ortho*-phenylene diamine/H_2O_2. The reaction was stopped by adding 50 µl 4 M H_2SO_4. Optical density was measured at a wavelength of 492 nm. As a reference for calculating antibody levels against PG, a pooled standard serum (≈ 500 bloodbank donors) selected for high anti-PG reactivity was used. On each plate this standard serum was included and the background absorption of the conjugate was tested in duplicate. Levels of antibodies were expressed as a ratio calculated as follows:

ratio = $OD_{sample} - OD_{background} / OD_{standard\ serum} - OD_{background}$

Data-analysis

First, the associations between IgM, IgA and IgG antibody levels (as continuous variables) against PG and cardiovascular outcome were quantified with the unweighted Cox proportional-hazards model by Prentice, particularly suitable to analyze case-cohort data and hazard ratios with robust 95% confidence interval are presented. A two-sided p-value<0.05 was considered statistically significant. This weighted method is incorporated in a SAS macro written by Barlow and Ichikawa, and made available through Statlib on the Internet http://lib.stat.cmu.edu/general/robphreg [19]. Subsequently, the analyses were repeated with adjustment for variables, known to potentially affect the association examined, notably gender, age, diabetes, hypertension and hyperlipidemia. The statistical package used was the SAS system for Windows, release 8.02 (SAS Institute Inc., Cary, NC).

RESULTS

Follow-up and cardiovascular outcome

Median follow-up time of patients was 2.6 years (range 0.2–4.6). A first cardiovascular event during follow-up occurred in 136 patients. Three patients died of cardiovascular disease: sudden death occurred in two patients and one patient died of congestive heart failure. In 26 patients a non-fatal myocardial infarction occurred, in 3 patients a non-fatal ischemic stroke, and 104 patients had a cardiovascular intervention. Baseline- and clinical characteristics of the 136 cases and the 101 non-cases that were studied are presented in table 1.

Characteristics of the study population	All patients (n=237)	Cases (n=136)	Non-cases (n=101)	p-value
Demographics:				
Age at index-PTCA (years)	58 ± 9	58 ± 9	57 ± 10	0.6
Male gender	198 (84)	119 (88)	79 (78)	0.07
Cardiovascular risk factors:				
Current smoking	59 (25)	30 (22)	29 (29)	0.4
Alcohol consumption:				
- never	35 (15)	21 (15)	14 (14)	0.6
- < 1-21 units/week	153 (65)	89 (66)	64 (63)	0.6
- ≥ 21 units/week	26 (11)	13 (10)	13 (13)	0.4
Diabetes mellitus	44 (19)	31 (23)	13 (13)	0.03 *
Hypertension	79 (33)	46 (34)	33 (33)	0.5
Hyperlipidemia	174 (73)	101 (74)	73 (72)	0.1

Number of patients with events (%) or mean ± SD

Table 1. Characteristics of the study population
PTCA = percutaneous transluminal coronary angioplasty
SD = standard deviation

Cardiovascular outcomes and antibody levels against PG

Table 2 summarizes the results of the analysis regarding the specific serum antibody levels against PG and the risk of a cardiovascular event after PTCA, both unadjusted and adjusted for gender, age, diabetes, hypertension and hyperlipidemia. The unadjusted risk of a first cardiovascular event was not associated with systemic IgM, IgG and IgA levels against PG (IgM, hazard ratio [HR] 0.88, 95% confidence interval [CI] 0.61-1.28, p=0.5; IgG, HR 1.30, 95% CI 0.67-2.51, p=0.4; and IgA, HR 1.07, 95% CI 0.71-1.62, p=0.8). Also, adjusted for gender, age, diabetes, hypertension and hyperlipidemia, the IgM, IgG and IgA antibody levels against PG at baseline were not related to a higher or lower risk of a cardiovascular event during follow-up (IgM, HR 1.05, 95% CI 0.95-1.16; p=0.4; IgG, HR 1.15, 95% CI 0.92-1.45, p=0.2; and IgA, HR 1.00, 95% CI 0.88-1.13, p=0.9).

	IgM	IgG	IgA
Unadjusted	0.88 (0.61-1.28)	1.30 (0.67-2.51)	1.07 (0.71-1.62)
p-value	0.50	0.44	0.75
Adjusted *	1.05 (0.95-1.16)	1.15 (0.92-1.45)	1.00 (0.88-1.13)
p-value	0.37	0.22	0.96

Hazard ratio (95% Confidence interval)

Table 2. Hazard ratio of first cardiovascular event after coronary angioplasty in relation to specific serum antibody levels against peptidoglycan
** adjusted for gender, age, diabetes mellitus, hypertension and hyperlipidemia*

DISCUSSION

Previously, we have demonstrated that the bacterial cell wall component PG is present in atherosclerotic plaques with histoimmunological features of a vulnerable phenotype [6]. Furthermore, in a clinical study, we observed that atherosclerotic patients had a significantly lower systemic IgM antibody level against PG than control patients with increased cardiovascular risk but without clinically manifest disease and closely matched for risk factors [7]. In addition, the level of IgM antibodies against PG negatively correlated with common carotid IMT. In the present case-cohort study among 655 patients with ischemic heart disease which were referred for elective PTCA, we investigated the predictive value of antibodies to PG for the occurrence of subsequent cardiovascular events. In concordance with our previous cross-sectional observations we expected that a low IgM antibody titer against PG would be predictive for the occurrence of adverse cardiovascular events. However, we found in the present study that the occurrence of cardiovascular events after coronary angioplasty was not associated with IgM, IgG and IgA antibody levels against PG. We demonstrated that, overall, systemic IgM antibody levels against PG were negatively correlated with common carotid IMT which confirms our previous observations (data not shown).

In our previous study we found that IgM antibody levels against PG were significantly lower in atherosclerotic patients compared to control patients with increased cardiovascular risk, but without clinically manifest disease. In this study, all patients were admitted for elective PTCA implying that domains of these studies differ significantly. In this study we can only make inferences concerning the predictive value of systemic antibody levels against PG for cardiovascular events in atherosclerotic patients undergoing PTCA. So, basically, patients included in this study can be considered as a sub-group of the atherosclerotic patients in our previous study, which makes direct comparisons difficult.

Development of atherosclerosis starts with the formation of a fatty streak consisting of monocytes, T-lymphocytes and smooth muscle cells. The fatty streak progresses into an advanced lesion with a necrotic core that is covered by a fibrous cap. Eventually the fibrous cap can rupture and the thrombogenic material inside the necrotic core is exposed to the blood. This rupture leads to formation of a thrombus

and in a later stage to unstable coronary syndromes or MI. Microbial antigens, like PG and *C. pneumoniae*, have been found to be present in atherosclerotic tissues. Previous studies suggest that an association exists between chronic infection with *C. pneumoniae* and manifestations of coronary heart disease (CHD) [20,21]. High-titre IgG antibodies against *C. pneumoniae* are associated with an increased risk of CHD death and MI [22,23]. Nishimura *et al* found that in patients with end-stage renal disease seropositivity for *C. pneumoniae* IgA but not IgG strongly associated with the presence of coronary stenosis [24]. In contrast, a strong body of evidence suggests that such an association does not exist [25-27]. Haim *et al* found in a prospective case-control study that IgG and IgA antibody levels against *C. pneumoniae* in patients with chronic stable CHD were not associated with an increased risk of acute coronary events [26]. Koh *et al* performed a study in Singapore in which they found no association between high IgG antibody titers against *C. pneumoniae* and ischemic heart disease [27]. Ieven and Hoymans summarized in a review that, although initial reports were positive, the later ones, often prospectively designed and adjusted for known cardiovascular risk factors, showed a negative or weak positive association overall between seropositivity for *C. pneumoniae* and atherosclerosis [28].

It has been postulated that the physiological function of antibodies to antigens is to participate in the removal of these agents from the artery wall and to have a protective role (eg, an antiatherogenic role of the humoral immune system), similar to the defense mechanisms in infectious disease. In support of the concept that the immune response might be antiatherogenic rather than proatherogenic are reports showing that immunization of experimental animals with oxidized LDL (OxLDL) leads to increased immunoglobulin levels and that immunization with both OxLDL and heat shock protein (HSP)60 inhibits the progression of atherosclerosis [29-31]. Chyu *et al* demonstrated that immunization of ApoE-/- mice aged 6-7 weeks with LDL did modulate cellular and humoral immune responses and did decrease plaque size, whereas immunization of 20-week old ApoE-/- mice did not reduce plaque size or composition, suggesting that anti-atherogenic efficacy of immunization depends on timing [32]. George *et al* previously studied the effects of immunization with recombinant HSP65 and HSP65-rich *Mycobacterium tuberculosis* on early atherogenesis in C57BL/6J mice fed either a normal chow diet or a high-cholesterol diet [33]. Early atherosclerosis was significantly enhanced in HSP65-immunized mice. Furthermore, serum antibodies to mycobacterial HSP65 have been found to be significantly higher in patients with established coronary or carotid atherosclerosis [34,35]. Increased antibody levels were also found to be predictive of the future development of severe atherosclerosis, and this was independent of several established coronary risk factors [36].

The current study demonstrates that IgM, IgA and IgG directed against PG (isolated from the healthy human gut) is not a useful prognostic marker for cardiovascular events in patients admitted for elective coronary angioplasty. However, this does not preclude a prognostic application of anti-PG antibodies in other patient groups. Finally, several lines of evidence from both human material [6] and from animal studies [14] do convincingly support the hypothesis that this bacterial compound plays a role in

atherosclerosis.
In summary, in patients with ischemic heart disease and referred for elective PTCA, risk of a second cardiovascular event is not associated with systemic IgM, IgG or IgA antibody levels against PG. In patients undergoing angioplasty, IgM, IgG and IgA levels against PG are no ideal markers for prognosis of a subsequent cardiovascular event.

ACKNOWLEDGEMENTS

This study was supported by the Dutch Heart Foundation (grant 2001-077) and the Netherlands Organisation for Health Research and Development (ZonMw, project 904-65-095), The Hague, The Netherlands, which had no involvement in the writing of this paper or its submission.

REFERENCES

1. Sullivan GW, Sarembock IJ, Linden J. The role of inflammation in vascular diseases. J Leukoc Biol 2000;67:591-602.
2. Springer TA. Traffic signals for lymphocyte recirculation and leukocyte emigration: the multistep paradigm. Cell 1994;76:301-14.
3. Ross R. Atherosclerosis--an inflammatory disease. N Engl J Med 1999;340:115-26.
4. Olson TS, Ley K. Chemokines and chemokine receptors in leukocyte trafficking. Am J Physiol Regul Integr Comp Physiol 2002;283:R7-28.
5. Grayston JT. Background and current knowledge of *Chlamydia pneumoniae* and atherosclerosis. J Infect Dis 2000;181 Suppl 3:S402-S410.
6. Laman JD, Schoneveld AH, Moll FL, van Meurs M, Pasterkamp G. Significance of peptidoglycan, a proinflammatory bacterial antigen in atherosclerotic arteries and its association with vulnerable plaques. Am J Cardiol 2002;90:119-23.
7. Oude Nijhuis MM, van der Graaf Y, Melief MJ, Schoneveld AH, de Kleijn DP, Laman JD, et al. IgM antibody level against proinflammatory bacterial peptidoglycan is inversely correlated with extent of atherosclerotic disease. Atherosclerosis 2004;173:245-51.
8. Schrijver IA, Melief MJ, Eulderink F, Hazenberg MP, Laman JD. Bacterial peptidoglycan polysaccharides in sterile human spleen induce proinflammatory cytokine production by human blood cells. J Infect Dis 1999;179:1459-68.
9. Wang JE, Jørgensen PF, Almlöf M, Thiemermann C, Foster SJ, Aasen AO, et al. Peptidoglycan and lipoteichoic acid from *Staphylococcus aureus* induce tumor necrosis factor alpha, interleukin 6 (IL-6), and IL-10 production in both T cells and monocytes in a human whole blood model. Infect Immun 2000;68:3965-70.
10. Yoshimura A, Lien E, Ingalls RR, Tuomanen E, Dziarski R, Golenbock D. Cutting edge: recognition of Gram-positive bacterial cell wall components by the innate immune system occurs via Toll-like receptor 2. J Immunol 1999;163:1-5.
11. Dziarski R, Gupta D. *Staphylococcus aureus* peptidoglycan is a toll-like receptor 2 activator: a reevaluation. Infect Immun 2005;73:5212-6.
12. Girardin SE, Travassos LH, Herve M, Blanot D, Boneca IG, Philpott DJ, et al. Peptidoglycan molecular requirements allowing detection by Nod1 and Nod2. J Biol Chem 2003;278:41702-8.
13. Martinon F, Agostini L, Meylan E, Tschopp J. Identification of bacterial muramyl dipeptide as activator of the NALP3/cryopyrin inflammasome. Curr Biol 2004;14:1929-34.
14. Schoneveld AH, Oude Nijhuis MM, van Middelaar B, Laman JD, de Kleijn DP, Pasterkamp G. Toll-like receptor 2 stimulation induces intimal hyperplasia and atherosclerotic lesion development. Cardiovasc Res 2005;66:162-9.
15. Simons PC, Algra A, van de Laak MF, Grobbee DE, van der Graaf Y. Second manifestations of ARTerial disease (SMART) study: rationale and design. Eur J Epidemiol 1999;15:773-81.
16. Koerselman J, de Jaegere PP, Verhaar MC, Grobbee DE, van der Graaf Y. Prognostic significance of coronary collaterals in patients with coronary heart disease having percutaneous transluminal coronary angioplasty. Am J Cardiol 2005;96:390-4.
17. Hazenberg MP, Pennock-Schroder AM, Wensinck F, Van de Merwe JP. Effect of a soluble bacterial carbohydrate fraction on the viscosity of intestinal contents in healthy subjects and patients with Crohn's disease. Eur J Clin Invest 1989;19:61-4.
18. Hazenberg MP, de Visser H, Bras MJ, Prins ME, Van de Merwe JP. Serum antibodies to peptidoglycan-polysaccharide complexes from the anaerobic intestinal flora in patients with Crohn's disease. Digestion 1990;47:172-80.
19. Barlow WE, Ichikawa L, Rosner D, Izumi S. Analysis of case-cohort designs. J Clin Epidemiol 1999;52:1165-72.
20. Higgins JP, Higgins JA, Higgins PM, Ahuja S, Higgins DL. *Chlamydia pneumoniae* and coronary artery disease: legitimized linkages? Expert Rev Cardiovasc Ther 2003;1:367-84.
21. Kinjo K, Sato H, Sato H, Ohnishi Y, Hishida E, Nakatani D, et al. Joint effects of *Chlamydia pneumoniae* infection and classic coronary risk factors on risk of acute myocardial infarction. Am Heart J 2003;146:324-30.

22. Siscovick DS, Schwartz SM, Corey L, Grayston JT, Ashley R, Wang SP, et al. *Chlamydia pneumoniae*, herpes simplex virus type 1, and cytomegalovirus and incident myocardial infarction and coronary heart disease death in older adults : the Cardiovascular Health Study. Circulation 2000;102:2335-40.

23. Sessa R, Di Pietro M, Santino I, del Piano M, Varveri A, Dagianti A, et al. *Chlamydia pneumoniae* infection and atherosclerotic coronary disease. Am Heart J 1999;137:1116-9.

24. Nishimura M, Hashimoto T, Kobayashi H, Fukuda T, Okino K, Yamamoto N, et al. Close association of *Chlamydia pneumoniae* IgA seropositivity by ELISA with the presence of coronary artery stenosis in haemodialysis patients. Nephrol Dial Transplant 2005;20:1944-50.

25. Coles KA, Knuiman MW, Plant AJ, Riley TV, Smith DW, Divitini ML. A prospective study of infection and cardiovascular diseases: the Busselton Health Study. Eur J Cardiovasc Prev Rehabil 2003;10:278-82.

26. Haim M, Tanne D, Battler A, Boyko V, Reshef T, Goldbourt U, et al. *Chlamydia pneumoniae* and future risk in patients with coronary heart disease. Int J Cardiol 2004;93:25-30.

27. Koh WP, Taylor MB, Chew SK, Phoon MC, Kang KL, Chow VT. *Chlamydia pneumoniae* IgG seropositivity and clinical history of ischemic heart disease in Singapore. J Microbiol Immunol Infect 2003;36:169-74.

28. Ieven MM, Hoymans VY. Involvement of *Chlamydia pneumoniae* in atherosclerosis: more evidence for lack of evidence. J Clin Microbiol 2005;43:19-24.

29. Ameli S, Hultgardh-Nilsson A, Regnstrom J, Calara F, Yano J, Cercek B, et al. Effect of immunization with homologous LDL and oxidized LDL on early atherosclerosis in hypercholesterolemic rabbits. Arterioscler Thromb Vasc Biol 1996;16:1074-9.

30. Maron R, Sukhova G, Faria AM, Hoffmann E, Mach F, Libby P, et al. Mucosal administration of heat shock protein-65 decreases atherosclerosis and inflammation in aortic arch of low-density lipoprotein receptor-deficient mice. Circulation 2002;106:1708-15.

31. Zhou X, Caligiuri G, Hamsten A, Lefvert AK, Hansson GK. LDL immunization induces T-cell-dependent antibody formation and protection against atherosclerosis. Arterioscler Thromb Vasc Biol 2001;21:108-14.

32. Chyu KY, Reyes OS, Zhao X, Yano J, Dimayuga P, Nilsson J, et al. Timing affects the efficacy of LDL immunization on atherosclerotic lesions in apo E (-/-) mice. Atherosclerosis 2004;176:27-35.

33. George J, Shoenfeld Y, Afek A, Gilburd B, Keren P, Shaish A, et al. Enhanced fatty streak formation in C57BL/6J mice by immunization with heat shock protein-65. Arterioscler Thromb Vasc Biol 1999;19:505-10.

34. Birnie DH, Holme ER, McKay IC, Hood S, McColl KE, Hillis WS. Association between antibodies to heat shock protein 65 and coronary atherosclerosis. Possible mechanism of action of *Helicobacter pylori* and other bacterial infections in increasing cardiovascular risk. Eur Heart J 1998;19:387-94.

35. Xu Q, Willeit J, Marosi M, Kleindienst R, Oberhollenzer F, Kiechl S, et al. Association of serum antibodies to heat-shock protein 65 with carotid atherosclerosis. Lancet 1993;341:255-9.

36. Xu Q, Kiechl S, Mayr M, Metzler B, Egger G, Oberhollenzer F, et al. Association of serum antibodies to heat-shock protein 65 with carotid atherosclerosis : clinical significance determined in a follow-up study. Circulation 1999;100:1169-74.

CHAPTER 5

Toll-like receptor 2 stimulation induces intimal hyperplasia and atherosclerotic lesion development

Arjan H Schoneveld[1,2], Manon M Oude Nijhuis[1,2],
Ben van Middelaar[1], Jon D Laman[3], Dominique PV de Kleijn[1,2],
Gerard Pasterkamp[1]

1. Experimental Cardiology Laboratory, University Medical Center Utrecht, Utrecht
2. Interuniversity Cardiology Institute of the Netherlands (ICIN), Utrecht
3. Department of Immunology and MS Center ErasMS, Erasmus MC, Rotterdam

Cardiovascular Research, 2005; 66(1): 162-169

ABSTRACT

Objective: Toll-like receptors (TLRs) are essential in activation of the innate immune system. We recently described that peptidoglycan, an exogenous TLR2 specific ligand, is present in human atherosclerotic plaques and is associated with histological markers for plaque vulnerability. Also, endogenous TLR2 ligands can be expressed in atherosclerotic tissues. Here, we determined whether TLR2 stimulation promotes pro-inflammatory cytokine/chemokine production *in vitro* and augments neointima formation and development of atherosclerotic plaques *in vivo*.

Methods and results: We detected TLR2 using Western blot and RT-PCR in human coronary arteries and primary adventitial fibroblasts. RNAse protection assay demonstrated significant induction of IL-1, IL-6, IL-8 and MCP-1 mRNA after TLR2 stimulation in human adventitial fibroblasts *in vitro*. ELISA demonstrated induction of IL-6, IL-8 and MCP-1. *In vivo*, application of $Pam_3Cys\text{-}SK_4$, a synthetic TLR2 ligand, on femoral arteries of C57BL/6 wild type mice using a peri-adventitial cuff, significantly enhanced neointima formation compared to control arteries. This increased inflammatory response was not observed in TLR2 knockout (TLR2-/-) mice. In ApoE knockout (ApoE-/-) mice, application of the same TLR2 ligand led to a significant increase in atherosclerotic plaque development.

Conclusion: TLR2 stimulation induces the expression of pro-inflammatory cytokines and chemokines by vascular cells. Furthermore, local arterial TLR2 stimulation induced neointima and atherosclerotic plaque formation in mouse femoral arteries. TLR2 stimulation may be an important mediator in arterial occlusive disease.

INTRODUCTION

As part of the innate immune system Toll-like receptors (TLRs) play a key role in the host defense against exposure to micro-organisms [1,2]. Eleven members of the TLR-family have been described and all recognize specific pathogen-associated molecular patterns (PAMPs) [3]. Upon activation, TLRs predominantly use a similar downstream nuclear factor (NF)-κB signaling pathway leading to the production of pro-inflammatory cytokines and chemokines, thereby enhancing the inflammatory response and influencing the adaptive immune response [4,5]. Clinical data that support the importance of the TLRs in human immune-deficiency have already emerged. TLR2 and TLR4 polymorphisms have been associated with susceptibility to *Staphylococcus aureus* infection [6] and endotoxin hypo-responsiveness [7]. With respect to cardiovascular disease it has been demonstrated that patients with an Asp299Gly TLR4 polymorphism showed less carotid intima-media thickness compared with non-carriers [8]. Patients with this polymorphism and suffering from coronary occlusive disease also showed significantly more benefit from pravastatin treatment, a lipid-lowering HMG-CoA reductase inhibitor, compared to non-carriers [9].
Previously, we demonstrated that TLR4 is expressed in adventitial fibroblasts and that TLR4 activation augments intimal lesion formation [10]. We also demonstrated that TLR4 plays a role in arterial geometrical remodeling [11].
To date, it has been demonstrated that TLR1, TLR2 and TLR4 expression is markedly enhanced in human atherosclerotic plaques [12]. Earlier, we demonstrated the presence of the bacterial cell wall component peptidoglycan (PG), a ligand that can be recognized by TLR2 and intracellular nucleotide-binding oligomerization domain (NOD) receptors [13,14], in atherosclerotic plaques. The presence of PG was associated with histological markers for plaque vulnerability [15]. Besides this exogenous PG, other publications indicate the presence of endogenous TLR2 ligands in the atherosclerotic plaque. Heat shock protein (HSP)60 and HSP70, that are considered potential endogenous ligands for TLR2 [16-18], are found in plaques of ApoE deficient mice [19] and human coronary bypass grafts [20]. Evidence for TLR2 involvement in vascular occlusive disease however is still not provided. Here we hypothesized that TLR2 activation in vascular cells promotes pro-inflammatory cytokine and chemokine production and that TLR2 ligand application *in vivo* augments neointima and enhances arterial plaque formation.

MATERIAL AND METHODS

Human cells and coronary tissue
Our study conformed to the Declaration of Helsinki and all volunteers gave written informed consent. Primary adventitial fibroblasts were isolated from freshly obtained thoracic aorta, dissected from donor and recipient hearts (n=5) during heart transplantation. Adventitial layers were stripped from the aorta and rinsed with PBS several times. The adventitial layer was cut into small pieces and treated with 2.5 μg/ml collagenase A (Roche, Basel, Switzerland). After 6 h, we spun debris for 5

min at 1000 rpm and took up the cell pellet in minimal essential medium (MEM), supplemented with 10% FBS, penicillin/streptomycin, minimum essential amino acids and sodium-pyruvate. Cells that attached in the first 6 h after transfer onto a 6-well plate were considered fibroblasts. Fibroblast origin of the cells was checked by an anti-vimentin staining as described earlier [10]. Cells were used for *in vitro* assays at 80-90% confluence.
All cells were cultured under standard conditions (5% CO_2, 37°C) using 10% FBS. One h before stimulation cells were cultured with medium containing only 1% FBS. All cells were stimulated for 6 h with 1% FBS medium containing 500 ng/ml Pam_3Cys-Ser-$(Lys)_4$, Hydrochloride (Pam_3Cys-SK_4, 3HCl; Novabiochem, Cambridge, MA), a synthetic TLR2 ligand and 15 µg/ml polymyxin B sulphate (Calbiochem, San Diego, CA), which blocks the effects of possible LPS contamination. After stimulation, medium was collected and cells were washed with PBS. RNA was isolated using Tri-pure reagent® (Roche) according to the manufacturer's protocol.

Tissue
Fresh coronary arteries were dissected from 2 individuals post mortem (1 left ascending and 1 right coronary artery; post mortem delay was ≤ 24 h). Arteries were frozen in liquid nitrogen and stored at -80°C until use. Tissue samples were pulverized under liquid nitrogen using a pestle and mortar and RNA and protein were isolated from the same sample using Tri-pure reagent® according to manufacturer's protocol.

RT-PCR
After isolation, total RNA was treated with DNAse (Amersham Pharmacia, Freiburg, Germany). The presence of genomic DNA was tested by PCR without reverse transcriptase. cDNA was created using 'Ready to go, You prime First system' (Amersham Pharmacia). For the amplification of TLR2 a specific primer set was used, derived from the indicated nucleotide positions in the EMBL accession number, using Prime (Caoscamm, Nijmegen, the Netherlands). Human TLR2: 5'gagacctatagtgactcccag3' (758-777, U88878), 5'tgatgatgacccccaagac3' (871-852, U88878), forward and reverse, respectively. After a hot start at 94°C for 2 min, amplification was performed during 30 cycles at 94°C for 30 s, 57°C for 30 s and 72 °C for 30 s, followed by a 7 min extension at 72°C. The identity of the amplified PCR product was confirmed by sequence analysis.

Western blot analysis
Denatured protein samples (10 µg/lane) were loaded on a 10% SDS-PAGE and blotted on a Hybond-P membrane (Amersham Pharmacia). Next, membranes were blocked overnight in 5% non-fat dried milk in PBS/0.1% Tween 20. Blots were incubated with 0.5 µg/ml goat anti-human TLR2 (Santa Cruz, San Diego, CA) followed by 0.1 µg/ml rabbit anti-goat-HRPO (Dakopatts, Glostrup, Denmark). Blots that were incubated with normal goat serum served as negative controls.

RNAse protection assay
RNAse protection assay was used to measure the pro-inflammatory cytokine and chemokine induction on mRNA level following TLR2 stimulation. In these assays

radioactive multi-probes detect different cytokines or chemokines simultaneously. RNAse protection assay was performed using manufacturer's protocol (BD Pharmacia, San Diego, CA). In short, ^{32}P UTP radioactive labeled RNA probes were generated using manufacturer's hCK-2 or hCK-5 cDNA multi-templates including 15 cytokines and chemokines and 6 house keeping genes (BD Pharmacia) and T7 RNA polymerase (Roche). Radioactive probes were used to hybridize 2 µg total RNA from cultured cells. Protected RNA fragments were quantified after film exposure using Quantity One software (Bio-Rad, Hercules, CA).

Enzyme-linked immunosorbent assay (ELISA)
Human interleukin (IL-1)α, IL-6, IL-8 and monocyte chemoattractant protein (MCP)-1 protein concentrations were determined using commercially available ELISA kits (IL-6 and IL-8 from Sanquin, Amsterdam, the Netherlands; IL-1α and MCP-1 from R&D Systems, Abingdon, UK) according to manufacturer's protocol. Cells were stimulated in cell culture medium containing only 1% FCS. This medium was collected after 6 h of incubation and used for the ELISA measurements.

Animal experiments
All experiments were approved by the local ethical committee on animal experiments, conformed to the *Guide for Care and Use of Laboratory Animals* published by the U.S. National Institutes of health (NIH publication No. 85-23, revised 1996). The following experiment was performed on 10 male C57BL/6 mice, 10 male ApoE-/- mice (both from Jackson Laboratory, Bar Harbor, ME) and 9 TLR2-/- mice (kindly provided by Dr. Shizuo Akira, Osaka University, Japan). All mice were 10-14 weeks of age. Mice were anesthetized through intraperitoneal injection using 0.025 ml/10 g body weight of KRA cocktail (ketaminehydrochloride 57.5 mg/ml, xylazine 10 mg/ml, atropine 0.5 mg/ml in NaCl 0.9%). Under sterile conditions, a non-constrictive polyethylene cuff (0.4 mm inner diameter, 0.8 mm outer diameter, length 2 mm, Portex, Kent, UK), cut longitudinally, was placed around the femoral arteries, as described before [21]. Two% gelatin with (right femoral) or without (left femoral; internal control) the synthetic TLR2 ligand $Pam_3Cys\text{-}SK_4$ (1 µg/µl), was injected between the cuff and artery. The skin was closed with a suture. After 21 days, the mice were anesthetized with KRA mix. The thorax was opened and the arterial system of the mice was perfused by cardiac puncture via the left ventricle with 0.9% NaCl containing 0.1 mg/ml nitroglycerin (3 min) and subsequently with 4% formalin with nitroglycerin (3 min). After perfusion, the left and right femoral arteries were harvested and paraffin embedded.
ApoE-/- mice were fed a high cholesterol-rich diet, containing at least 1% cholesterol (USP), 0.5% cholic acid, 2% choline (CL 50%), 20% acid casein, 15% cacao butter, 10% corn starch, 40.5% sucrose and minerals (purified diet N, Hope farms, Woerden, the Netherlands). The diet was fed for 3 weeks before cuff placement, to enhance the process of atherosclerotic plaque formation.

Histology
Serial cross-sections (5 µm thick) were obtained over the entire length of the cuffed femoral artery (200 µm intervals) for histological analysis. The sections were stained

by elastin van Giesson staining to visualize the internal and external elastic lamina. In each section, we measured the luminal area (LA), area inside the inner elastic lamina (IEL), and the area inside the outer elastic lamina (EEL) by using image-analyzing software (Soft Imaging Systems, Munster, Germany). The intimal area was defined as IEL-LA. The medial area was defined as EEL-IEL. Due to technical problems we lost 2 ApoE-/- treated arteries and 4 of the TLR2-/- mice (non-perpendicular embedding of tissue; n=4 and non-detectable cuff at termination; n=2).

Statistics

Values are presented as mean ± standard deviation (SD). A Mann-Whitney test was used to compare differences among groups. A paired T-test was performed to compare differences between left and right arteries within one animal. A p-value <0.05 was considered significant.

Figure 1. Human coronary arteries and primary adventitial fibroblasts express TLR2
Western blot showing TLR2 in human coronary artery tissue samples; (A) and (C) represent proximal samples of two different arteries, (B) and (D) represent the distal samples of the same arteries. (E) shows TLR2 expression on human primary adventitial fibroblasts. TLR2 bands are detected at ± 78 kD as predicted.

RESULTS

TLR2 expression in vascular cells and arteries

Western blot demonstrated the presence of TLR2 in human coronary arteries and non-stimulated primary adventitial fibroblasts (*Figure 1*). To determine if TLR2 expression was distributed equally over the whole artery, we investigated both a proximal and distal part of the same artery. As shown in *Figure 1*, TLR2 protein was not homogenously expressed in the atherosclerotic arteries. RT-PCR confirmed these results (data not shown).

	Control	$Pam_3Cys\text{-}SK_4$
IL-1Ra	3168 ± 404	6995 ± 1660 *
IL-1β	1924 ± 213	3990 ± 750 *
IL-1α	785 ± 416	1136 ± 256 *
IL-6	10674 ± 2792	19545 ± 3456 *
IL-8	1324 ± 623	7859 ± 1144 *
MCP-1	4208 ± 1502	10432 ± 221 *

Table 1. Induction of pro-inflammatory factors after TLR2 stimulation in vitro
*Cytokine and chemokine mRNA upregulation after $Pam_3Cys\text{-}SK_4$ stimulation of human primary adventitial fibroblasts for 6 h (mean OD ± SD); n=3. *p<0.05*

Cytokine/chemokine expression in vascular cells after TLR2 stimulation

Next, we questioned if TLR2, expressed on the vascular cells, could be activated. After stimulation of the adventitial fibroblasts with the synthetic TLR2 ligand, $Pam_3Cys\text{-}SK_4$, we observed significant increments of IL-1α, IL-1β, IL-1Ra, IL-6, IL-8 and MCP-1 mRNA using RNAse protection assays (*Figure 2*/table 1).

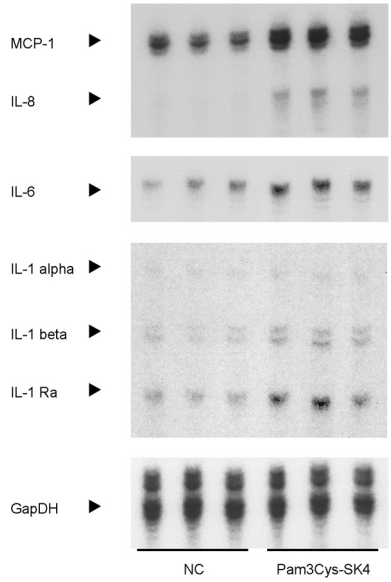

Figure 2. TLR2 stimulation leads to mRNA upregulation of pro-inflammatory factors in vitro
Detection of cytokine and chemokine mRNA induction after TLR2 stimulation in cultured primary adventitial fibroblasts using RNAse protection assay (n=3). NC --- negative control: non-stimulated human adventitial fibroblasts, $Pam_3Cys\text{-}SK_4$ --- stimulated human adventitial fibroblasts

In addition to the mRNA cytokine detection we tried to demonstrate that stimulation via TLR2 would also induce protein expression of pro-inflammatory cytokines/ chemokines in primary adventitial fibroblasts. Using ELISA, we demonstrated significant increments of IL-8 (*Figure 3A*), IL-6 (*Figure 3B*) and MCP-1 (*Figure 3C*) in stimulated versus non-stimulated primary adventitial fibroblasts. We were unable to observe detectable levels of IL-1α. ELISAs for IL-1β and IL-1Ra were not performed.

Intimal hyperplasia formation after $Pam_3Cys\text{-}SK_4$ stimulation

Having demonstrated that TLR2 can be activated in vascular cells *in vitro*, we performed *in vivo* TLR2 stimulation studies in mice to investigate neointima or atherosclerotic plaque formation. After 21 days both intimal hyperplasia and intima-media ratio were significantly increased (p=0.004 and p=0.005, respectively) after $Pam_3Cys\text{-}SK_4$ application in C57BL/6 wild type mice (*Figures 4A* and *4C*, *5A* and *5B*). At this time point, the TLR2-/- mice showed no significant increase in intimal hyperplasia in $Pam_3Cys\text{-}SK_4$ treated versus untreated arteries (p=0.46; *Figures 4A* and *4C*, *5C* and *5D*), confirming that *in vivo* effects of $Pam_3Cys\text{-}SK_4$ are mediated trough its specific receptor TLR2. In addition, intimal hyperplasia and intima-media ratio in wild type mice showed a significant increase compared to the TLR2 -/- mice in $Pam_3Cys\text{-}SK_4$ treated arteries (p=0.013 and p=0.003, respectively; *Figures 4A* and *4C*). The medial area of the TLR2-/- animals was significantly larger in both the untreated (7746 ± 1206 μm^2 versus 4052 ± 231 μm^2 in TLR2-/- versus wild type) and treated (8303 ± 797 μm^2 versus 4282 ± 883 μm^2 in TLR2-/- versus wild type) arteries (p<0.001 in both cases).

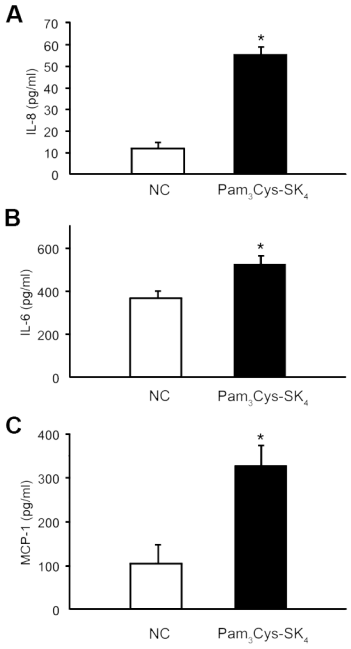

Figure 3. TLR2 stimulation leads to protein upregulation of IL-6, IL-8 and MCP-1 in vitro
*IL-6 (A), IL-8 (B) and MCP-1 (C) protein expression after TLR2 stimulation measured by ELISA. NC --- negative control: non-stimulated human adventitial fibroblasts, $Pam_3Cys\text{-}SK_4$ --- stimulated human adventitial fibroblasts (mean pg/ml ± SD); n=6. *p<0.05*

Atherosclerotic plaque formation after $Pam_3Cys\text{-}SK_4$ stimulation

To investigate TLR2 involvement in atherosclerotic disease we performed *in vivo* $Pam_3Cys\text{-}SK_4$ application experiments in ApoE-/- mice that were put on a high cholesterol diet for 3 weeks prior to operation. Treatment of the right femoral artery with $Pam_3Cys\text{-}SK_4$ resulted in a significant increase in atherosclerotic plaque formation and plaque-media ratio (both p=0.005; *Figures 4B* and *4D*, *5E* and *5F*). The medial area of the $Pam_3Cys\text{-}SK_4$-treated arteries (16087 ± 4323 μm^2) in ApoE-/- mice was significantly larger compared to the untreated artery (10087 ± 1541 μm^2, p=0.004). Arterial size was significantly larger in the $Pam_3Cys\text{-}SK_4$-treated artery (33722 ± 10243 μm^2) compared to the untreated artery (16796 ± 3928 μm^2, p=0.002).

DISCUSSION

The present study, in which we locally applied a specific TLR2 ligand on the femoral artery of the C57BL/6 and the TLR2-/- mice, demonstrates that TLR2 stimulation induces vascular intimal hyperplasia formation.

Previous research on the role of the TLR in arterial occlusive disease primarily focused on the TLR4. Although the role of TLR4 in atherosclerotic and injury-related luminal narrowing is appreciated, the role of TLR2 in atherosclerotic luminal narrowing is unknown. We previously demonstrated that TLR4 was involved in development of intimal

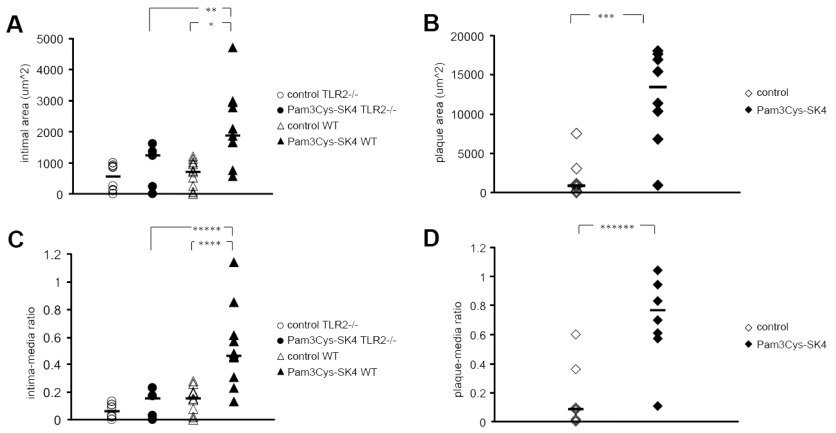

Figure 4. Neointima and atherosclerotic plaque formation after TLR2 stimulation in vivo
*Intimal hyperplasia area (A) and intima-media ratio (C) of wild type (rectangles, n=10) mice and TLR2-/- (circles, n=5) mice treated with peri-adventitial cuff containing only gelatin (open circles and rectangles) or cuff and gelatin containing $Pam_3Cys\text{-}SK_4$ (closed circles and rectangles) after 21 days. (B) and (D) demonstrate plaque area and plaque-media ratio in ApoE-/- mice after gelatin (open diamonds) or gelatin with $Pam_3Cys\text{-}SK_4$ (closed diamonds) treatment (n=8). Bar represents median, *p=0.004, **p=0.013, ***p=0.005, ****p=0.005, *****p=0.003, ******p=0.005.*

lesions [10]. Besides neointima formation, recent studies also suggest an important role for TLR4 in atherogenesis. These studies demonstrate the reduction of arterial plaque formation in TLR4/ApoE double knockout mice [22]. This was accompanied by a more stable plaque phenotype. Knocking out MyD88, an essential downstream adaptor molecule in the TLR signaling pathway in the ApoE-/- mice, reduced the number of aortic atherosclerotic lesions by 40-65% and decreased macrophage content compared to ApoE-/- mice [22,23]. So not only TLR4, but also the downstream TLR signaling molecule MyD88, is a key player in atherosclerosis.

In this study, using the ApoE-/- atherosclerotic mouse model, we show that triggering TLR2 activation dramatically increases atherosclerotic plaque formation and plaque-media ratio. So here we provide evidence that TLR2 is involved not only in intimal lesion formation but also in development of atherosclerotic occlusive disease. How TLR2 stimulation exactly leads to neointima and atherosclerotic plaque formation, remains to be elucidated. However, in the present study, we demonstrated that TLR2 stimulation of adventitial fibroblasts induces IL-1α, IL-1β, IL-1Ra, IL-6, IL-8 and MCP-1 production. This points not only to TLR2 expression but also to pro-inflammatory effects upon TLR2 stimulation in vascular cells. Earlier studies have shown that pro-inflammatory cytokine production after TLR2 stimulation is a direct effect of NF-κB translocation [24,25]. Genetic elimination of some chemokines and cytokines can prevent atherosclerosis [26], demonstrating the importance of these inflammation-associated proteins in relation to arterial occlusive disease. Most cytokines and chemokines are pro-inflammatory factors, favoring cell migration, proliferation [27,28] and chemoattraction of inflammatory cells, like monocytes/macrophages and T-lymphocytes [29-31]. TLR2 deficiency may well prevent these atherosclerotic favoring signals. Indeed, the earlier paper of Björkbacka *et al* [23] indicates that deletion of MyD88 resulted in reduced chemokine expression in ApoE-/- mice, which leads to

reduced macrophage attraction and reduction of atherosclerotic lesion development. TLR2 deficiency may also reduce matrix metalloproteinase (MMP) activity during neointima and plaque formation. IL-6 and IL-8, but also MCP-1, which are elevated after TLR2 stimulation in this study, are activators of MMP-2 and MMP-9 [32]. Both MMP-2 and MMP-9 are important enzymes associated with atherosclerotic occlusive disease [33] and facilitate smooth muscle cell migration and proliferation [34-38].

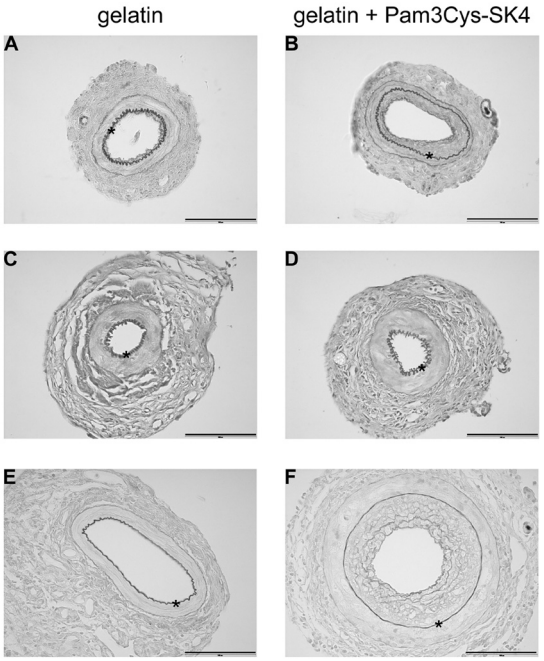

Figure 5. Pathology of neointima and atherosclerotic plaque formation induced by TLR2 stimulation in vivo
*Elastin van Giesson staining on cross-sections of mouse femoral arteries treated with peri-adventitial cuff and gelatin (panels A, C and E), or with cuff and gelatin with $Pam_3Cys\text{-}SK_4$ (panels B, D and F). Panels (A) and (B) depict C57BL/6 femoral arteries. Panels (C) and (D) depict TLR2-/- femoral arteries. Panels (E) and (F) depict ApoE-/- femoral arteries. *Internal elastic lamina. Bar is 100 μm.*

How ligands are able to trigger TLR2 stimulation in the arterial wall is another question that needs to be elucidated. In an earlier study we demonstrated that PG, a ligand that can be recognized by TLR2, NOD1 and NOD2, is present in atherosclerotic plaques [15]. However, the ligand-repertoire for TLR2 is expanding. Endogenous TLR2 ligands [39] like necrotic cells [40] but also HSP60 [18] and HSP70 [16], which are expressed after tissue injury, are found in the atherosclerotic plaque [19]. Possible exogenous triggers for the arterial TLR2 cannot be excluded. Redistribution of microbial antigens from for instance the intestinal mucosa, where abundant numbers of commensal microbes are present, by macrophages or dendritic cells to other sides of the body is possible [41].
For our experiments we used a selective TLR2 agonist, $Pam_3Cys\text{-}SK_4$. $Pam_3Cys\text{-}SK_4$ is a cell-permeable, water soluble synthetic cationic lipohexapeptide analog of the immunological active N-terminal portion of bacterial lipoprotein [42]. The *in vivo* experiments in ApoE-/- mice presented in this paper have been reproduced with

the natural TLR2 ligand PG isolated from *S. aureus* (data not shown). Also, the *in vitro* RNAse protection assays have been reproduced with the natural TLR2 ligand lipoteichoic acid (LTA) from *S. aureus* (n=1, data not shown). However, since LPS contamination could not be excluded, which is the case for most natural TLR2 ligands [43] and since recent reports raised doubts about the TLR2 specificity of PG [17,44], we chose to activate TLR2 with a synthetic ligand.

In this study we made an additional interesting observation. The medial area of the TLR2-/- mice was significantly larger in the control artery compared to the wild type mice ($p<0.001$), accounting for an almost significant lower intima-media ratio in the TLR2-/- mice compared to the wild type ($p=0.062$). The reason for this increased media size is unknown. Our observation merits careful consideration, since other investigators reported no differences in medial area between TLR2-/- and wild type mice [45].

In summary, this study demonstrates that TLR2 stimulation on vascular cells induces the expression of pro-inflammatory cytokines and chemokines. Moreover, intima and atherosclerotic plaque formation can be induced by TLR2 stimulation *in vivo*, pointing to a potential role for TLR2 in local augmentation of arterial obstruction.

ACKNOWLEDGEMENTS

This study is supported by the Dutch Heart Foundation grants 99-209, 2001-077, and 2001-162.

REFERENCES

1. Sieling PA, Modlin RL. Toll-like receptors: mammalian “taste receptors” for a smorgasbord of microbial invaders. Curr Opin Microbiol 2002;5:70-5.
2. Underhill DM, Ozinsky A. Toll-like receptors: key mediators of microbe detection. Curr Opin Immunol 2002;14:103-10.
3. Akira S, Takeda K, Kaisho T. Toll-like receptors: critical proteins linking innate and acquired immunity. Nat Immunol 2001;2:675-80.
4. Barton GM, Medzhitov R. Toll-like receptors and their ligands. Curr Top Microbiol Immunol 2002;270:81-92.
5. Medzhitov R, Janeway CA, Jr. Innate immunity: the virtues of a nonclonal system of recognition. Cell 1997;91:295-8.
6. Lorenz E, Mira JP, Cornish KL, Arbour NC, Schwartz DA. A novel polymorphism in the toll-like receptor 2 gene and its potential association with staphylococcal infection. Infect Immun 2000;68:6398-401.
7. Arbour NC, Lorenz E, Schutte BC, Zabner J, Kline JN, Jones M, et al. TLR4 mutations are associated with endotoxin hyporesponsiveness in humans. Nat Genet 2000;25:187-91.
8. Kiechl S, Lorenz E, Reindl M, Wiedermann CJ, Oberhollenzer F, Bonora E, et al. Toll-like receptor 4 polymorphisms and atherogenesis. N Engl J Med 2002;347:185-92.
9. Boekholdt SM, Agema WR, Peters RJ, Zwinderman AH, van der Wall EE, Reitsma PH, et al. Variants of toll-like receptor 4 modify the efficacy of statin therapy and the risk of cardiovascular events. Circulation 2003;107:2416-21.
10. Vink A, Schoneveld AH, van der Meer JJ, van Middelaar BJ, Sluijter JP, Smeets MB, et al. *In vivo* evidence for a role of toll-like receptor 4 in the development of intimal lesions. Circulation 2002;106:1985-90.
11. Hollestelle SC, De Vries MR, Van Keulen JK, Schoneveld AH, Vink A, Strijder CF, et al. Toll-like receptor 4 is involved in outward arterial remodeling. Circulation 2004;109:393-8.
12. Edfeldt K, Swedenborg J, Hansson GK, Yan ZQ. Expression of toll-like receptors in human atherosclerotic lesions: a possible pathway for plaque activation. Circulation 2002;105:1158-61.
13. Iwaki D, Mitsuzawa H, Murakami S, Sano H, Konishi M, Akino T, et al. The extracellular toll-like receptor 2 domain directly binds peptidoglycan derived from *Staphylococcus aureus*. J Biol Chem 2002;277:24315-20.
14. Takeuchi O, Hoshino K, Kawai T, Sanjo H, Takada H, Ogawa T, et al. Differential roles of TLR2 and TLR4 in recognition of gram-negative and gram-positive bacterial cell wall components. Immunity 1999;11:443-51.
15. Laman JD, Schoneveld AH, Moll FL, van Meurs M, Pasterkamp G. Significance of peptidoglycan, a proinflammatory bacterial antigen in atherosclerotic arteries and its association with vulnerable plaques. Am J Cardiol 2002;90:119-23.
16. Asea A, Rehli M, Kabingu E, Boch JA, Bare O, Auron PE, et al. Novel signal transduction pathway utilized by extracellular HSP70: role of toll-like receptor (TLR) 2 and TLR4. J Biol Chem 2002;277:15028-34.
17. Tsan MF, Gao B. Endogenous ligands of Toll-like receptors. J Leukoc Biol 2004;76:514-9.
18. Zanin-Zhorov A, Nussbaum G, Franitza S, Cohen IR, Lider O. T cells respond to heat shock protein 60 via TLR2: activation of adhesion and inhibition of chemokine receptors. FASEB J 2003;17:1567-9.
19. Kanwar RK, Kanwar JR, Wang D, Ormrod DJ, Krissansen GW. Temporal expression of heat shock proteins 60 and 70 at lesion-prone sites during atherogenesis in ApoE-deficient mice. Arterioscler Thromb Vasc Biol 2001;21:1991-7.
20. Hammerer-Lercher A, Mair J, Bonatti J, Watzka SB, Puschendorf B, Dirnhofer S. Hypoxia induces heat shock protein expression in human coronary artery bypass grafts. Cardiovasc Res 2001;50:115-24.
21. Moroi M, Zhang L, Yasuda T, Virmani R, Gold HK, Fishman MC, et al. Interaction of genetic deficiency of endothelial nitric oxide, gender, and pregnancy in vascular response to injury in mice. J Clin Invest 1998;101:1225-32.
22. Michelsen KS, Wong MH, Shah PK, Zhang W, Yano J, Doherty TM, et al. Lack of Toll-like receptor 4 or myeloid differentiation factor 88 reduces atherosclerosis and alters plaque phenotype in mice deficient in apolipoprotein E. Proc Natl Acad Sci U S A 2004;101:10679-84.
23. Bjorkbacka H, Kunjathoor VV, Moore KJ, Koehn S, Ordija CM, Lee MA, et al. Reduced atherosclerosis in MyD88-null mice links elevated serum cholesterol levels to activation of innate immunity signaling pathways. Nat Med 2004;10:416-21.

24. Schwandner R, Dziarski R, Wesche H, Rothe M, Kirschning CJ. Peptidoglycan- and lipoteichoic acid-induced cell activation is mediated by toll-like receptor 2. J Biol Chem 1999;274:17406-9.

25. Wang T, Lafuse WP, Zwilling BS. NFkappaB and Sp1 elements are necessary for maximal transcription of toll-like receptor 2 induced by *Mycobacterium avium*. J Immunol 2001;167:6924-32.

26. Gosling J, Slaymaker S, Gu L, Tseng S, Zlot CH, Young SG, et al. MCP-1 deficiency reduces susceptibility to atherosclerosis in mice that overexpress human apolipoprotein B. J Clin Invest 1999;103:773-8.

27. Barillari G, Albonici L, Incerpi S, Bogetto L, Pistritto G, Volpi A, et al. Inflammatory cytokines stimulate vascular smooth muscle cells locomotion and growth by enhancing alpha5beta1 integrin expression and function. Atherosclerosis 2001;154:377-85.

28. Yue TL, Wang X, Sung CP, Olson B, McKenna PJ, Gu JL, et al. Interleukin-8. A mitogen and chemoattractant for vascular smooth muscle cells. Circ Res 1994;75:1-7.

29. Reape TJ, Groot PH. Chemokines and atherosclerosis. Atherosclerosis 1999;147:213-25.

30. Shah PK. Circulating markers of inflammation for vascular risk prediction: are they ready for prime time. Circulation 2000;101:1758-9.

31. Shin WS, Szuba A, Rockson SG. The role of chemokines in human cardiovascular pathology: enhanced biological insights. Atherosclerosis 2002;160:91-102.

32. Katrib A, Tak PP, Bertouch JV, Cuello C, McNeil HP, Smeets TJ, et al. Expression of chemokines and matrix metalloproteinases in early rheumatoid arthritis. Rheumatology (Oxford) 2001;40:988-94.

33. Kossakowska AE, Edwards DR, Prusinkiewicz C, Zhang MC, Guo D, Urbanski SJ, et al. Interleukin-6 regulation of matrix metalloproteinase (MMP-2 and MMP-9) and tissue inhibitor of metalloproteinase (TIMP-1) expression in malignant non-Hodgkin's lymphomas. Blood 1999;94:2080-9.

34. Aguilera CM, George SJ, Johnson JL, Newby AC. Relationship between type IV collagen degradation, metalloproteinase activity and smooth muscle cell migration and proliferation in cultured human saphenous vein. Cardiovasc Res 2003;58:679-88.

35. Auge N, Maupas-Schwalm F, Elbaz M, Thiers JC, Waysbort A, Itohara S, et al. Role for matrix metalloproteinase-2 in oxidized low-density lipoprotein-induced activation of the sphingomyelin/ceramide pathway and smooth muscle cell proliferation. Circulation 2004;110:571-8.

36. Shah PK, Falk E, Badimon JJ, Fernandez-Ortiz A, Mailhac A, Villareal-Levy G, et al. Human monocyte-derived macrophages induce collagen breakdown in fibrous caps of atherosclerotic plaques. Potential role of matrix-degrading metalloproteinases and implications for plaque rupture. Circulation 1995;92:1565-9.

37. Siwik DA, Chang DL, Colucci WS. Interleukin-1beta and tumor necrosis factor-alpha decrease collagen synthesis and increase matrix metalloproteinase activity in cardiac fibroblasts *in vitro*. Circ Res 2000;86:1259-65.

38. Yamamoto T, Eckes B, Mauch C, Hartmann K, Krieg T. Monocyte chemoattractant protein-1 enhances gene expression and synthesis of matrix metalloproteinase-1 in human fibroblasts by an autocrine IL-1 alpha loop. J Immunol 2000;164:6174-9.

39. Beg AA. Endogenous ligands of Toll-like receptors: implications for regulating inflammatory and immune responses. Trends Immunol 2002;23:509-12.

40. Li M, Carpio DF, Zheng Y, Bruzzo P, Singh V, Ouaaz F, et al. An essential role of the NF-kappa B/Toll-like receptor pathway in induction of inflammatory and tissue-repair gene expression by necrotic cells. J Immunol 2001;166:7128-35.

41. Brandtzaeg P. The role of humoral mucosal immunity in the induction and maintenance of chronic airway infections. Am J Respir Crit Care Med 1995;151:2081-6.

42. Muller MR, Pfannes SD, Ayoub M, Hoffmann P, Bessler WG, Mittenbuhler K. Immunostimulation by the synthetic lipopeptide P3CSK4: TLR4-independent activation of the ERK1/2 signal transduction pathway in macrophages. Immunology 2001;103:49-60.

43. Gao B, Tsan MF. Endotoxin contamination in recombinant human heat shock protein 70 (Hsp70) preparation is responsible for the induction of tumor necrosis factor alpha release by murine macrophages. J Biol Chem 2003;278:174-9.

44. Travassos LH, Girardin SE, Philpott DJ, Blanot D, Nahori MA, Werts C, et al. Toll-like receptor 2-dependent bacterial sensing does not occur via peptidoglycan recognition. EMBO Rep 2004;5:1000-6.

45. Shishido T, Nozaki N, Takahashi H, Arimoto T, Yakeishi Y, Kubota I. Effect of TLR-2 deficiency on inhibition of neointimal formation after vascular injury [Abstract].

CHAPTER 6

Peptidoglycan increases firm adhesion of monocytes to endothelial cells under flow and primes monocyte chemotaxis

Manon M Oude Nijhuis[1,2], Gerard Pasterkamp[1], Nienke I Sluis[1], Dominique PV de Kleijn[1,2], Jon D Laman[3], Laurien H Ulfman[4]

1. Experimental Cardiology Laboratory, University Medical Center Utrecht, Utrecht
2. Interuniversity Cardiology Institute of the Netherlands (ICIN), Utrecht
3. Department of Immunology and MS Center ErasMS, Erasmus MC, Rotterdam
4. Department of Pulmonary Diseases, University Medical Center Utrecht, Utrecht

SUBMITTED FOR PUBLICATION

ABSTRACT

Objective: The Toll-like receptor (TLR)2/nucleotide-binding oligomerization domain (NOD) ligand peptidoglycan (PG) has been shown to be present in macrophage-rich regions within atherosclerotic lesions. Furthermore, it is known that TLR2 ligation stimulates atherosclerotic plaque and intima formation in *in vivo* mouse models. Monocytes play an important role in the initiation and progression of atherosclerosis but it is as yet largely unknown whether PG affects adhesion and migration processes. We therefore determined the effect of PG and $Pam_3Cys\text{-}SK_4$, a synthetic TLR2 activator, on I) adhesion molecule expression by flow cytometry, II) monocyte adhesion to endothelial cells under flow conditions and III) monocyte migration using a Boyden chamber assay.

Methods and results: Total adhesion (=rolling and firm adhesion) of the PG-stimulated monocytes to L-cells, constitutively expressing ICAM-1 and E-selectin, was decreased (shear stresses 0.8-1.6 dyn/cm^2). This was most likely due to the L-selectin shedding, since monocyte incubation with a blocking L-selectin antibody resulted in a comparable number of adherent monocytes as PG-stimulated cells. Stimulation by PG resulted in an increase in the percentage of firmly adherent, polarized cells. Furthermore, PG stimulated a β_2-integrin-dependent increase in binding of monocytes to ICAM-1 coated beads. Interestingly, PG-activated monocytes were primed for increased migration towards the chemoattractant C5a which was TLR2- and β_2-integrin-dependent. Next to PG stimulation, monocytes were stimulated with $Pam_3Cys\text{-}SK_4$, which gave identical results in all assays tested.

Conclusion: This study demonstrates that PG activation of monocytes results in an increase in adhesive and migratory capacities of these cells. This might be a mechanism by which PG promotes atherosclerotic disease *in vivo*.

INTRODUCTION

One of the first steps in the series of events underlying atherosclerotic plaque formation is the recruitment of monocytes to the site of vascular damage with subsequent uptake of oxidized lipid particles [1]. Atherogenic lesions have been observed to arise at regions of the vessel wall exhibiting endothelial activation. The adhesion of circulating monocytes to the activated endothelium is an important first event in the inflammatory response. Like for all leukocytes, monocyte adhesion to activated endothelial cells is a multistep process [2]. First, L-selectin (CD62L) and the P-selectin glycoprotein ligand-1 (CD162) expressed on monocytes and E-selectin (CD62E) and P-selectin (CD62P) expressed on activated endothelial cells mediate the initial tethering and rolling interactions of these cells. When a rolling monocyte encounters chemokines presented by the activated endothelial cells, integrins get activated, a process that is called inside-out signaling [3]. Not only chemokines, but also other stimuli like growth factors, cytokines, and bacterial-derived products such as lipopolysaccharide (LPS), a Toll-like receptor (TLR)4 ligand [4], and R-848, a TLR7 ligand [5], are able to activate integrins on leukocytes. However, these stimuli act much slower (minutes) than chemokines (seconds) in integrin activation. Activated β_2-integrins and α_4-integrins on the monocyte ensure firm adhesion by binding to intercellular adhesion molecule-1 (ICAM-1/CD54) and vascular cell adhesion molecule-1 (VCAM-1/CD106) on the activated endothelium, respectively [2, 6]. Finally, the cells spread and migrate into the site of inflammation where in the case of monocyte migration to the atherosclerotic plaque the cells develop into large lipid-laden macrophages [7].

Monocytes are part of the innate immune system that provides a first line of defense against invading pathogens. This response is initiated by host pattern recognition receptors (PRRs) [8]. The most widely studied class of PRRs are the TLRs. Eleven members of the TLR-family have been described and all recognise specific microbial agents, also known as pathogen–associated molecular patterns [9]. The latter include, among others, LPS, lipoteichoic acid (LTA), peptidoglycan (PG) and flagellin. TLRs are transmembrane proteins that have been shown to initiate signaling cascades that ultimately regulate the immune response via nuclear factor (NF)-κB. Of the eleven members of the TLR-family, TLR1, TLR2 and TLR4 expression is markedly enhanced in human atherosclerotic plaques [10]. Recently, we observed that TLR4 [11] and TLR2 [12] ligation accelerates neointima formation in arteries of mice. In addition, we previously observed that PG, a bacterial TLR2/nucleotide-binding oligomerization domain (NOD) receptor ligand, is prevalent in atherosclerotic lesions with an inflammatory unstable phenotype. The presence of PG was mainly observed in macrophage-rich atheromatous regions [13]. PG is the major constituent of Gram-positive bacteria and is known to induce production of pro-inflammatory cytokines. In a clinical study, we demonstrated a significant relation between PG-specific IgM antibody levels and carotid intima-media thickness [14].

The mechanisms by which TLR2 ligation could enhance initiation or progression of atherosclerotic plaque formation are unknown. Considering the aforementioned relation of PG staining with vulnerable inflammatory plaque characteristics, we hypothesized that stimulation of monocytes by PG affects the adhesive and migratory

properties of these cells contributing to lesion growth. Some controversy exists whether PG is a genuine TLR2 ligand, in addition to its uncontested NOD engagement [15, 16]. Therefore, we systematically compared activity of our *Staphylococcus aureus* PG preparation [17] with the commercial synthetic TLR2 ligand, $Pam_3Cys\text{-}SK_4$. In the present study, we investigated the effect of PG and $Pam_3Cys\text{-}SK_4$ on adhesion molecule expression, adhesion to endothelial cells in an *in vitro* flow chamber model and on migration of primary human monocytes.

MATERIALS AND METHODS

Antibodies

mAb's IB4, DREG56 and control antibody W6/32 (anti HLA-A, -B, -C) were isolated from the supernatant of hybridoma obtained from the American Type Culture Collection (Rockville, MD). BBA2 (clone 5D11) was purchased from R&D systems (Minneapolis, MN) and ENA2 was kindly provided by Dr W.A. Buurman [18]. TLR2 blocking antibody (clone TLR2.1) was obtained from eBiosciences (San Diego, CA). Anti-CD18-FITC, anti-CD11b-PE and anti-CD62L-FITC antibodies were purchased from DAKO (Glostrup, Denmark). mAb's were incubated with monocytes ($4x10^6$ cells/ml) or with confluent L-cell layers on cover slips at 10 µg/ml during 15 min before the experiments. The cell suspensions were diluted once with HEPES incubation buffer (final concentration of 5 µg/ml mAb at $2x10^6$ cells/ml). HEPES incubation buffer contained 20 mM HEPES, 132 mM NaCl, 6 mM KCl, 1 mM $MgSO_4$, 1.2 mM KH_2PO_4, supplemented with 5 mM glucose, 1.0 mM $CaCl_2$ and 0.5% (w/v) human serum albumin (HSA).

TLR2 ligands

Since commercial PG preparations are routinely contaminated with LPS (and vice versa), PG was isolated from *S. aureus* using a special methodology, as described previously [19] and kindly provided by Prof. Dr. U. Zähringer (Leibniz-Center for Medicine and Biosciences, Borstel, Germany). This isolated PG is a crude PG preparation containing lipopeptides that show TLR2 activity as well but most likely not related to LTA. Importantly, the preparation was rigorously checked for LPS contamination which was below detection limit (15 pg/mg). We compared activity of our PG preparation with the commercial synthetic TLR2 ligand, $Pam_3Cys\text{-}SK_4$ (Novabiochem, Cambridge, MA). This is a cell-permeable, water soluble cationic lipohexapeptide analog of the immunologically active N-terminal portion of bacterial lipoprotein known for its ability to activate monocytes and macrophages [20]. Monocytes ($2x10^6$ cells/ml) were stimulated with the PG preparation (3 µg/$1x10^6$ cells) and $Pam_3Cys\text{-}SK_4$ (1 µg/$1x10^6$ cells) for 30 min at 37°C unless stated otherwise.

Isolation of monocytes

Blood, anti-coagulated with 0.4% trisodium-citrate (pH 7.4), was obtained from healthy volunteers from the University Medical Center Utrecht, The Netherlands. Mononuclear cells were separated from granulocytes and red blood cells by centrifugation over isotonic 1.077 g/ml ficoll-paque (Pharmacia, Uppsala, Sweden). Monocytes were

further purified from peripheral blood mononuclear cells by depletion of non-monocytes (negative selection) via magnetic cell sorting using a human monocyte isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany). After isolation the cells were resuspended in HEPES incubation buffer ($4x10^6$ cells/ml) and kept on ice.

Flow cytometry
Flow cytometry analysis was carried out as described before [21]. In short, monocytes were incubated with either PG or $Pam_3Cys\text{-}SK_4$ for 5-60 min at 37°C. After washing with cold PBS, antibodies against $\alpha_m\beta_2$-integrins (αCD11b-PE and αCD18-FITC) and L-selectin (αCD62L-FITC) were added to the cells and incubated in the dark for 45 min at 4°C. After washing the cells, fluorescence was quantified for 10.000 cells with a FACSvantage flowcytometer (Becton Dickinson immunocytometry systems, Mountain View, CA). To exclude the possibility of LPS contamination in the PG batch, 15 μg/ml polymyxin B sulphate (Calbiochem, San Diego, CA) was added to control and PG-activated monocytes. Monocytes were incubated with the antibodies described above and fluorescence was quantified with the flowcytometer. The results showed no decrease in fluorescence when polymyxin B was added, further confirming that our PG batch did not contain biologically relevant traces of LPS.

L-cells
L-cells were kindly provided by Dr. W. Smith (Baylor College of Medicine, Houston, TX). These L-cells transfected with ICAM-1 and E-selectin were cultured in RPMI-1640 medium supplemented with glutamine and neomycin. L-cells coated on Thermanox coverslips (18x18 mm, confluent cell layer) were used in perfusion assays.

Monocyte perfusion and evaluation
Perfusions under steady flow were performed in a modified transparent parallel-plate perfusion chamber as described by Van Zanten *et al* [22]. *In vitro* flow chamber experiments were performed as described previously [23]. In short, monocytes in suspension ($2x10^6$ cells/ml in HEPES incubation buffer, preincubated with PG, $Pam_3Cys\text{-}SK_4$, or control mAb W6/32 for 30 min at 37°C) were aspirated from a reservoir through the perfusion chamber (for 5 min at shear stresses of 0.8 dyn/cm^2 and 1.6 dyn/cm^2) and were recorded on videotape. Video images were evaluated for the number of adherent cells, cluster index and percentage of rolling cells using dedicated routines made in the image-analysis software Optimas 6.1 (Media Cybernetics systems, Silverspring, MD).

Fluorescent beads adhesion assay
Functionality of β_2-integrins was measured using ICAM-1 coated fluorescent microbeads. TransFluorSpheres (488/645 nm, 1.0 μm; Molecular Probes) were coated with adhesion ligands and the fluorescent beads adhesion assay was performed according to Geijtenbeek *et al* [24]. In short, monocytes were resuspended in HEPES incubation buffer. Fifty thousand cells were preincubated with control anti HLA-ABC (W6/32) mAb or with anti β_2-integrin blocking mAb IB4 (10 μg/ml) and 0.6 μg/100 μl PG or 0.2 μg/100 μl $Pam_3Cys\text{-}SK_4$ for 30 min at 37°C. The ligand-coated beads

(40 beads/cell) were washed twice and added together with the recombinant human complement factor C5a (10^{-8} M) (Sigma, St. Louis, MO) in a 96-well V-shaped–bottom plate. Next, the preincubated monocytes were added and incubated for 30 min at 37°C. The cells were washed and resuspended in HEPES incubation buffer (4°C) and kept on ice until measurement. Binding of the fluorescent beads to the monocytes was determined by flow cytometry using the FACSvantage and is depicted as the percentage of monocytes positive for ICAM-1-coated beads.

Boyden chamber migration assay

Monocyte migration was measured in the modified Boyden chamber assay as described [25]. Briefly, monocytes ($1x10^6$ cells/ml) were preincubated with control mAb (W6/32), β_2-integrin blocking mAb IB4, or a TLR2 blocking mAb for 10 min at 37°C, followed by incubation with PG, $Pam_3Cys\text{-}SK_4$ or control mAb W6/32 for 30 min at 37°C. The chemoattractant C5a (at the physiologically relevant concentration of 10^{-8}M) and control HEPES incubation buffer were put in the bottom chamber and the monocytes in the upper chamber. The monocytes were incubated for 3 h in the Boyden chamber at 37°C. Filters were fixed in 80% EtOH/20% BuOH, stained with Weigert solution and embedded in malinol. Analysis of the filters was done by an image analysis system (Quantimet 570 C) and an automated microscope to score the number of cells at 15 intervals of 10 µm in the Z-direction of the filters. The results are expressed as the mean migrated distance in µm, excluding cells with migration 0.

		CD18	
Time (min)	**control**	**PG**	**$Pam_3Cys\text{-}SK_4$**
0	384 ± 12	384 ± 12	384 ± 12
5	398 ± 24	419 ± 23	446 ± 39
15	452 ± 23	566 ± 48 *	631 ± 10 *
30	484 ± 21	693 ± 73 *	680 ± 42 *
60	521 ± 31	657 ± 37 *	661 ± 34 *
		CD11b	
Time (min)	**control**	**PG**	**$Pam_3Cys\text{-}SK_4$**
0	419 ± 85	419 ± 85	419 ± 85
5	494 ± 92	593 ± 33	830 ± 66
15	591 ± 97	926 ± 20 *	1167 ± 179 *
30	772 ± 238	1467 ± 90 *	1551 ± 23 *
60	929 ± 55	1551 ± 14 *	1568 ± 30 *
		CD62L	
Time (min)	**control**	**PG**	**$Pam_3Cys\text{-}SK_4$**
0	224 ± 21	224 ± 21	224 ± 21
5	183 ± 21	147 ± 21	122 ± 11
15	144 ± 18	45 ± 4 *	22 ± 4 *
30	144 ± 37	30 ± 3 *	27 ± 5 *
60	134 ± 26	27 ± 6 *	26 ± 3 *

Table 1. Peptidoglycan stimulation induces L-selectin (CD62L) shedding from the monocyte surface and increases $a_m\beta_2$-integrin (CD18 and CD11b) expression

*The expression of CD18, CD11b and CD62L on monocytes was measured after 5-60 min of PG or $Pam_3Cys\text{-}SK_4$ stimulation at 37°C. After stimulation with both ligands, monocytes were stained for the adhesion molecules, washed, and measured by flow cytometry (n=7). The results are expressed as mean fluorescence intensity ± SEM. *p<0.05 compared to control*

Statistical analysis

Results are expressed as mean ± standard error of the mean (SEM). Statistical analysis of the data was performed using Student's T test. A p-value <0.05 was considered to be significant.

RESULTS

Peptidoglycan stimulation increases $\alpha_m\beta_2$-integrin expression on and induces L-selectin shedding from monocytes

To investigate whether PG and $Pam_3Cys\text{-}SK_4$ affect expression levels of adhesion molecules on monocytes, the cells were incubated with these compounds at 37°C for 5-60 min. The expression of adhesion molecules CD18, CD11b and CD62L was measured by flow cytometry. Table 1 shows expression levels of CD18, CD11b and CD62L in time. PG as well as $Pam_3Cys\text{-}SK_4$ activation resulted in a time-dependent increase in expression of $\alpha_m\beta_2$-integrins CD18 and CD11b with a maximal expression at 30 min. CD62L expression decreased upon stimulation with PG and $Pam_3Cys\text{-}SK_4$, with a maximal inhibition that was reached after 30 min of incubation. The pattern of adhesion molecule expression on the PG-stimulated monocytes was comparable to the pattern of $Pam_3Cys\text{-}SK_4$-stimulated cells, suggesting that stimulation with both ligands resulted in an increased functionality state of the adhesion molecules.

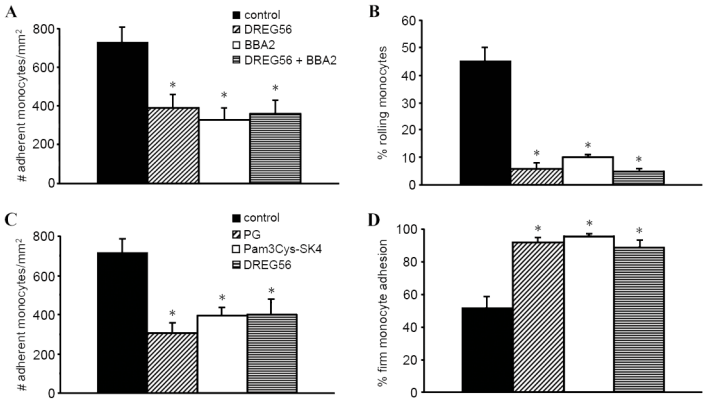

Figure 1. Peptidoglycan stimulation results in decreased total adhesion but increased firm adhesion of monocytes to L-cell layers

*Monocytes ($2x10^6$ cells/ml, 30 min, 37°C) were perfused over confluent L-cell layers at a shear stress of 0.8 dyn/cm². A) depicts the total number of adherent cells (=rolling and firmly adherent cells) and B) shows the percentage of rolling cells after blocking of L-selectin (DREG56) on monocytes, E-selectin (BBA2) on L-cells or blocking both (n=3). Figure C) depicts the total number of adherent cells and D) shows the firm adhesion of control, PG- or $Pam_3Cys\text{-}SK_4$-stimulated monocytes and monocytes incubated with the L-selectin blocking antibody DREG56 to confluent L-cell layers (n=10-16). Results are expressed as mean ± SEM. *p<0.05 compared to control*

Peptidoglycan leads to a decrease in total adhesion but increase in firm adhesion and polarisation of monocytes under flow conditions

The observed L-selectin shedding by PG stimulation suggested that this would affect monocyte recruitment to endothelial cells. L-cells constitutively expressing E-selectin and ICAM-1 were used to test this hypothesis since this surface is known to support

L-selectin mediated rolling of neutrophils [26]. Indeed, *Figure 1A* shows that blocking L-selectin (DREG56) on monocytes or E-selectin (BBA2) on L-cells or blocking both, resulted in a decrease in total adhesion compared to control mAb-treated cells (389 ± 69 cells/mm^2, 327 ± 63 cells/mm^2, and 358 ± 71 cells/mm^2 versus 729 ± 74 cells/mm^2; p=0.01, p=0.006, and p=0.01, respectively). Also, a significant decrease in percentage of rolling cells was seen when L-selectin, E-selectin or both were inhibited (*Figure 1B*). When the monocytes were pre-stimulated with PG or $Pam_3Cys\text{-}SK_4$ for 30 min at 37°C the total number of adherent cells to the L-cell layer decreased to similar levels as DREG56-treated monocytes (PG, 309 ± 50 cells/mm^2, p<0.001; $Pam_3Cys\text{-}SK_4$, 394 ± 44 cells/mm^2, p=0.003; DREG56, 402 ± 78 cells/mm^2, p=0.004, compared to control, 718 ± 71 cells/mm^2; *Figure 1C*). This suggests that the PG- and $Pam_3Cys\text{-}SK_4$-induced L-selectin shedding is responsible for the decrease in total adhesion. Similar experiments were performed at a shear stress of 1.6 dyn/cm^2 and the results were comparable to 0.8 dyn/cm^2 (PG, 212 ± 34 cells/mm^2, p<0.001; $Pam_3Cys\text{-}SK_4$, 172 ± 63 cells/mm^2, p<0.001; DREG56, 216 ± 78 cells/mm^2 p=0.004, compared to control, 538 ± 52 cells/mm^2). Furthermore, the percentage firm adhesion of monocytes to the L-cell layer of PG- or $Pam_3Cys\text{-}SK_4$- treated cells compared to control monocytes was greatly increased (92 ± 3%, 96 ± 1% versus 52 ± 7%, p<0.001 and p=0.004, respectively (*Figure 1D*). This was comparable to the DREG56-treated monocytes (89 ± 4%, p=0.01 compared to control).

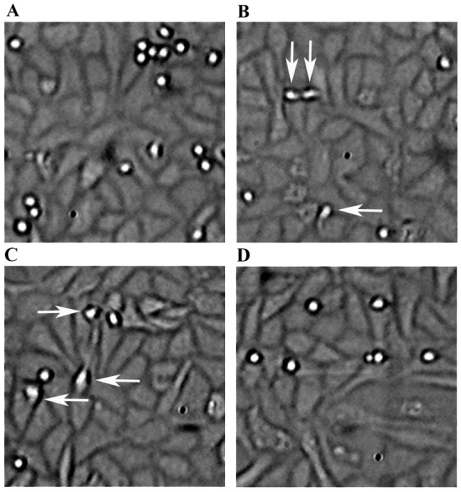

Figure 2. Spreading of monocytes on endothelial cells under flow is induced by peptidoglycan stimulation
Video microscopy of adherent monocytes on L-cells under flow conditions. The monocytes were untreated (A), pre-treated with PG (B) or $Pam_3Cys\text{-}SK_4$ (C), or incubated with DREG56, an L-selectin blocking antibody (D), and perfused over the L-cells at a shear stress of 0.8 dyn/cm^2. Images were recorded on video during 5 min. The images depicted were taken after 3 min of monocyte perfusion. The firmly adhering control monocytes remained round, white-centered cells, whereas the PG- or $Pam_3Cys\text{-}SK_4$-stimulated monocytes spread within a few moments (arrows indicate spreading cells) indicative of active migration. Once firmly adhered, the DREG56-incubated monocytes remained rounded.

However, a clear difference between the DREG56-treated and the PG-treated cells was the appearance of the cells. Unstimulated, control mAb-treated monocytes appeared as round, bright white-centered cells (*Figure 2A*), indicating that these cells were non-

activated. Similarly, the DREG56-treated cells that were firmly attached also had a rounded morphology (*Figure 2D*). In contrast, the PG- and Pam_3Cys-SK_4-stimulated cells polarized immediately after attachment to the endothelium (*Figures 2B* and *2C* for PG- and Pam_3Cys-SK_4-stimulated cells, respectively). This suggests that TLR2 ligation in addition to its effect on functional L-selectin shedding also induces activation of the cells resulting in cell spreading. This prompted us to further determine the activation of integrins and the migration capacities of PG- and Pam_3Cys-SK_4-ligated monocytes.

Peptidoglycan-induced inside-out signaling leads to activation of β_2-integrins.
It has been well documented that not the increased expression levels of β_2-integrins but instead the activation of the integrins via inside-out signaling leads to increased adhesion of monocytes to ICAM-1. Therefore, we determined the binding of monocytes to ICAM-1-coated fluorescent beads using a flow cytometry based adhesion assay. Monocytes were pre-incubated with a control mAb or a blocking β_2-integrin mAb and then stimulated with PG or Pam_3Cys-SK_4. Stimulation by PG or Pam_3Cys-SK_4 significantly increased binding of monocytes to the ICAM-1 coated beads compared to control monocytes (38 ± 6% and 37 ± 3% versus 18 ± 3%, p=0.02 and p=0.01, respectively; *Figure 3*). Incubation of the monocytes with the β_2-integrin blocking mAb IB4 abrogated monocyte binding to the fluorescent beads showing that the interaction is β_2-integrin-specific (PG, 7.3 ± 2.0%, p=0.002; Pam_3Cys-SK_4, 6.0 ±0.5%, p=0.007; control, 4.5 ± 0.7%, p=0.002, compared to control mAb).

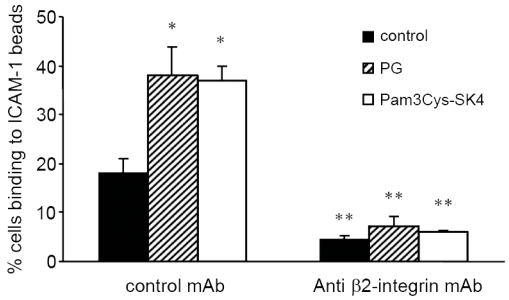

Figure 3. Peptidoglycan stimulation increases monocyte binding to ICAM-1 coated fluorescent beads
*The percentage binding of the control, and TLR2-stimulated monocytes to fluorescent ICAM-1 coated beads, measured by flow cytometry, is shown. Monocytes were pre-incubated with a control mAb or a β_2-integrin blocking mAb and were either not stimulated or stimulated with PG or Pam_3Cys-SK_4 for 30 min at 37°C. Results are expressed as mean ± SEM (n=6). *p<0.05 compared to control monocytes and **p<0.05 compared to control mAb*

TLR2-dependent priming of monocytes by peptidoglycan results in increased β_2-integrin-dependent migration towards C5a
Activated integrins are a prerequisite for chemotaxis. Although chemokines can activate integrins this does not always lead to optimal migration. For eosinophils for example it is known that priming with IL-5 is needed for migration to suboptimal concentrations of PAF. Therefore, we tested whether PG and Pam_3Cys-SK_4 could prime the monocytes for enhanced migration towards C5a in a Boyden chamber assay. Indeed, PG or Pam_3Cys-SK_4 greatly enhanced migration towards C5a (10^{-8}M) compared to unstimulated cells (PG; 45 ± 5 µm and Pam_3Cys-SK_4; 61 ± 9 µm versus unstimulated; 24 ± 3 µm, p=0.01 and p=0.02, respectively; *Figure 4A*). A trend in increased chemotaxis of the control

monocytes towards C5a compared to HEPES incubation buffer was observed (24 ± 3 µm versus 18 ± 3 µm). Moreover, chemotaxis of the monocytes stimulated with PG or $Pam_3Cys\text{-}SK_4$ towards C5a was significantly increased compared to HEPES incubation buffer (PG; 45 ± 5 µm versus 19± 3 µm, p=0.01 and $Pam_3Cys\text{-}SK_4$; 61 ± 9 µm versus 23 ± 4 µm, p=0.007).

Since PG is known to bind to TLR2 but also contains minimal motifs that can bind to intracellular NOD receptors leading to cell activation [27], we determined whether the priming-effect of our PG preparation was TLR2-specific. *Figure 4B* shows that incubation of monocytes with a blocking TLR2 mAb (PG, 25 ± 3 µm versus 41 ± 2 µm, p=0.004; $Pam_3Cys\text{-}SK_4$, 26 ± 3 µm versus 43 ± 6 µm, p=0.036; anti-TLR2 mAb versus control mAb, respectively) or a blocking β_2-integrin mAb (PG, 18 ± 2 µm versus 41 ± 2 µm, p<0.001; $Pam_3Cys\text{-}SK_4$, 22 ± 1 µm versus 43 ± 6 µm, p=0.001; anti-β_2-integrin mAb versus control mAb, respectively) resulted in a significant decrease in PG- and $Pam_3Cys\text{-}SK_4$-primed migration towards C5a. Thus, the PG and $Pam_3Cys\text{-}SK_4$ effects are TLR2-specific and totally β_2-integrin-dependent.

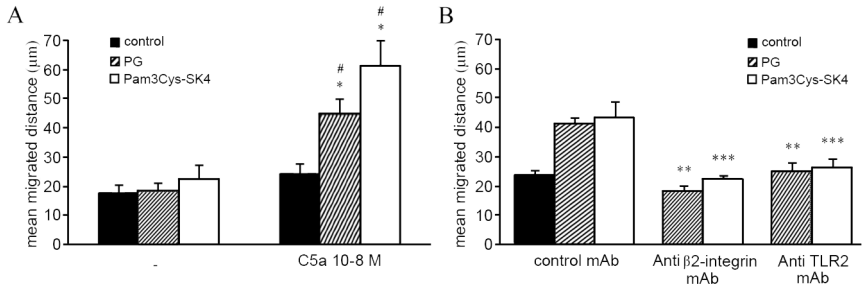

Figure 4. Migratory capacity of monocytes is increased by TLR2 ligation
The mean migrated distance (µm) of the control and PG- or $Pam_3Cys\text{-}SK_4$-stimulated monocytes towards HEPES incubation buffer and C5a (10^{-8}M) in a Boyden chamber assay is shown. A) shows mean migrated distance of monocytes that were left unstimulated or were stimulated with PG or $Pam_3Cys\text{-}SK_4$ for 30 min at 37°C and incubated for 3 h in the Boyden chamber (n=7). Figure B) depicts migration of monocytes towards C5a. Monocytes were pre-incubated for 10 min at 37°C with control mAb (W6/32), a β_2-integrin blocking mAb, or a TLR2 blocking mAb, followed by incubation with W6/32, PG or $Pam_3Cys\text{-}SK_4$ for 30 min at 37°C and 3 h in the Boyden chamber (n=4). Results are expressed as mean ± SEM.
**p<0.05 compared to control monocytes, #p<0.05 compared to HEPES incubation buffer, **p<0.05 compared to PG-stimulated monocytes, and ***p<0.05 compared to $Pam_3Cys\text{-}SK_4$-stimulated monocytes*

DISCUSSION

In this study, we examined the effect of PG and $Pam_3Cys\text{-}SK_4$, which are known TLR2 ligands, on monocyte-endothelial adhesion and on monocyte migration. We showed that PG and $Pam_3Cys\text{-}SK_4$ stimulation leads to I) shedding of L-selectin from the monocyte surface resulting in a decrease in E-selectin-mediated rolling interactions under flow conditions, II) an increased expression and function of β_2-integrins on monocytes leading to polarization of the cells and III) priming of the monocytes resulting in increased chemotaxis towards C5a. This study reveals a mechanism by which the presence of the TLR2/NOD ligand PG, that is associated with histological markers for plaque vulnerability [13], could influence atherosclerotic plaque development and progression via monocyte recruitment.

L-selectin is constitutively expressed on leukocytes, but is shed by proteolytic cleavage following cellular activation [28]. Indeed, after 30 min of stimulation by either PG or $Pam_3Cys\text{-}SK_4$, L-selectin was shed from the monocyte surface. Furthermore, β_2-integrin expression on the monocyte surface was increased. These data are consistent with a paper by Sabroe *et al* showing decreased L-selectin and increased integrin expression after TLR2 stimulation on neutrophils [29]. In contrast, Saetre *et al* showed that *Streptococcus pyogenes* PG amplified the LTA-induced decrease in expression of L-selectin on monocytes, but PG stimulation alone had no effect [30]. An important difference with our PG is the bacterial source (*S. aureus* versus *S. pyogenes*) suggesting that the effects of the cell wall components might vary between different bacterial species. Using an *in vitro* flow chamber model, we analyzed the adhesive behaviour of freshly isolated monocytes to E-selectin and ICAM-1 expressing L-cell layers which mediate L-selectin-dependent rolling of neutrophils on E-selectin [26]. We obtained similar results for monocytes and could therefore asses the role of L-selectin/E-selectin-dependent interactions after stimulation. Stimulation of monocytes by PG or $Pam_3Cys\text{-}SK_4$ decreased the total adhesive capacities (=rolling plus firm adhesion) to L-cells to a similar extent as when L-selectin on monocytes was inhibited. However, a clear difference was observed in the polarization of the cells since the PG- and $Pam_3Cys\text{-}SK_4$-treated cells all showed a polarized phenotype after 5 min of perfusion whereas the residual firm adherent cells in the anti-L-selectin-treated group remained rounded. Indeed, we showed that activation of the monocytes by PG and $Pam_3Cys\text{-}SK_4$ resulted not only in an upregulation of β_2-integrin expression as detected by flow cytometry but also in a functional increase as detected by the increased binding to ICAM-1-coated fluorescent beads. Interesting, PG and $Pam_3Cys\text{-}SK_4$ activation also resulted in priming of the monocytes thereby increasing their chemotactic behaviour. This effect was TLR2-specific and dependent on β_2-integrins. Previously, Ogawa *et al* showed that bacterial cell walls and PG specimens from bacterial walls caused an increase in migration of peripheral blood mononuclear cells towards FMLP [31]. Yipp *et al* [32] showed that systemic admission of PG in mice resulted in an increased firm adhesion and migration of total leukocytes compared to sham-treated mice after 4 hours. This at least shows that PG induced similar effects *in vivo* as we observed *in vitro*. A discrepancy is the observed increase in rolling flux *in vivo* rather than the decrease in rolling cells in our system which might be explained by the difference in experimental set up (e.g. time frame of the experiment, total versus isolated cells). The increased chemotactic effect upon PG stimulation resembles GM-CSF and IL-5 induced priming of chemotaxis of neutrophils and eosinophils, respectively [33]. It is generally accepted that β_2-integrins play an important role in the migration of monocytes into atherosclerotic sites [34] and therefore it might be that PG and $Pam_3Cys\text{-}SK_4$ promote this mechanism.

Dziarski *et al* demonstrated that TLR2 is the receptor for isolated *S. aureus* PG [15] whereas $Pam_3Cys\text{-}SK_4$ has been shown to bind and signal through TLR2/6 heterodimers. TLR2 recognizes several other molecules as well including LTA, lipoproteins and lipoarabinomannan. In a recent study, Travassos *et al* [16] concluded that non-PG contaminants of PG preparations (lipoproteins and LTA) are responsible

for recognition by TLR2 and that PG detection is more likely to occur through NOD2 (CARD15), an intracellular protein. NOD2 is a general sensor for both Gram-positive and Gram-negative bacteria, through the recognition of muramyl dipeptide (MDP), the minimal motif found in all PGs [35]. In contrast to NOD2, NOD1 (CARD4) specifically recognizes DAP-type PG, which is present in all Gram-negative bacteria [36]. In this study, we used PG derived from *S. aureus*, which does not contain the motif required for NOD1 ligation, but does contain the motif necessary for NOD2 ligation. In addition, our crude PG preparation most likely contains lipopeptides that have potential TLR2-activating capacities since the isolation procedure was performed as described in [19]. At this moment the exact identification of these lipoproteins is under investigation but it can be excluded that the observed TLR2 activity is dependent on LTA contamination. Theoretically, PG could exert its function via NOD2 activation of monocytes in our study. However, this is unlikely since we showed that stimulation of the monocytes with the PG preparation and the TLR2 agonist $Pam_3Cys\text{-}SK_4$ resulted in the exact similar effects in all assays that were performed. Furthermore, we showed PG-stimulatory effects on the monocytes after short incubation-time, suggesting that processing of PG into small fragments that are necessary for NOD2 engagement is unlikely. Moreover, the $Pam_3Cys\text{-}SK_4$- and PG-induced priming of migration was abrogated by inhibiting the TLR2 receptor on monocytes.

In previous studies, we found that PG was present in atherosclerotic plaques. PG was mainly present in macrophage-rich regions in plaques that displayed all features of rupture prone vulnerable plaques [13]. PG is composed of two repeating alternating amino sugars, N-acetylglucosamine and N-acetylmuramic acid, attached to each other by glycosidic bonds. Muramic acid is a unique structural component of PG and can therefore be used as a marker for PG detection. Lehtonen *et al* described that muramic acid can be observed in mononuclear and polymorphonuclear cells in the peripheral blood of healthy adults [37]. Recently, we demonstrated that TLR2 is present on vascular cells and that TLR2 ligation by $Pam_3Cys\text{-}SK_4$ is able to induce expression of pro-inflammatory cytokines and chemokines by vascular cells [12]. Furthermore, using an *in vivo* mouse model, local adventitial triggering via TLR2 ligation by both $Pam_3Cys\text{-}SK_4$ and the same *S. aureus* PG preparation as used here in the arterial wall resulted in increased intimal formation, suggesting an important role for TLR2 in arterial obstruction.
TLR2 has also been shown to bind other bacterial structures like fimbriae from *Porphyromonas gingivalis*, a microorganism described to accelerate atherosclerosis in an *in vivo* mouse model [38]. *P. gingivalis*-derived fimbriae structures induced activation of β_2-integrins determined by the binding of the CBRM1/5 antibody, recognizing the activation epitope of CD11b/CD18 [39]. However, this study did not further address the adhesive and migratory implications of this activation. Furthermore, *Chlamydia pneumoniae* has also been implicated in the development of atherosclerosis and functionally increased β_2-integrin-mediated adhesion and migration of monocytes and macrophages *in vivo* [40]. Interestingly, *C. pneumoniae* activates monocytes via TLR2 [41]. These data support the hypothesis that TLR2-mediated activation of monocytes via PG (our study) or other structures [38] contributes to the development

of atherosclerosis.
In this study, we show a potential new role for PG in stimulating increased firm adhesion of monocytes to ICAM-1 expressing cell layers under flow conditions and increased migratory capacities. Despite the observation that the L-selectin shedding caused by PG and $Pam_3Cys\text{-}SK_4$ stimulation leads to reduced monocyte adhesion to the endothelium, the fraction of cells that did bind to the endothelium showed strong binding, spreading and TLR2-specific and β_2-integrin-dependent migration. The present results suggest that TLR2 activation may enhance accumulation of monocytes at sites of inflammation like the atherosclerotic arterial wall.

ACKNOWLEDGEMENTS

This work was supported by the Dutch Heart Foundation (grant 2001-077) and the Dutch Foundation for MS Research (grant 04-540 MS). L.H. Ulfman was supported by a grant of the Netherlands Organisation for Scientifc Research (NWO, 016.036.051). The authors thank Prof. Dr. Ulrich Zähringer for critically reading the manuscript and helpful discussions.

REFERENCES

1. Gerrity RG. The role of the monocyte in atherogenesis: I. Transition of blood-borne monocytes into foam cells in fatty lesions. Am J Pathol 1981;103:181-90.
2. Springer TA. Traffic signals for lymphocyte recirculation and leukocyte emigration: the multistep paradigm. Cell 1994;76:301-14.
3. Ginsberg MH, Du X, Plow EF. Inside-out integrin signalling. Curr Opin Cell Biol 1992;4:766-71.
4. Haugen TS, Skjonsberg OH, Nakstad B, Lyberg T. Modulation of adhesion molecule profiles on alveolar macrophages and blood leukocytes. Respiration 1999;66:528-37.
5. Nagase H, Okugawa S, Ota Y, Yamaguchi M, Tomizawa H, Matsushima K, et al. Expression and function of Toll-like receptors in eosinophils: activation by Toll-like receptor 7 ligand. J Immunol 2003;171:3977-82.
6. Chuluyan HE, Issekutz AC. VLA-4 integrin can mediate CD11/CD18-independent transendothelial migration of human monocytes. J Clin Invest 1993;92:2768-77.
7. Ross R. Atherosclerosis--an inflammatory disease. N Engl J Med 1999;340:115-26.
8. Medzhitov R, Janeway CA, Jr. Innate immunity: the virtues of a nonclonal system of recognition. Cell 1997;91:295-8.
9. Janeway CA, Jr. The immune system evolved to discriminate infectious nonself from noninfectious self. Immunol Today 1992;13:11-6.
10. Edfeldt K, Swedenborg J, Hansson GK, Yan ZQ. Expression of toll-like receptors in human atherosclerotic lesions: a possible pathway for plaque activation. Circulation 2002;105:1158-61.
11. Vink A, Schoneveld AH, van der Meer JJ, van Middelaar BJ, Sluijter JP, Smeets MB, et al. *In vivo* evidence for a role of toll-like receptor 4 in the development of intimal lesions. Circulation 2002;106:1985-90.
12. Schoneveld AH, Oude Nijhuis MM, van Middelaar B, Laman JD, de Kleijn DP, Pasterkamp G. Toll-like receptor 2 stimulation induces intimal hyperplasia and atherosclerotic lesion development. Cardiovasc Res 2005;66:162-9.
13. Laman JD, Schoneveld AH, Moll FL, van Meurs M, Pasterkamp G. Significance of peptidoglycan, a proinflammatory bacterial antigen in atherosclerotic arteries and its association with vulnerable plaques. Am J Cardiol 2002;90:119-23.
14. Oude Nijhuis MM, van der Graaf Y, Melief MJ, Schoneveld AH, de Kleijn DP, Laman JD, et al. IgM antibody level against proinflammatory bacterial peptdioglycan is inversely correlated with extent of atherosclerotic disease. Atherosclerosis 2004;173:245-51.
15. Dziarski R, Gupta D. *Staphylococcus aureus* peptidoglycan is a toll-like receptor 2 activator: a reevaluation. Infect Immun 2005;73:5212-6.
16. Travassos LH, Girardin SE, Philpott DJ, Blanot D, Nahori MA, Werts C, et al. Toll-like receptor 2-dependent bacterial sensing does not occur via peptidoglycan recognition. EMBO Rep 2004;5:1000-6.
17. de Jonge BL, Chang YS, Gage D, Tomasz A. Peptidoglycan composition of a highly methicillin-resistant *Staphylococcus aureus* strain. The role of penicillin binding protein 2A. J Biol Chem 1992;267:11248-54.
18. Leeuwenberg JF, Van Damme J, Meager T, Jeunhomme TM, Buurman WA. Effects of tumor necrosis factor on the interferon-gamma-induced major histocompatibility complex class II antigen expression by human endothelial cells. Eur J Immunol 1988;18:1469-72.
19. Rosenthal RS, Dziarski R. Isolation of peptidoglycan and soluble peptidoglycan fragments. Methods Enzymol 1994;235:253-85.
20. Muller MR, Pfannes SD, Ayoub M, Hoffmann P, Bessler WG, Mittenbuhler K. Immunostimulation by the synthetic lipopeptide P3CSK4: TLR4-independent activation of the ERK1/2 signal transduction pathway in macrophages. Immunology 2001;103:49-60.
21. Bracke M, Dubois GR, Bolt K, Bruijnzeel PL, Vaerman JP, Lammers JW, et al. Differential effects of the T helper cell type 2-derived cytokines IL-4 and IL-5 on ligand binding to IgG and IgA receptors expressed by human eosinophils. J Immunol 1997;159:1459-65.
22. van Zanten HG, Saelman EU, Schut-Hese KM, Wu YP, Slootweg PJ, Nieuwenhuis HK, et al. Platelet adhesion to collagen type IV under flow conditions. Blood 1996;88:3862-71.
23. Ulfman LH, Kuijper PH, van der Linden JA, Lammers JW, Zwaginga JJ, Koenderman L. Characterization of eosinophil adhesion to TNF-alpha-activated endothelium under flow conditions: alpha 4 integrins mediate initial attachment, and E-selectin mediates rolling. J Immunol 1999;163:343-50.

24. Geijtenbeek TB, van Kooyk Y, van Vliet SJ, Renes MH, Raymakers RA, Figdor CG. High frequency of adhesion defects in B-lineage acute lymphoblastic leukemia. Blood 1999;94:754-64.

25. Schweizer RC, Kessel-Welmers BA, Warringa RA, Maikoe T, Raaijmakers JA, Lammers JW, et al. Mechanisms involved in eosinophil migration. Platelet-activating factor-induced chemotaxis and interleukin-5-induced chemokinesis are mediated by different signals. J Leukoc Biol 1996;59:347-56.

26. Abbassi O, Kishimoto TK, McIntire LV, Anderson DC, Smith CW. E-selectin supports neutrophil rolling *in vitro* under conditions of flow. J Clin Invest 1993;92:2719-30.

27. Fritz JH, Girardin SE, Fitting C, Werts C, Mengin-Lecreulx D, Caroff M, et al. Synergistic stimulation of human monocytes and dendritic cells by Toll-like receptor 4 and. Eur J Immunol 2005;35:2459-70.

28. Blankenberg S, Barbaux S, Tiret L. Adhesion molecules and atherosclerosis. Atherosclerosis 2003;170:191-203.

29. Sabroe I, Prince LR, Jones EC, Horsburgh MJ, Foster SJ, Vogel SN, et al. Selective roles for Toll-like receptor (TLR)2 and TLR4 in the regulation of neutrophil activation and life span. J Immunol 2003;170:5268-75.

30. Saetre T, Kahler H, Foster SJ, Lyberg T. Peptidoglycan and lipoteichoic acid, components of the streptococcal cell wall, have marked and differential effects on adhesion molecule expression and the production of reactive oxygen species in human whole blood leukocytes. Scand J Clin Lab Invest 2000;60:311-21.

31. Ogawa T, Kotani S, Fukuda K, Tsukamoto Y, Mori M, Kusumoto S, et al. Stimulation of migration of human monocytes by bacterial cell walls and muramyl peptides. Infect Immun 1982;38:817-24.

32. Yipp BG, Andonegui G, Howlett CJ, Robbins SM, Hartung T, Ho M, et al. Profound differences in leukocyte-endothelial cell responses to lipopolysaccharide versus lipoteichoic acid. J Immunol 2002;168:4650-8.

33. Warringa RA, Mengelers HJ, Kuijper PH, Raaijmakers JA, Bruijnzeel PL, Koenderman L. *In vivo* priming of platelet-activating factor-induced eosinophil chemotaxis in allergic asthmatic individuals. Blood 1992;79:1836-41.

34. Huo Y, Ley K. Adhesion molecules and atherogenesis. Acta Physiol Scand 2001;173:35-43.

35. Girardin SE, Boneca IG, Viala J, Chamaillard M, Labigne A, Thomas G, et al. Nod2 is a general sensor of peptidoglycan through muramyl dipeptide (MDP) detection. J Biol Chem 2003;278:8869-72.

36. Girardin SE, Boneca IG, Carneiro LA, Antignac A, Jehanno M, Viala J, et al. Nod1 detects a unique muropeptide from gram-negative bacterial peptidoglycan. Science 2003;300:1584-7.

37. Lehtonen L, Eerola E, Oksman P, Toivanen P. Muramic acid in peripheral blood leukocytes of healthy human subjects. J Infect Dis 1995;171:1060-4.

38. Gibson FC, III, Hong C, Chou HH, Yumoto H, Chen J, Lien E, et al. Innate immune recognition of invasive bacteria accelerates atherosclerosis in apolipoprotein E-deficient mice. Circulation 2004;109:2801-6.

39. Harokopakis E, Hajishengallis G. Integrin activation by bacterial fimbriae through a pathway involving CD14, Toll-like receptor 2, and phosphatidylinositol-3-kinase. Eur J Immunol 2005;35:1201-10.

40. May AE, Redecke V, Gruner S, Schmidt R, Massberg S, Miethke T, et al. Recruitment of *Chlamydia pneumoniae*-infected macrophages to the carotid artery wall in noninfected, nonatherosclerotic mice. Arterioscler Thromb Vasc Biol 2003;23:789-94.

41. Netea MG, Kullberg BJ, Galama JM, Stalenhoef AF, Dinarello CA, Van Der Meer JW. Non-LPS components of *Chlamydia pneumoniae* stimulate cytokine production through Toll-like receptor 2-dependent pathways. Eur J Immunol 2002;32:1188-95.

CHAPTER 7

Peptidoglycan promotes atherosclerotic plaque progression and homing of macrophages into atherosclerotic plaques

Manon M Oude Nijhuis[1,2], Margreet R de Vries[3], Paul H Quax[3], Jon D Laman[4], Dominique PV de Kleijn[1,2], Gerard Pasterkamp[1]

1. Experimental Cardiology Laboratory, University Medical Center Utrecht, Utrecht
2. Interuniversity Cardiology Institute of the Netherlands (ICIN), Utrecht
3. TNO-Quality of Life, Gaubius Laboratory, Leiden
4. Department of Immunology and MS Center ErasMS, Erasmus MC, Rotterdam

IN PREPARATION

ABSTRACT

Objective: Pattern recognition receptors (PRRs), like Toll-like receptors (TLRs) and intracellular nucleotide-binding oligomerization domain (NOD) proteins, play an important role in activation of the innate immune system. We previously demonstrated that peptidoglycan (PG), an exogenous TLR2/NOD ligand, is present in human atherosclerotic lesions that reveal an inflammatory vulnerable plaque phenotype. Furthermore, activation of both TLR2 and TLR4 *in vivo* results in increased neointima formation and arterial remodeling. In the present study, we determined whether PG and $Pam_3Cys\text{-}SK_4$, a synthetic TLR2 ligand, promote atherosclerotic plaque progression and characteristics in cuff-induced atherosclerotic lesions of the ApoE*3Leiden transgenic mice.

Methods and results: Application of PG and $Pam_3Cys\text{-}SK_4$ in a periadventitial cuff around the femoral artery in ApoE*3Leiden transgenic mice significantly increased atherosclerotic plaque formation, medial area and total vessel area. Furthermore, PG and $Pam_3Cys\text{-}SK_4$ administration resulted in atherosclerotic plaques that were enriched in smooth muscle cells (SMC) and collagen, whereas macrophage content was not changed. Peritoneal macrophages isolated from GFP-transgenic mice and stimulated *ex vivo* with the two ligands or saline (control) were administered intravenously in ApoE-/- mice. Only stimulated GFP cells were observed in atherosclerotic plaques in ApoE-/- mice, demonstrating that activation promotes macrophage migration into the plaque.

Conclusion: Progression of atherosclerotic plaques, enriched in SMC and collagen, is promoted by PG and $Pam_3Cys\text{-}SK_4$ stimulation *in vivo*. Furthermore, PG and $Pam_3Cys\text{-}SK_4$ stimulated macrophages show increased homing into atherosclerotic plaques, suggesting a mechanism by which PG promotes atherosclerotic disease *in vivo*.

INTRODUCTION

Toll-like receptors (TLRs) are part of the innate immune system that provides a first line of defense against invading pathogens. An innate immune response can be initiated by pattern recognition receptors (PRRs), of which TLRs are the most widely studied class [1]. TLRs are able to recognize pattern associated molecular patterns (PAMPs) carried by micro-organisms and pathogens [2]. TLRs are transmembrane proteins that are involved in responses to infection and play an important role in the development of sepsis [3]. Until now, eleven human TLRs have been identified [4]. Expression of TLR1, TLR2, and TLR4 is significantly enhanced in human atherosclerotic plaques [5]. TLR4 is the receptor for exogenous lipopolysaccharide (LPS), but also for endogenous ligands like heat shock protein (HSP)60 [6] and extra domain A of fibronectin (EDA) [7]. Upon ligand binding TLR2 forms a dimer with either TLR1 or TLR6. The heterodimer TLR2/1 detects tris-acylated lipopeptides [8], whereas the TLR2/6 heterodimer detects bis-acylated lipopeptides [9] and peptidoglycan (PG) [10]. PAMPs can also be recognized by nucleotide-binding oligomerization domain (NOD) receptors, which are intracellular PRRs. Two NOD receptors, NOD1 (CARD4) and NOD2 (CARD15), are involved in the recognition of PG [11].

PG is present in the cell walls of most bacteria; in Gram-positive bacteria PG constitutes 30-70% of the total bacterial cell wall mass and in Gram-negative bacteria only 10% of the total wall mass [12]. Stimulation of the TLR2 by PG results in production and secretion of pro-inflammatory cytokines, like IL-1, IL-6 and TNF-α, by monocytes and macrophages via activation of Nuclear Factor-κB (NF-κB). We previously observed that PG is prevalent in human atherosclerotic lesions that reveal an inflammatory unstable phenotype [13]. The presence of PG was mainly observed in macrophage-rich atheromatous regions. In a clinical study, we showed that systemic IgM antibody levels against PG are significantly lower in atherosclerotic patients compared to control patients and that the IgM antibody levels against PG are inversely related with common carotid intima-media thickness [14]. Functionally, our group showed *in vivo* that local application of $Pam_3Cys\text{-}SK_4$, a synthetic TLR2 ligand, and the TLR4 ligand LPS both resulted in accelerated neointima formation [15,16]. In a more recent paper Mullick *et al* showed that injection of $Pam_3Cys\text{-}SK_4$ in low-density lipoprotein receptor-deficient (LDLr-/-) mice exacerbates atherosclerosis. Furthermore, in the absence of known exogenous ligands, cells not of bone marrow-derived origin but expressing TLR2 enhance atherosclerotic disease, whereas cells deficient in TLR2 reduce atherosclerosis, suggesting that endogenous TLR2 agonists influence lesion progression.

The effect of PG on plaque growth and plaque characteristics is unknown. In addition, it remains to be investigated if and how PG, which is present in most mucosa and abundantly in the gut, is capable of eliciting a pro-inflammatory response that induces atherosclerotic plaque formation and destabilization.
ApoE*3Leiden transgenic mice develop diet-induced hyperlipidemia. Following placement of a non-constrictive cuff, the ApoE*3Leiden transgenic mice develop

atherosclerotic plaques with a necrotic core and a fibrous cap in the aortic arch within 14 days [17]. After 3 days monocytes start to adhere, followed by foam cell accumulation and near-total occlusion of the lumen after 14 days. In the current study, we investigated whether the local TLR2 ligands PG and $Pam_3Cys\text{-}SK_4$ stimulate plaque development and characteristics after placement of non-constrictive polyethylene cuffs around the femoral arteries of atherosclerosis-susceptible ApoE*3Leiden transgenic mice. Furthermore, we studied whether PG and $Pam_3Cys\text{-}SK_4$ stimulation of isolated peritoneal macrophages harvested from Green Fluorescent Protein (GFP) transgenic mice increases homing of these cells into the atherosclerotic plaque upon administration in the atherosclerotic ApoE -/- mice.

MATERIAL AND METHODS

Peptidoglycan

Commercial PG preparations are routinely contaminated with LPS (and vice versa). To prevent such a contamination PG was isolated from *Staphylococcus aureus* using a special methodology, as described previously [18] and kindly provided by Prof. Dr. U. Zähringer (Leibniz-Center for Medicine and Biosciences, Borstel, Germany). Importantly, the preparation was rigorously checked for LPS contamination which was below detection limit (15 pg/mg). However, this crude PG preparation contains lipopeptides that show TLR2 activity not related to LTA. Furthermore, since some controversy exists whether PG is a genuine TLR2 ligand [19], in addition to its uncontested NOD engagement [20], we systematically compared activity of our *S. aureus* PG preparation with the commercial synthetic TLR2 ligand, $Pam_3Cys\text{-}SK_4$ (Novabiochem, Cambridge, MA). This is a cell-permeable, water soluble cationic lipohexapeptide analog of the immunologically active N-terminal portion of bacterial lipoprotein known for its ability to activate monocytes and macrophages.

*ApoE*3Leiden transgenic mice*

All experiments were approved by the committee on animal welfare of TNO (The Organization for Applied Scientific Research) and University Utrecht conformed to the *Guide for Care and Use of Laboratory Animals* published by the U.S. National Institutes of Health (NIH publication No. 85-23, revised 1996). Pathogen-free transgenic ApoE*3Leiden mice were crossbred for 18 generations with C57Bl/6 mice. Male animals, age 8 to 10 weeks, were fed a cholesterol-enriched high-fat diet containing 0.5% cholate to improve intestinal cholesterol uptake and suppress bile acid synthesis, both leading to increased plasma cholesterol levels. This diet was given 4 weeks before operation and continued after operation. The high fat and 0.5% cholate-enriched [HFC] diet contained: casein 20%, choline chloride 1%, methionine 0.2%, cocoa butter 15%, cholate 0.5%, cholesterol 1%, sucrose 40.5%, cornstarch 10%, corn oil 1%, cellulose 5.1%, and mineral mixture 5.1%.

Cuff placement

After 4 weeks of 0.5% HFC diet ApoE*3Leiden transgenic mice were anesthetized with Hypnorm (fentanyl citrate 0.315 mg/ml / fluanisone 10 mg/ml; Bayer, 25 mg/kg IP) and Dormicum (midazolam 5 mg/ml; Roche, 25 mg/kg IP). The right and left femoral arteries were dissected from their surroundings. A non-constrictive polyethylene cuff (0.4 mm inner diameter, 0.8 mm outer diameter, and length 1.5 mm, Portex, Kent, UK), cut longitudinally, was placed loosely around the femoral arteries, as described before [21]. PG (1 µg/µl) or $Pam_3Cys\text{-}SK_4$ (1 µg/µl) dissolved in 2% gelatin was administered between the cuff and the right femoral artery. PBS dissolved in gelatin was injected as control between the cuff and the left femoral artery. Mice were terminated 2 weeks following cuff placement (PG, n=10; $Pam_3Cys\text{-}SK_4$, n=8).

Harvesting and administration of GFP peritoneal macrophages

GFP-mice were sacrificed and their macrophages were harvested by peritoneal lavage. The peritoneal cavity was injected with 5 ml of cold isolation buffer, containing Hanks' Balanced Salt Solution (HBSS; Invitrogen, Carlsbad, CA), bicarbonate (pH 7.3) and heparin. The fluid-distended peritoneal cavity was massaged, and the cells were collected and centrifuged at 1500 rpm for 10 min at 4°C. Cells were washed twice with RPMI 1640 medium and resuspended in complete RPMI medium, consisting of RPMI 1640 supplemented with 5% heat-inactivated fetal bovine serum, 1% non-essential amino acids and penicillin/streptomycin. Cells were plated as to adhere to culture plates and incubated at 37°C in a humified atmosphere containing 5% CO_2. After incubation for 2 h, the plates were washed twice with RPMI 1640 medium to remove non-adherent cells and the adherent cells were referred to as macrophages. Macrophages were cultured in the presence or absence of PG or $Pam_3Cys\text{-}SK_4$ for 6 hours. Cells were washed, trypsinized and resuspended in PBS.

ApoE-/- mice 20-25 weeks of age (Jackson Laboratories, Bar Harbor, Me) were divided into 3 groups. The mice were anesthetized with isoflurane and $2x10^6$ macrophages were injected in the tail vein. The first group received intravenous injections of peritoneal macrophages stimulated with PG at day 1 and day 5. The second group received injections of peritoneal macrophages stimulated with $Pam_3Cys\text{-}SK_4$ at day 1 and day 5. Group 3 receiving control peritoneal macrophages at day 1 and day 5 served as control. Recipient mice from the 3 groups were euthanized after 8 days. The mice were anesthetized with KRA cocktail containing ketaminehydrochloride 57.5 mg/ml, xylazine 10 mg/ml, atropine 0.5 mg/ml in NaCl 0.9%. The aortic arch was harvested and longitudinal cross-sections (7 µm thick) were cut and stained for histological analysis.

Histology

ApoE*3Leiden transgenic mice were anesthetized with Hypnorm/Dormicum. The thorax was opened, and mild pressure-perfusion (100 mm Hg) with 3.7% formaldehyde in 0.9% NaCl (wt/vol) for 10 min was performed by cardiac puncture. After perfusion, the femoral artery was harvested, fixed overnight in 3.7% formaldehyde in PBS, and paraffin-embedded. Serial cross-sections (5 µm thick) throughout the entire length

of the cuffed femoral artery (200 μm intervals) were used for histological analysis. All samples were routinely stained with hematoxylin–eosin. A Lawson (Elastin-von-Giesson) staining was used to visualize the internal and external elastic laminae. In each section, we measured the luminal area (LA), area inside the inner elastic lamina (IEL), and the area inside the outer elastic lamina (EEL) by using image-analyzing software (Soft Imaging Systems, Münster, Germany). The plaque area was defined as IEL-LA. The medial area was defined as EEL-IEL.
Immunohistochemical stainings were performed for α-SMC actin (monoclonal anti-α-SMC actin antibody, dilution 1:800, Sigma, St Louis, MO), and Mac-3 (monoclonal anti-mouse Mac-3, dilution 1:30, Becton Dickinson) to detect monocytes/macrophages. The percentage of SMC and monocytes/macrophages was determined by morphometry as the percentage α-SMC actin-positive or Mac-3-positive area of the medial and plaque area. Extracellular matrix deposition was determined using Sirius Red (collagen) staining. To assess the amount of proliferation we stained the sections with a monoclonal antibody that recognizes the cell cycle associated protein Ki-67. Ki-67 staining was determined as number of Ki-67 positive cells divided by total number of cells in the plaque area.
Elastin-von-Giesson stained sections were examined for the discontinuities of the internal elastic lamina. In each mouse the number of breakdowns of elastic lamina was measured in 3 slides and the average number of lamina ruptures/mouse was calculated.

Longitudinal-cut cross-sections of the aortic arch of ApoE-/- mice were stained with haematoxylin-eosin. In consecutive sections, Hoechst was used to stain cell nuclei in the plaques. GFP-containing macrophages and Hoechst staining can be visualized by immunofluorescence microscopy.

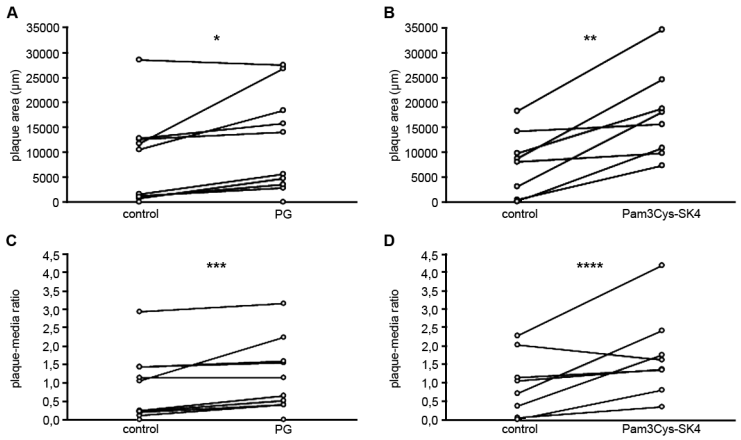

Figure 1. PG and Pam$_3$Cys-SK$_4$ stimulation induce atherosclerotic plaque development
*Plaque area (A and B) and plaque-media ratio (C and D) of ApoE*3Leiden transgenic mice treated with peri-adventitial cuff containing only gelatin (control) or cuff and gelatin containing PG (A and C, n=10) or Pam$_3$Cys-SK$_4$ (B and D, n=8) after 14 days. The contra-lateral side was used as internal control for the treated arteries and therefore data are presented as lines connecting control arteries and PG- or Pam$_3$Cys-SK$_4$-treated arteries within one mouse. *p=0.011, **p=0.012, ***p=0.008, ****p=0.05.*

Statistics

All data are presented as lines connecting control arteries and PG- or $Pam_3Cys\text{-}SK_4$-treated arteries within one mouse. Statistical analysis of the data was performed using Wilcoxon signed rank test. A value of $p<0.05$ was regarded as statistically significant.

RESULTS

Atherosclerotic plaque formation after stimulation with PG and $Pam_3Cys\text{-}SK_4$

To investigate the involvement of TLR2 in atherosclerotic plaque formation we performed *in vivo* experiments on ApoE*3Leiden transgenic mice. These mice were put on a high-cholesterol and high-fat diet for 4 weeks, followed by placement of a non-constrictive cuff with gelatin containing PG or $Pam_3Cys\text{-}SK_4$ around the right femoral artery and a cuff with only gelatin around the left femoral artery (control). Treatment of the right femoral artery with both PG and $Pam_3Cys\text{-}SK_4$ resulted in a significant increase in atherosclerotic plaque formation (PG p=0.011; $Pam_3Cys\text{-}SK_4$ p=0.012 versus control) and plaque-media ratio (PG p=0.008; $Pam_3Cys\text{-}SK_4$ p=0.05 versus control) (*Figure 1*). Furthermore, the medial area and arterial size (EEL area) of the PG- or $Pam_3Cys\text{-}SK_4$-treated arteries were significantly larger compared to the untreated arteries (medial area: PG p=0.009; $Pam_3Cys\text{-}SK_4$ p=0.05 versus control and EEL area: PG p=0.017; $Pam_3Cys\text{-}SK_4$ p=0.036 versus control). Lumen area did not differ between groups (median PG, 8515 μm^2 versus control, 7896 μm^2; $Pam_3Cys\text{-}SK_4$, 5895 μm^2 versus control, 9426 μm^2).

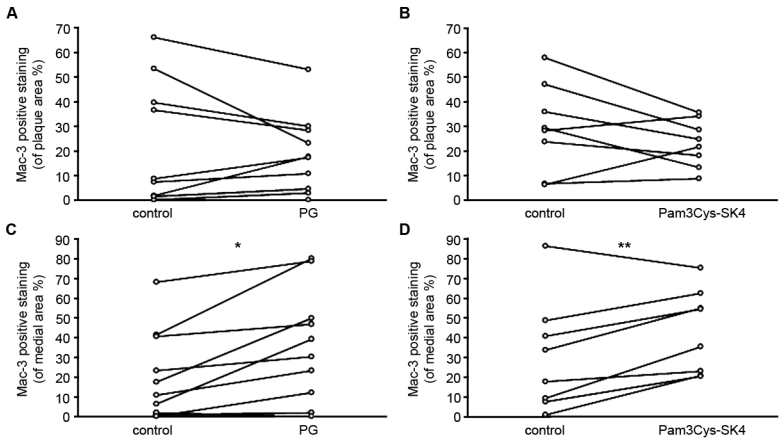

Figure 2. Macrophage content in plaque and media after PG and $Pam_3Cys\text{-}SK_4$ stimulation
*Atherosclerotic lesions in ApoE*3Leiden transgenic mice treated with a peri-adventitial cuff containing gelatin (control), gelatin and PG (A and C) or gelatin and $Pam_3Cys\text{-}SK_4$ (B and D) for 14 days were stained for Mac-3 (macrophages). Panels (A) and (B) demonstrate percentage of the plaque area that stained positive for Mac-3. Percentage of the medial area that stained positive for Mac-3 after PG and $Pam_3Cys\text{-}SK_4$ treatment is shown in (C) and (D), respectively. *p=0.009, **p=0.025*

Plaque phenotype after stimulation with PG and $Pam_3Cys\text{-}SK_4$

No significant difference was observed in macrophage content in the arterial plaques of arteries treated with PG or $Pam_3Cys\text{-}SK_4$ compared to control arteries (p=0.8 and

p=0.2 versus control, respectively, *Figure 2*). However, in the media, the macrophage positive area was significantly larger after stimulation with PG and $Pam_3Cys\text{-}SK_4$ (p=0.009 and p=0.025 versus control, respectively).

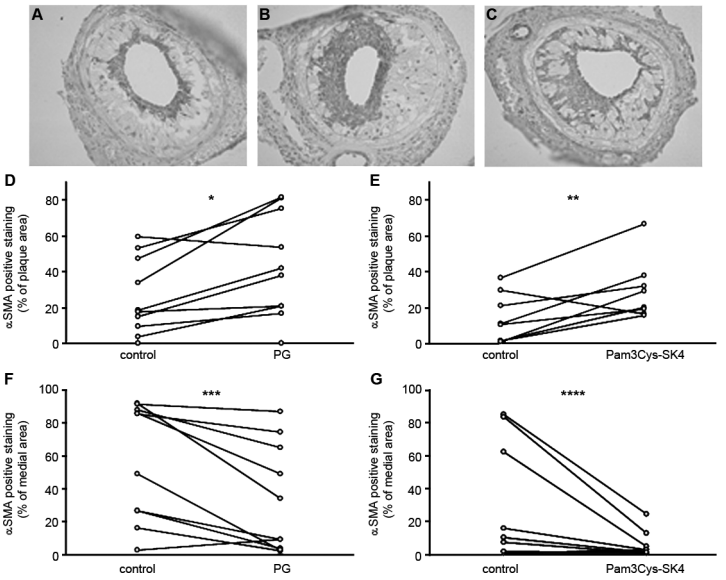

Figure 3. SMC content is increased in plaque area and decreased in medial area after PG and $Pam_3Cys\text{-}SK_4$ stimulation
*Immunohistochemistry with α-SMC actin of arteries treated with only gelatin (control, A), gelatin and PG (B) or gelatin and $Pam_3Cys\text{-}SK_4$ (C) for 14 days after cuff placement around the femoral arteries. Percentage of plaque area that stained positive for α-SMC actin in mice that were treated with PG or $Pam_3Cys\text{-}SK_4$ is shown in panels D and E. Panels (F) and (G) depict the percentage of the medial area that stained positive for α-SMC actin after placement of the cuff with gelatin and PG or gelatin and $Pam_3Cys\text{-}SK_4$, respectively. *p=0.015, **p=0.036, ***p=0.009, ****p=0.025*

The area that stained positive for α-SMC actin in the plaque was significantly increased after stimulation with both PG and $Pam_3Cys\text{-}SK_4$ (p=0.015 and p=0.036 versus control, respectively, *Figure 3*). In contrast, the medial α-SMC actin-positive area was significantly smaller after stimulation of the arteries with both PG and $Pam_3Cys\text{-}SK_4$ (p=0.009 and p=0.025 versus control, respectively).
We also measured the amount of extracellular matrix deposition in the vessel wall. For this we used a Sirius Red staining, specifically staining collagen. Both PG and $Pam_3Cys\text{-}SK_4$ stimulated collagen deposition in the atherosclerotic plaque (*Figure 4*). Extracellular matrix deposition seemed to co-localize with the presence of SMC.
Fragmentation of the internal elastic lamina is indicated in *Figures 5A* and *5B*. Arteries treated with PG or $Pam_3Cys\text{-}SK_4$ featured a significantly increased frequency of internal elastic lamina fragmentation (PG p=0.038; and $Pam_3Cys\text{-}SK_4$ p=0.046 versus control, *Figures 5C* and *5D*).
The percentage of Ki-67 positive-stained cells in the plaque of PG- or $Pam_3Cys\text{-}SK_4$-stimulated arteries did not significantly differ from control arteries (*Figure 6*).

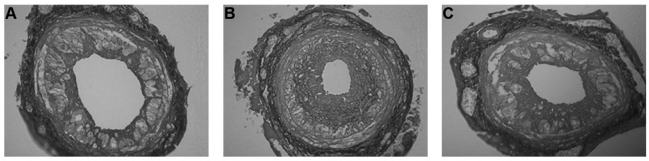

Figure 4. Extracellular matrix deposition in atherosclerotic lesions after PG and Pam_3Cys-SK_4 stimulation
Sirius Red staining of arteries after cuff placement and treatment with only gelatin (A), gelatin and PG (B), or gelatin and Pam_3Cys-SK_4 (C) for 14 days.

Macrophage homing after PG and Pam_3Cys-SK_4 stimulation

To determine whether peripheral stimulation of macrophages with PG and Pam_3Cys-SK_4 would result in homing of these cells into the atherosclerotic plaque, peritoneal macrophages were isolated from GFP-transgenic mice, stimulated or left unstimulated and injected into ApoE-/- mice. In the whole aortic arch multiple atherosclerotic lesions could be found.

Unstimulated GFP macrophages injected in the ApoE-/- mouse were rarely detected in the atherosclerotic plaques; only a few macrophages could be found in the whole aortic arch. However, stimulation with PG and Pam_3Cys-SK_4 increased homing of the macrophages compared to control macrophages. We did not see clusters of more than 10 macrophages in an atherosclerotic lesion within the aortic arch, but in each captured picture of the atherosclerotic plaque a few macrophages could be found. Representative immunofluorescencent pictures of GFP macrophages, stimulated with or without PG and Pam_3Cys-SK_4, present in the plaque are shown in *Figure 7*.

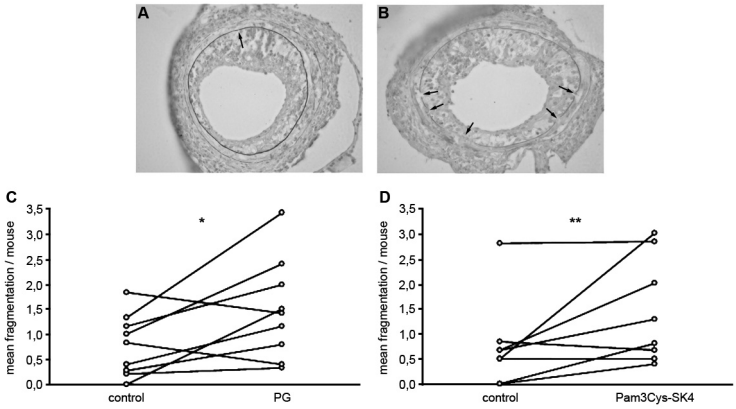

Figure 5. PG and Pam_3Cys-SK_4 induce fragmentation of the internal elastic lamina
*Fragmentation of internal elastic lamina in control (A) or PG (B) treated arteries. Arrows show sites of internal elastic lamina disruption. Quantification of mean number of fragmentations per mouse after stimulation with PG and Pam_3Cys-SK_4 in ApoE*3Leiden transgenic mice after cuff placement around the femoral arteries is shown in (C) and (D), respectively. *p=0.038, **p=0.046*

DISCUSSION

To address the hypothesis that PG stimulates atherosclerotic plaque progression and development, we studied advanced lesions in an ApoE*3Leiden transgenic mice model that shows accelerated plaque formation due to the use of a non-constrictive polyethylene cuff placed around the femoral arteries. PG and $Pam_3Cys\text{-}SK_4$ both significantly increased atherosclerotic plaque formation, enlargement of both the media and the EEL area, whereas the luminal area did not change. Both PG- and $Pam_3Cys\text{-}SK_4$-treated arteries revealed plaques enriched in SMC and collagen, suggesting that both PG and $Pam_3Cys\text{-}SK_4$ stimulation induce a stable plaque phenotype. Macrophage content in the atherosclerotic plaque was not changed after PG or $Pam_3Cys\text{-}SK_4$ stimulation. Interestingly, a switch in cellular content seemed evident in the media: the number of α-SMC actin-positive cells was strongly reduced following PG and $Pam_3Cys\text{-}SK_4$ stimulation, whereas the number of Mac-3 positive macrophages clearly increased.

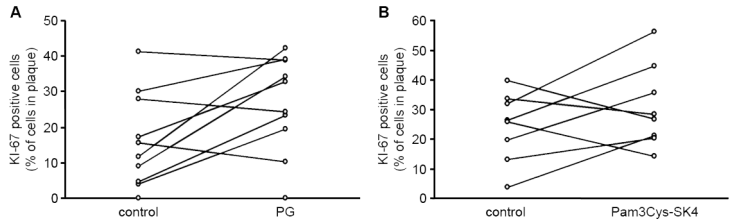

Figure 6. PG and $Pam_3Cys\text{-}SK_4$ do not stimulate proliferation of cells in the plaque
*Percentage of Ki-67 positive-stained cells (proliferating cells) in the plaque of ApoE*3Leiden transgenic mice treated with peri-adventitial cuff containing gelatin (control) or gelatin and PG (A) or $Pam_3Cys\text{-}SK_4$ (B) after 14 days.*

Previously, we demonstrated that TLR2 stimulation on vascular cells induces the expression of pro-inflammatory cytokines and chemokines [15]. Furthermore, *in vivo* cuff placement around the femoral artery with local stimulation of either TLR2 or TLR4 resulted in increased neointima formation [15,16]. The role of TLR2 in atherosclerotic lesion formation is relatively unexplored until recently. Mullick *et al* showed that in atherosclerosis-susceptible LDLr-/- mice, deficiency of TLR2 reduced development of atherosclerosis whereas systemic exposure to $Pam_3Cys\text{-}SK_4$ dramatically increased lesion severity. However, with bone marrow transplantation, loss of TLR2 expression in cells not of bone marrow origin (such as endothelial cells and SMC) reduces atherosclerosis in the absence of a known exogenous ligand, whereas expression of TLR2 had no impact on disease. This suggests that an unknown TLR2 agonist influences lesion progression. We previously showed that PG is observed in atherosclerotic vulnerable plaques [13]. In the current study we hypothesized that PG and the synthetic TLR2 ligand $Pam_3Cys\text{-}SK_4$ influence advanced atherosclerotic lesion progression and plaque characteristics in an atherosclerotic mouse model.
In accordance with our previous results, histological analysis showed a significant increase in plaque and media size following PG and $Pam_3Cys\text{-}SK_4$ application. Importantly, total vessel area was also increased by the PG and $Pam_3Cys\text{-}SK_4$ treatment, thereby preventing a decrease in luminal area. This indicates that TLR2 ligation by PG and $Pam_3Cys\text{-}SK_4$ might promote outward arterial remodeling. Previously, our lab

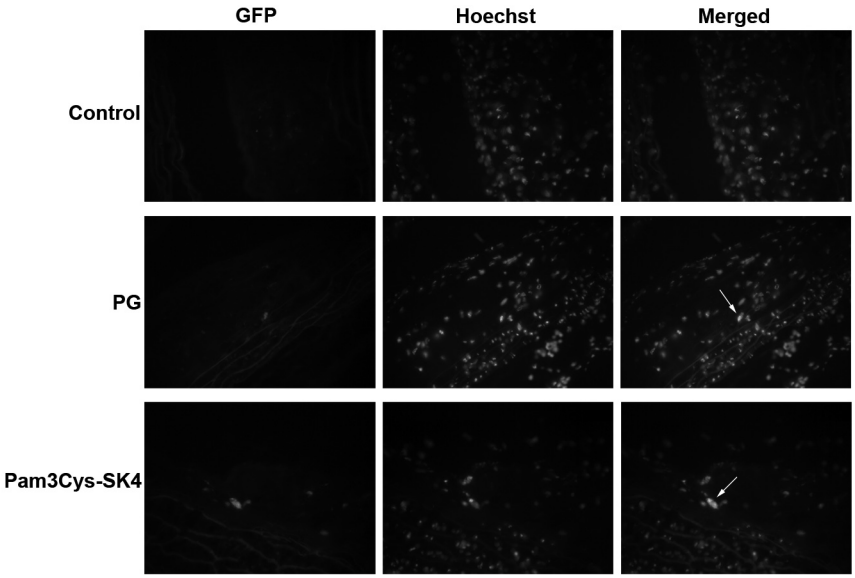

Figure 7. PG and Pam_3Cys-SK_4 increase macrophage homing into atherosclerotic plaques
*The ability of macrophages to migrate into the atherosclerotic plaque upon PG and Pam_3Cys-SK_4 stimulation was investigated in ApoE-/- mice. Control GFP-macrophages could hardly be found in the atherosclerotic plaques (only a few cells in the whole aortic arch), whereas macrophages that were stimulated with either PG or Pam_3Cys-SK_4 were found easier (a few macrophages in each atherosclerotic plaque within the aortic arch). The left panel shows GFP cells in the arterial wall, the middle panel shows Hoechst nuclear staining and the right panel shows the merged picture. **

demonstrated that TLR4 activation with LPS in ApoE*3Leiden mice induces outward arterial remodeling [22]. Outward remodeling in arteries is an important determinant for lumen loss because it can compensate for plaque accumulation in the arterial lumen. However, although the luminal area is preserved, in human atherosclerosis the plaque beneath the surface of the lumen often has a vulnerable phenotype [23,24]. Next to a vulnerable plaque phenotype, outward remodeling is associated with aneurysm formation and shear stress–induced arteriogenesis [25,26].

Although we found an increase in total vessel area after PG and Pam_3Cys-SK_4 stimulation, the plaques did not show an increase in macrophage content compared to control (one of the main features of a vulnerable plaque). The plaques were enriched in collagen, mainly in the cap but also in the core of the plaques, and in α-actin-positive SMC content. Furthermore, in the media of PG- and Pam_3Cys-SK_4-treated arteries α-actin-positive SMC seemed to be diminished and replaced by Mac-3-positive macrophages. The SMC are, together with macrophages, the first cells that migrate into the subendothelial layer and form the initial plaque. An increase in SMC content in atherosclerotic plaques is caused by SMC migration from the media followed by proliferation. Our results suggest that in PG and Pam_3Cys-SK_4 treated arteries SMC initially present in the media have been triggered to migrate to the plaque for participation in growth of the atherosclerotic plaque. To our knowledge, a decrease in arterial medial SMC content as we have observed has not been described earlier in atherosclerotic mouse models. Our observations merit careful consideration since, from previous studies it is known that vascular SMC are able to re-differentiate towards an

** see color supplement*

α-SMC actin-negative phenotype and that those cells have been reported to be present in atherosclerotic lesions [27]. Therefore, another explanation might be that the SMC in the media of arteries treated with PG and $Pam_3Cys\text{-}SK_4$ differentiated in an α-actin negative phenotype, which has been shown to be a feature of migrating SMC. The substitution of medial SMC by macrophages was an unexpected observation. This is a novel observation for which we do not have an explanation at yet. However, it is known that there is ample evidence that ligation of TLR and NOD can result in enhanced apoptosis of cells [11].

Adventitial fibroblasts can act as innate immune cells and signaling through TLR4 and TLR2 on adventitial fibroblasts leads to production of pro-inflammatory cytokines, like IL-1α, IL-1β, IL-6, IL-8 and MCP-1 [15,16]. These cytokines are able to activate matrix metalloproteinase (MMP)-2 and MMP-9 [28-31]. Both MMP-2 and MMP-9 are important enzymes associated with development of atherosclerosis [32] and aneurysm formation [33,34], and they facilitate SMC migration and proliferation [35,36]. Although we did not measure MMP levels in the atherosclerotic plaques, both PG and $Pam_3Cys\text{-}SK_4$, present around the femoral artery in the cuff, might be able to stimulate adventitial fibroblasts. Thereby these ligands promote the production and secretion of pro-inflammatory cytokines which are able to activate MMPs, and induce the migration and proliferation of SMC, resulting in increased atherosclerotic plaque development identical to what we demonstrated in the present study. This notion is supported by fragmentation of the internal elastica lamina, which may indicate enhanced elastase activity. Fragmentation of the lamina thereby facilitates migration of the SMC from the media into the atherosclerotic plaques at sites were the elastic lamina is ruptured [37,38]. PG and $Pam_3Cys\text{-}SK_4$ significantly stimulated fragmentation of the lamina compared to control arteries, suggesting better opportunities for SMC migration from media to plaque and therefore resulting in an increased atherosclerotic plaque.

Previously, we demonstrated that stimulation of monocytes with both PG and $Pam_3Cys\text{-}SK_4$ resulted in increased integrin expression on the monocyte surface. We also showed that those β_2-integrins were not only upregulated, but were also functional and able to bind to ICAM-1 coated beads. Furthermore, stimulated monocytes showed increased firm adhesion to endothelial cells under flow conditions as well as increased migratory capacities. In the present study we demonstrated *in vivo* that macrophages stimulated with PG and $Pam_3Cys\text{-}SK_4$ were able to home to atherosclerotic plaques more efficiently than unstimulated cells. These results suggest once more that TLR2 stimulation of monocytes/macrophages by PG might enhance accumulation of these cells at sites of inflammation, such as atherosclerotic plaques.

Based on the previous study by Laman *et al* which showed that PG is present in plaques that reveal a vulnerable phenotype and that PG can mainly be found in macrophage-rich regions [13], we hypothesized that after PG and $Pam_3Cys\text{-}SK_4$ stimulation in ApoE*3Leiden transgenic mice, plaques would reveal a more vulnerable phenotype. Surprisingly, we observed that after PG and $Pam_3Cys\text{-}SK_4$ stimulation plaques revealed a stable phenotype, harboring more SMC and collagen compared

with control arteries. In the present study we applied the ligands at the outer layer of the artery and thereby probably stimulated the cells that are present in the adventitia, such as fibroblasts. These cells are able to produce and secrete cytokines that play an important role in SMC migration and proliferation. In contrast, stimulation of cells in the adventitia might not result in activation of cells in the bloodstream, and thereby might not recruit more monocytes and macrophages to the atherosclerotic plaque than in the arteries that were treated with only gelatin. However, in the present study we showed that macrophages stimulated with PG and $Pam_3Cys\text{-}SK_4$ and injected into the bloodstream more efficiently home into atherosclerotic plaques. We therefore postulate that monocytes and macrophages that are stimulated with PG or $Pam_3Cys\text{-}SK_4$ at mucosal or non-mucosal sites and afterwards enter the bloodstream might be able to participate in development of vulnerable plaques. It is now established that antigen-presenting cells can take up bacterial antigens at the mucosa, including sampling of the gut [39,40] and can subsequently carry the antigen to other sites of the body, including the atherosclerotic plaque.

In summary, this study demonstrates that progression of atherosclerotic plaques is promoted by PG and $Pam_3Cys\text{-}SK_4$ stimulation *in vivo* and that those atherosclerotic plaques are enriched in collagen and SMC, suggesting that stimulation is associated with formation of a stable plaque. Furthermore, macrophages stimulated with PG or $Pam_3Cys\text{-}SK_4$ showed increased homing towards atherosclerotic plaques, suggesting that PG and $Pam_3Cys\text{-}SK_4$ stimulation enhance accumulation of macrophages, via the bloodstream, at sites of inflammation like the atherosclerotic arterial wall.

ACKNOWLEDGEMENTS

This study is supported by the Dutch Heart Foundation grant 2001-077. JD Laman received support from the Dutch MS Research Foundation.

REFERENCES

1. Medzhitov R, Janeway CA, Jr. Innate immunity: the virtues of a nonclonal system of recognition. Cell 1997;91:295-8.
2. Janeway CA, Jr. The immune system evolved to discriminate infectious nonself from noninfectious self. Immunol Today 1992;13:11-6.
3. Akira S, Takeda K. Functions of toll-like receptors: lessons from KO mice. C R Biol 2004;327:581-9.
4. Abreu MT, Arditi M. Innate immunity and toll-like receptors: clinical implications of basic science research. J Pediatr 2004;144:421-9.
5. Edfeldt K, Swedenborg J, Hansson GK, Yan ZQ. Expression of toll-like receptors in human atherosclerotic lesions: a possible pathway for plaque activation. Circulation 2002;105:1158-61.
6. Ohashi K, Burkart V, Flohe S, Kolb H. Cutting edge: heat shock protein 60 is a putative endogenous ligand of the toll-like receptor-4 complex. J Immunol 2000;164:558-61.
7. Okamura Y, Watari M, Jerud ES, Young DW, Ishizaka ST, Rose J, et al. The extra domain A of fibronectin activates Toll-like receptor 4. J Biol Chem 2001;276:10229-33.
8. Takeuchi O, Sato S, Horiuchi T, Hoshino K, Takeda K, Dong Z, et al. Cutting edge: role of Toll-like receptor 1 in mediating immune response to microbial lipoproteins. J Immunol 2002;169:10-4.
9. Takeuchi O, Kawai T, Muhlradt PF, Morr M, Radolf JD, Zychlinsky A, et al. Discrimination of bacterial lipoproteins by Toll-like receptor 6. Int Immunol 2001;13:933-40.
10. Ozinsky A, Underhill DM, Fontenot JD, Hajjar AM, Smith KD, Wilson CB, et al. The repertoire for pattern recognition of pathogens by the innate immune system is defined by cooperation between toll-like receptors. Proc Natl Acad Sci U S A 2000;97:13766-71.
11. Inohara N, Nunez G. NODs: intracellular proteins involved in inflammation and apoptosis. Nat Rev Immunol 2003;3:371-82.
12. Snowden MA, Perkins HR. Peptidoglycan cross-linking in *Staphylococcus aureus*. An apparent random polymerisation process. Eur J Biochem 1990;191:373-7.
13. Laman JD, Schoneveld AH, Moll FL, van Meurs M, Pasterkamp G. Significance of peptidoglycan, a proinflammatory bacterial antigen in atherosclerotic arteries and its association with vulnerable plaques. Am J Cardiol 2002;90:119-23.
14. Oude Nijhuis MM, van der Graaf Y, Melief MJ, Schoneveld AH, de Kleijn DP, Laman JD, et al. IgM antibody level against proinflammatory bacterial peptidoglycan is inversely correlated with extent of atherosclerotic disease. Atherosclerosis 2004;173:245-51.
15. Schoneveld AH, Oude Nijhuis MM, van Middelaar B, Laman JD, de Kleijn DP, Pasterkamp G. Toll-like receptor 2 stimulation induces intimal hyperplasia and atherosclerotic lesion development. Cardiovasc Res 2005;66:162-9.
16. Vink A, Schoneveld AH, van der Meer JJ, van Middelaar BJ, Sluijter JP, Smeets MB, et al. *In vivo* evidence for a role of toll-like receptor 4 in the development of intimal lesions. Circulation 2002;106:1985-90.
17. Lardenoye JH, Delsing DJ, De Vries MR, Deckers MM, Princen HM, Havekes LM, et al. Accelerated atherosclerosis by placement of a perivascular cuff and a cholesterol-rich diet in ApoE*3Leiden transgenic mice. Circ Res 2000;87:248-53.
18. Rosenthal RS, Dziarski R. Isolation of peptidoglycan and soluble peptidoglycan fragments. Methods Enzymol 1994;235:253-85.
19. Dziarski R, Gupta D. *Staphylococcus aureus* peptidoglycan is a toll-like receptor 2 activator: a reevaluation. Infect Immun 2005;73:5212-6.
20. Travassos LH, Girardin SE, Philpott DJ, Blanot D, Nahori MA, Werts C, et al. Toll-like receptor 2-dependent bacterial sensing does not occur via peptidoglycan recognition. EMBO Rep 2004;5:1000-6.
21. Moroi M, Zhang L, Yasuda T, Virmani R, Gold HK, Fishman MC, et al. Interaction of genetic deficiency of endothelial nitric oxide, gender, and pregnancy in vascular response to injury in mice. J Clin Invest 1998;101:1225-32.
22. Hollestelle SC, De Vries MR, Van Keulen JK, Schoneveld AH, Vink A, Strijder CF, et al. Toll-like receptor 4 is involved in outward arterial remodeling. Circulation 2004;109:393-8.
23. Ivan E, Khatri JJ, Johnson C, Magid R, Godin D, Nandi S, et al. Expansive arterial remodeling is associated with increased neointimal macrophage foam cell content: the murine model of macrophage-rich carotid artery lesions. Circulation 2002;105:2686-91.

24. Shah PK. Mechanisms of plaque vulnerability and rupture. J Am Coll Cardiol 2003;41:15S-22S.

25. Mulvany MJ. Determinants of vascular structure. J Cardiovasc Pharmacol 1992;19 Suppl 5:S1-S6.

26. Anidjar S, Kieffer E. Pathogenesis of acquired aneurysms of the abdominal aorta. Ann Vasc Surg 1992;6:298-305.

27. Mitani Y, Ueda M, Komatsu R, Maruyama K, Nagai R, Matsumura M, et al. Vascular smooth muscle cell phenotypes in primary pulmonary hypertension. Eur Respir J 2001;17:316-20.

28. Xie Z, Singh M, Singh K. Differential regulation of matrix metalloproteinase-2 and -9 expression and activity in adult rat cardiac fibroblasts in response to interleukin-1beta. J Biol Chem 2004;279:39513-9.

29. Chu SC, Yang SF, Lue KH, Hsieh YS, Wu CL, Lu KH. Regulation of gelatinases expression by cytokines, endotoxin, and pharmacological agents in the human osteoarthritic knee. Connect Tissue Res 2004;45:142-50.

30. Li A, Dubey S, Varney ML, Dave BJ, Singh RK. IL-8 directly enhanced endothelial cell survival, proliferation, and matrix metalloproteinases production and regulated angiogenesis. J Immunol 2003;170:3369-76.

31. Reape TJ, Groot PH. Chemokines and atherosclerosis. Atherosclerosis 1999;147:213-25.

32. Pasterkamp G, Schoneveld AH, Hijnen DJ, de Kleijn DP, Teepen H, van der Wal AC, et al. Atherosclerotic arterial remodeling and the localization of macrophages and matrix metalloproteases 1, 2 and 9 in the human coronary artery. Atherosclerosis 2000;150:245-53.

33. Palombo D, Maione M, Cifiello BI, Udini M, Maggio D, Lupo M. Matrix metalloproteinases. Their role in degenerative chronic diseases of abdominal aorta. J Cardiovasc Surg (Torino) 1999;40:257-60.

34. Lamblin N, Bauters C, Hermant X, Lablanche JM, Helbecque N, Amouyel P. Polymorphisms in the promoter regions of MMP-2, MMP-3, MMP-9 and MMP-12 genes as determinants of aneurysmal coronary artery disease. J Am Coll Cardiol 2002;40:43-8.

35. Aguilera CM, George SJ, Johnson JL, Newby AC. Relationship between type IV collagen degradation, metalloproteinase activity and smooth muscle cell migration and proliferation in cultured human saphenous vein. Cardiovasc Res 2003;58:679-88.

36. Auge N, Maupas-Schwalm F, Elbaz M, Thiers JC, Waysbort A, Itohara S, et al. Role for matrix metalloproteinase-2 in oxidized low-density lipoprotein-induced activation of the sphingomyelin/ceramide pathway and smooth muscle cell proliferation. Circulation 2004;110:571-8.

37. Sukhova GK, Wang B, Libby P, Pan JH, Zhang Y, Grubb A, et al. Cystatin C deficiency increases elastic lamina degradation and aortic dilatation in apolipoprotein E-null mice. Circ Res 2005;96:368-75.

38. Oho S, Rabinovitch M. Post-cardiac transplant arteriopathy in piglets is associated with fragmentation of elastin and increased activity of a serine elastase. Am J Pathol 1994;145:202-10.

39. Rescigno M, Urbano M, Valzasina B, Francolini M, Rotta G, Bonasio R, et al. Dendritic cells express tight junction proteins and penetrate gut epithelial monolayers to sample bacteria. Nat Immunol 2001;2:361-7.

40. Macpherson AJ, Uhr T. Induction of protective IgA by intestinal dendritic cells carrying commensal bacteria. Science 2004;303:1662-5.

CHAPTER 8

Involvement of intracellular pattern recognition receptors NOD1 and NOD2 in atherosclerosis

* Manon M. Oude Nijhuis[1,2], * Kristina Edfeldt[3], Anna Bergquist[3], Göran K. Hansson[3], Jon D. Laman[4], Dominique P.V. de Kleijn[1,2], Gerard Pasterkamp[1], Zhong-qun Yan[3]

1. Experimental Cardiology Laboratory, University Medical Center Utrecht, Utrecht
2. Interuniversity Cardiology Institute of the Netherlands (ICIN), Utrecht
3. Cardiovascular Research Unit, Karolinska University Hospital, Stockholm
4. Department of Immunology and MS Center ErasMS, Erasmus MC, Rotterdam

* Oude Nijhuis and Edfeldt contributed equally to this article.

IN PREPARATION

ABSTRACT

Objective: Nucleotide-binding oligomerization domain (NOD)1 and NOD2 proteins are recently recognized cytosolic pattern recognition receptors that sense the muropeptide derivatives of bacterial peptidoglycan and constitute an important part of innate immunity. Furthermore, NOD proteins are involved in apoptosis control. The innate immune system plays an important role in atherosclerosis and the current study aimed at elucidating the implication of NOD in this disease.

Methods and results: mRNA levels of NOD2 assessed by real-time RT-PCR were markedly elevated in human atherosclerotic lesions compared with non-atherosclerotic mammary arteries, whereas NOD1 levels were not significantly altered. Western blots revealed that both NOD1 and NOD2 protein expression was significantly increased in human atherosclerotic lesions compared to normal mammary arteries. Characterization of NOD1 and NOD2 protein by western blot in 120 carotid atherosclerotic plaques revealed that high NOD1 levels were more prevalent in asymptomatic rather than symptomatic patients, as well as in plaques enriched in SMC α-actin and collagen. An increase in NOD1 levels therefore seemed to be linked with a stable plaque phenotype. Despite of increased expression in atherosclerotic plaques, NOD2 was not specifically associated with characteristics that have been associated with a vulnerable plaque phenotype. Immunohistochemistry revealed that NOD2 protein was expressed in macrophages and endothelial cells.

Functionally, *in vitro* stimulation of endothelial cells with pro-inflammatory cytokines such as TNF-α induced increased expression of NOD2 transcript and protein.

Conclusion: In atherosclerosis, NOD1 and NOD2 protein expression is increased. In the atherosclerotic lesions NOD1 and NOD2 can be detected in macrophages and endothelial cells. Furthermore, NOD2 activation in endothelial cells might even promote the inflammatory phenotype of the atherosclerotic lesion.

INTRODUCTION

Atherosclerosis is characterized by chronic inflammation in conjunction with lipid retention and lipid accumulation in the vessel wall. Infections have been implicated in the pathogenesis of atherosclerosis based on seroepidemiological data and the presence of microbial DNA and protein within lesions [1-4]. Infectious agents are able to influence atherogenesis by triggering innate immune responses such as secretion of cytokines and chemokines by antigen-presenting cells and recruitment of macrophages and neutrophils to the site of inflammation. This is followed by induction of an adaptive immune reaction, notably activation and expansion of antigen-specific T cells.

Sensing of infectious agents by the host is mediated by the recognition of microbial agent-specific pathogen-associated molecular patterns (PAMPs). These PAMPs can be recognized by pattern recognition receptors (PRRs), including Toll-like receptors (TLRs) and nucleotide-binding oligomerization domain (NOD) proteins. Mammals have two closely related NOD family members -NOD1 and NOD2-, containing 1 or 2 CARD domains, respectively. NOD1 (CARD4) and NOD2 (CARD15) are members of the CATERPILLER [CArd, Transcription Enhancer, R (purine)-binding, Pyrin, Lots of LEucine Repeats] superfamily [5]. Both NOD1 and NOD2 are intracellular proteins containing three functional domains: a C-terminal leucine-rich repeat (LRR) domain serving as ligand-recognition domain, a centrally located nucleotide-binding domain, and an N-terminal protein/protein interaction domain involved in signaling. NODs are general sensors for both Gram-positive and Gram-negative bacteria, through the recognition of minimal motifs found in the bacterial cell wall component peptidoglycan (PG). Specifically, NOD1 responds to a Gram-negative-specific ligand, a diaminopimelic acid containing dipeptide [6,7] whereas NOD2 is a more general sensor recognizing muramyl dipeptide (MDP), a degradation product of PG from virtually all bacteria [8,9]. NOD1 is ubiquitously expressed in a variety of adult tissues [10,11], as opposed to NOD2 which has been found mainly in macrophages, dendritic cells and Paneth and epithelial cells of the intestine [12-15]. Stimulation of the receptors leads to activation of the transcription factor nuclear factor-kappa B (NF-κB) [10-12]. This stimulation probably occurs through the serine/threonine kinase RIP2 (also known as RICK or CARDIAK), thereby inducing the expression of pro-inflammatory mediators [16]. The NF-κB pathway plays an important role in inflammation and atherosclerosis [17].

A well-described ligand for TLR2/NOD is PG, a component of the wall of Gram-positive bacteria and to lesser extent Gram-negative bacteria. PG is able to induce production and secretion of pro-inflammatory cytokines, such as interleukin (IL)-1, IL-6 and tumor necrosis factor (TNF)-α, by monocytes and macrophages *in vitro* via activation of NF-κB [18,19]. That PG may stimulate the production of pro-inflammatory cytokines in atherosclerotic lesions is supported by our observation that PG is prevalent in human atherosclerotic lesions with an inflammatory unstable phenotype. The presence of PG is mainly observed in macrophage-rich atheromatous regions [20]. In addition, in a clinical study, we demonstrated that systemic IgM-specific antibody levels against PG are significantly lower in atherosclerotic patients compared to control patients [21].

Furthermore, the IgM antibody levels against PG are inversely related with common carotid intima-media thickness.

Pathophysiologic functions of NODs are only beginning to be understood. NOD2 has been implicated in Crohn's disease (CD) [22,23], an inflammatory bowel disease characterized by elevated levels of pro-inflammatory cytokines and inflammation in the intestine [24,25]. Individuals with one of the three major disease-associated NOD2 alleles have a 2- to 4-fold increased risk of developing CD, whereas homozygous or compound heterozygous carriers have a 15- to 40-fold increase in risk [26]. Furthermore, at least three reported NOD2 mutations have been shown to cause Blau syndrome [27].
To understand the molecular basis of innate immunity involved in atherosclerosis, we investigated expression of NOD1 and NOD2 in human atherosclerotic tissue. Our results demonstrate that both NOD1 and NOD2 protein expression is increased in human atherosclerotic lesions and that NOD2 is present within macrophages and endothelial cells in atherosclerotic plaques. Furthermore, NOD2 functions as an innate immune receptor in endothelial cells.

MATERIAL AND METHODS

Study population
Patients are participants of the Atherosclerotic plaque Expression study (Athero-Express), which is an ongoing longitudinal multicenter cohort study, initiated in 2002 and currently being executed in two Dutch hospitals: the University Medical Center Utrecht and Sint Antonius hospital Nieuwegein. Recruitment of patients undergoing carotid endarterectomy started in April 2002. All cohort members will be followed for the occurrence of adverse cardiovascular events for a minimum of 3 years. The objective of Athero-Express is to evaluate differential atherosclerotic plaque expression of protein in relation to future cardiovascular events and patient characteristics [28]. Patients in this study are either symptomatic or asymptomatic. Symptoms associated with carotid atherosclerotic disease are (minor) stroke, transient ischemic attack and amaurosis fugax.

In another independent study conducted in the Karolinska Institute in Stockholm, 13 human atherosclerotic lesions were collected from patients undergoing carotid endarterectomy. Ten human internal mammary arteries were obtained from patients undergoing coronary artery bypass surgery and used as non-atherosclerotic control arteries.

These studies were approved by the Medical Ethical Committees of the participating hospitals, and written informed consent was obtained from each patient. The investigation conforms to the principles outlined in the Declaration of Helsinki.

Carotid atherosclerotic plaque characterization
Segments of the culprit lesion of each plaque were cut into 5 μm sections and stained for collagen (Sirius Red), smooth muscle cell (SMC) α-actin, macrophages (CD68), and hematoxylin and eosin. All stained sections were examined semi-quantitatively and the degree of staining was classified as no, minor, moderate or heavily stained [28].
- Sirius Red using polarized light microscopy:
1) no or minor staining= staining along part of the luminal border;
2) moderate or heavy staining= staining along the entire luminal border.
- Alpha-actin positive cells:
1) no or minor staining = discontinuous over the entire circumference with absent staining at parts of the circumference of the arterial wall;
2) moderate or heavy staining = positive cells along the entire circumference of the luminal border, with at least minor staining locally with few scattered cells.
- CD68-positive cells:
1) absent or minor staining = no or few scattered positive cells;
2) moderate or heavy staining = stained clusters of cells with >10 cells present.

An overall plaque phenotype was assigned to each lesion based on overall appearance. A plaque is considered more active and unstable when it reveals a strong staining for macrophages, a large atheroma and when it lacks collagen and SMC. The more fibrous stable lesion typically lacks inflammatory cells and fat and reveals strong staining for collagen and SMC. Three groups were considered based on the percentage of atheroma in the plaque being present: fibrous plaques containing <10% fat; fibro-atheromatous plaques containing 10-40% fat; or atheromatous plaques containing >40% fat.

RNA and protein isolation carotid plaques
The segment adjacent to the culprit lesion was used to isolate total RNA and protein. For this purpose, frozen arterial segments were crushed in liquid nitrogen. Protein was isolated using two techniques. One part of the segment was treated with 1 ml Tripure Isolation Reagent (Boehringer, Mannheim, Germany) according to manufacturer's protocol. The other part was dissolved in TrisHCl (pH 7.5 at 4°C). RNA was isolated using Tripure Isolation Reagent. After isolation, total RNA was treated with DNAse (Amersham Pharmacia, Freiburg, Germany). The presence of genomic DNA was tested by PCR without reverse transcriptase. cDNA was created using 'Ready to go, You prime First system' (Amersham Pharmacia).

Total matrix metalloproteinase (MMP) activities were measured using the Biotrak activity assays RPN2631 (MMP-2), RPN2635 (MMP-8), and RPN2634 (MMP-9) (Amersham Biosciences, Buckinghamshire, United Kingdom). Extracellular MMP inducer (EMMPRIN) expression levels were determined by Western blotting (sc-9753, Santa Cruz Biotechnology, CA) [29]. EMMPRIN has been identified as a surface glycoprotein on tumor cells that is able to stimulate the production of different MMPs. Recently we investigated the relation of EMMPRIN with plaque characteristics and the association of EMMPRIN with MMP activity in the atherosclerotic plaque [29]. Expression levels of the less glycosylated form of EMMPRIN (45kD) were found to be

higher in SMC rich lesions and related to MMP-2 activity, whereas expression of the more glycosylated form of EMMPRIN (58kD) was associated with MMP-9 activity.

Western blotting for NOD protein in carotid plaques
NOD1 and NOD2 protein expression levels were assessed in 120 patient samples from the Athero-Express study. Six human internal mammary arteries were used as non-atherosclerotic control arteries. Samples (15 µg/lane) were separated on a 5% SDS polyacrylamide gel and blotted to a Hybond-C membrane (Amersham Biosciences). Blocking and incubation steps were done in 5% non-fat dry milk in PBS/0.1% Tween 20. Blots were incubated with either rabbit anti-human NOD1 (Alpha Diagnostics International, San Antonio, TX) or mouse anti-human NOD2 (Cayman Chemical Company, Ann Arbor, MI), followed by goat anti-rabbit-HRPO (1:2000, Dako, Glostrup, Denmark) or rabbit anti-mouse-HRPO (1:2000, Dako). Bands were visualized with the use of ECL kit (Amersham Biosciences). Optical densities of NOD1- and NOD2-positive bands were measured with the use of the GelDoc system (Biorad, Hercules, CA) and expressed in arbitrary units.

Immunohistochemistry and immunofluorescence
Acetone-fixed 10 µm cryosections were preincubated for 30 min with 5% normal serum. After incubation with mouse anti-human NOD2 at 4°C overnight, sections were incubated with biotinylated goat-anti rabbit 7.5 µg/ml or horse anti-mouse 7.5 µg/ml followed by avidin-biotin peroxidase complex and developed with diaminobenzidine (all from Vector Laboratories, Burlingame, CA). The specificity of the NOD2 antibody was confirmed by incubation with isotype-matched control IgG (DakoCytomation, Glostrup, Denmark).

Double-staining was performed using immunofluorescence. After preincubation with 5% normal serum in 50 mM NH_4Cl for 30 minutes, primary antibodies were incubated overnight at 4°C followed by goat anti-mouse biotin or goat anti-mouse Texas Red 10 µg/ml (Molecular Probes Inc, Eugene, Oregon) for 30 min at RT. Subsequently Oregon green-streptavidin 1 µg/ml (Molecular Probes) was incubated for 30 min at RT when appropriate. A biotin blocking kit (Vector Laboratories) was used after completion of staining for the first set of antibodies and prior to incubation of the second set of primary and secondary antibodies. Cross-binding between the different sets of antibodies was controlled for by incubating consecutive sections without the second primary antibody. No difference was observed in staining patterns between consecutive single or double-labelled sections. Sections were incubated with 0.003% Sudan black B (BDH Laboratory Supplies, Poole, England) in 70% alcohol to block lipid autofluorescence followed by thorough washing in PBS. 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) (Sigma) was used to stain cell nuclei in the tissue. The following antibodies were used for immunofluorescence: mouse anti-human NOD2 (Cayman Chemical), mouse anti-human von Willebrand factor, anti-human CD163, anti-human α-smooth muscle actin (all from DakoCytomation) and anti-human CD3 (Becton Dickinson, BD Biosciences, San Jose, CA).

Cell culture

Human umbilical vein endothelial cells (HUVEC) (Clonetics, Bio Whittaker, Walkersville, MD) of passages 5-8 were used. Cells were maintained at 37°C, 5% CO_2 in EGM-2 medium (Clonetics, Bio Whittaker) and seeded into 6-well plates for stimulation experiments. HUVEC (80% confluent) were stimulated in EGM-2 with different doses or time points of TNF-α, IFN-γ, IL-1β or TNF-α together with IFN-γ (all from PeproTech EC Ltd., London, UK). Proteins were extracted in RIPA buffer supplemented with the following protease inhibitors (final concentration): phenylmethylsulfonyl fluoride (1 mM), pepstatin A (10 μM), EDTA (1 mM) and E64 (10 μM) (all from Sigma). Samples (10 or 20 μg/lane) were separated on a 7.5% SDS polyacrylamide gel and blotted to polyvinylidene difluoride membranes. NOD2 protein expression was determined as described in the paragraph Western blotting for NOD protein in carotid plaques. In functional experiments, HUVEC were treated with different concentrations of MDP (Sigma-Aldrich, St. Louis, MI) for 24 h. Supernatants were collected and stored at -80°C.

Quantitative real-time RT-PCR

RNA was isolated with RNeasy Total RNA Mini Kit (Qiagen Inc., Valencia, CA) according to the manufacturer's instructions, with an additional phenol/chloroform extraction step for tissue samples, and treated with RNase free DNase I (Qiagen). Subsequently, 0.2 or 1 μg RNA was used in a 40 μl cDNA synthesis using hexanucleotides and Superscript II reverse transcriptase (Invitrogen Life Technologies, Paisley, UK). Real-time PCR on 3 μl cDNA was performed in an ABI 7700 Sequence Detector (Perkin-Elmer Applied Biosystems, Foster City, CA) using Assay on demand primer and probe for human NOD1, NOD2, intercellular adhesion molecule-1 (ICAM-1), monocyte chemoattractant protein-1 (MCP-1) and cyclooxygenase-2 (COX-2) (ABI, Foster City, CA). Cyclophilin A was used as housekeeping gene with the following sequence for; forward primer 5'-TGC TGG ACC CAA CAC AAA TG-3' reverse primer 5'-TGC CAT CCA ACC ACT CAG TC-3' and probe 6-FAM-TTC CCA GTT TTT CAT CTG CAC TGC CA-TAMRA. The data was analyzed using Sequence Detection System v 1.9.1 (ABI).

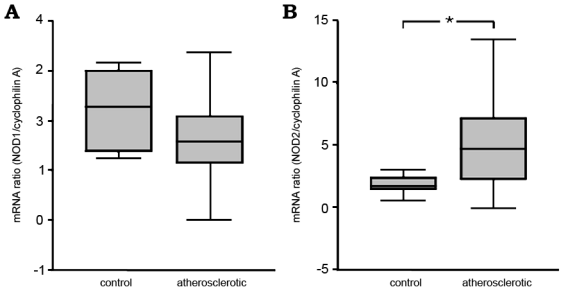

Figure 1. NOD1 and NOD2 mRNA expression level in atherosclerotic lesions
*Quantitative real-time RT-PCR assessment of human NOD1 and NOD2 transcripts in normal vessels (normal, n=10) and atherosclerotic lesions (atherosclerotic, n=38). Box plots show relative abundance of A) NOD1 transcript and B) NOD2 transcript normalized to cyclophilin A. *p<0.05*

Statistical analysis

Data are presented as mean and 95% confidence interval (CI). The Mann-Whitney test was used to compare mRNA and protein levels in atherosclerotic lesions and control arteries. One-way ANOVA, Mann-Whitney test, or Kruskal-Wallis test was used to compare differences among histological sections and NOD1 and NOD2 levels. Data from *in vitro* cell culture experiments are shown as mean ± standard error of the mean (SEM) of three different experiments. A p-value <0.05 was considered significant.

RESULTS

Characteristics of NOD1 and NOD2 transcripts in atherosclerotic lesions

Levels of NOD1 and NOD2 transcripts in atherosclerotic lesions and mammary arteries were determined by quantitative real-time RT-PCR. Our data showed no significant change in NOD1 transcript in atherosclerotic lesions compared to normal vessels (*Figure 1A*). The level of NOD2 transcript, on the other hand, increased 2.5-fold in the atherosclerotic lesions compared to the control arteries (*Figure 1B*).

Patient characteristics		NOD1 expression p-value	NOD2 expression p-value
Total number of patients	120		
Statin use	69%	0.88	0.37
Hypertension	59%	0.30	0.48
Hyperlipidemia	54%	0.87	0.88
Diabetes mellitus	18%	0.74	0.85
Smoking	60%	0.11	0.22
Symptomatic patients	72%	0.02 *	0.12

Table1. Characteristics of the study population
*P-values of correlations between NOD1 and NOD2 expression and baseline characteristics of the patients are shown. *: p<0.05*

NOD1 and NOD2 protein levels are increased in carotid atherosclerotic plaques and associated with plaque phenotype and MMP expression

Characteristics of the 120 patients are shown in table 1. NOD1 and NOD2 expression levels were not associated with patient risk factors or statin use. However, NOD1 protein levels were higher in asymptomatic patients compared with symptomatic patients (p=0.02).

We measured levels of NOD1 and NOD2 protein expression in samples of the Athero-Express and control mammary arteries. NOD1 and NOD2 protein levels were significantly increased in atherosclerotic lesions compared to control arteries (NOD1; 2.5-fold increase, p=0.009 and NOD2; 2-fold increase, p=0.009, compared to control mammary arteries, *Figure 2*).

NOD1 expression levels were increased in plaques that revealed more collagen staining (2-fold increase, p=0.01) and SMC α-actin staining (3-fold increase, p=0.006, *Figure 3*). There was no relation between NOD1 expression and macrophage content or plaque

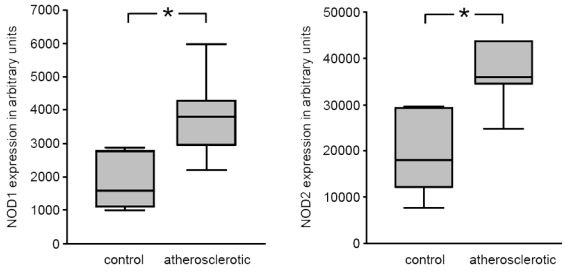

Figure 2. NOD1 and NOD2 protein expression in normal mammary arteries and atherosclerotic plaques
*NOD1 and NOD2 protein expression levels were measured by western blot in atherosclerotic lesions and control mammary arteries. Data are presented as arbitrary units and shown as mean and 95% CI. *p<0.05*

phenotype (atheromatous, fibro-atheromatous, or fibrous plaques). Since, in general, SMC are abundantly present in atherosclerotic plaques and NOD1 expression was highly associated with SMC staining, we also investigated whether NOD1 expression was associated with macrophage staining in cross-sections that revealed minor or no SMC staining. Indeed, we observed that moderate/heavy macrophage staining was associated with higher levels of NOD1 expression in this selected group of plaques that lacked SMC (2-fold increase, p=0.021, *Figure 3*). NOD2 expression in the plaque was not associated with either one of these variables although a tendency was observed for SMC α-actin (p=0.131, *Figure 3*).

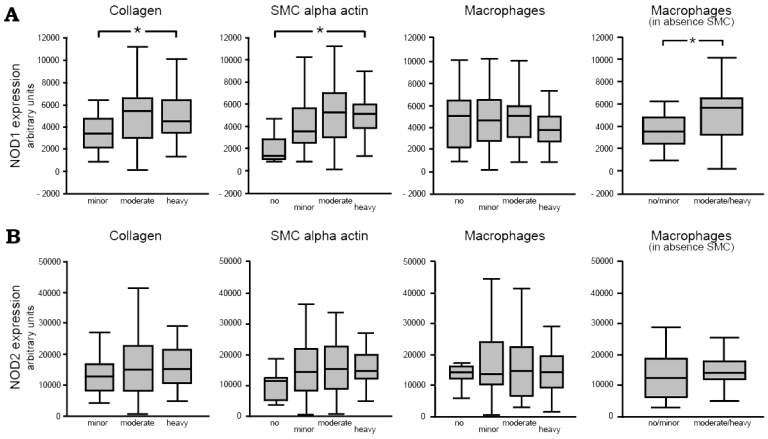

Figure 3. NOD1 and NOD2 protein expression in atherosclerotic plaques
*NOD1 (A) and NOD2 (B) protein expression levels were measured by western blot in sections stained for collagen, α-actin (SMC) and CD68 (macrophages). Data are presented as arbitrary units and shown as mean and 95% CI. *p<0.05*

An inverse relation was observed between NOD1 expression levels and MMP-2 levels in the plaque (p=0.012), while a significant positive relation could be seen with MMP-8 (p=0.008) and MMP-9 levels (p=0.031, *Figure 4A*). Furthermore, NOD1 expression was related with EMMPRIN 58kD (p=0.007) and inversely related with EMMPRIN 45kD (p=0.009) which is in line with the previously described association between EMMPRIN 58kD and MMP-9 as well as EMMPRIN 45kD and MMP-2. In line with NOD1

expression, NOD2 expression levels were inversely correlated with MMP-2 expression (p=0.04) and EMMPRIN 45kD (p=0.016), (*Figure 4B*). In addition, NOD2 protein levels were positively associated with both MMP-8 (p=0.015) and MMP-9 levels (p=0.039).

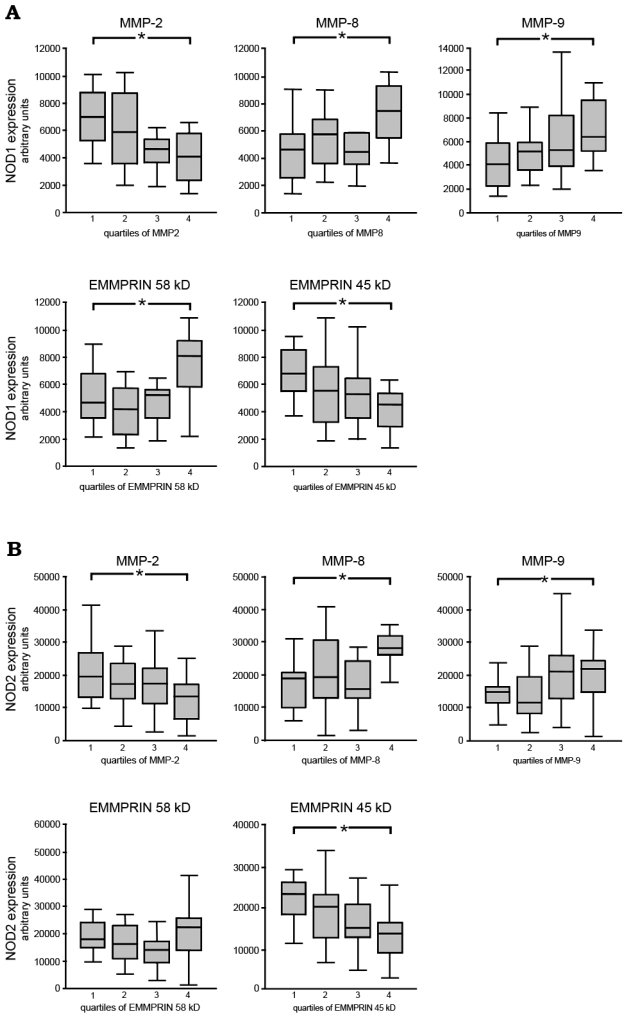

Figure 4. Correlations between NOD1 and NOD2 protein expression and quantitative MMP activity levels, EMMPRIN 45kD and EMMPRIN 58kD levels
*Continuous MMP and EMMPRIN variables were categorized into percentile groups (quartiles) and plotted against NOD1 (A) and NOD2 (B) protein expression. Data are presented as arbitrary units and shown as mean and 95% CI. *p<0.05*

NOD2 expression is induced in endothelial cells and predominantly located in infiltrating macrophages in atherosclerotic lesions

By the use of immunohistochemistry we found strong staining for NOD2 protein distributed in the inflamed areas inside the atherosclerotic lesion as well as in endothelium lining the lumen of the vessel (*Figures 5B* and *5C*). However, we could not confirm NOD2 protein expression in normal arteries, as would be predicted to

occur on basis of mRNA transcript (*Figure 5A*). Further investigation allowed us to identify which cell types were expressing NOD2 in the atherosclerotic lesion by the use of immunofluorescence staining. Macrophages are the most prominent NOD2-expressing cells as evident by the co-localization of NOD2 staining with that of CD163 (*Figure 6*). Some endothelial cells were also positive for NOD2 (*Figure 6*) but few CD3 expressing T-lymphocytes and SMC stained positive. Detecting NOD1 protein in normal and diseased vessels was unsuccessful in the current study due to lack of a specific antibody for immunohistochemistry.

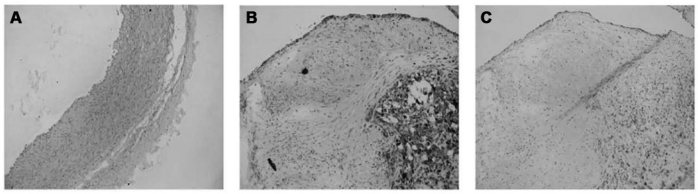

Figure 5. Immunohistochemical detection of NOD2 protein in normal vessels and human atherosclerotic lesions
The pictures show an overview of NOD2 protein expression and distribution in normal vessels (A) and human atherosclerotic lesions (B). (C) shows a consecutive section to (B) that is stained with isotype-matched control IgG as negative control. Cell nuclei are counterstained with hematoxylin. Original magnification: 200x.

NOD2 expression is induced in HUVEC by TNF-α alone and in synergy with IFN-γ

To further investigate the induction of NOD2 expression in endothelial cells, HUVEC were stimulated with different doses of TNF-α, IFN-γ or both. Similar to effects seen in intestinal epithelial cells [30], TNF-α induced the expression of NOD2 in a dose-dependent manner with a 20-fold increase in NOD2 transcript at 200 U/ml (*Figure 7A*). In another experiment, IL-1β could also induce NOD2 expression (data not shown). A time-course study showed that NOD2 expression could be induced quickly, starting already at 3 h after TNF-α stimulation (*Figure 7B*). Furthermore, these experiments suggest that expression of NOD2 seems to be a biphasic response with the second phase peaking at 9 h after stimulation. In addition, co-treatment of TNF-α with IFN-γ even further increased NOD2 expression (*Figure 7C*), whereas IFN-γ alone did not induce NOD2 expression in HUVEC (data not shown).
Consistent with the mRNA data, a similar pattern of NOD2 protein expression was confirmed by western blot using a mouse anti-human NOD2 antibody on protein extracts from HUVEC treated with or without different doses of TNF-α or TNF-α together with IFN-γ for 24 h (*Figure 7D*). Furthermore, basal levels of NOD2 protein could be found in HUVEC. No NOD1 mRNA expression could be detected.

DISCUSSION

The involvement of NODs in human diseases has been investigated by comparison of the incidence of disease among people having different polymorphisms in genes that participate in NOD signaling. In particular, mutations in the NOD2 gene have

been associated with increased susceptibility to CD [22,23], Blau syndrome [27] and risk of infant sepsis [31], indicating that alteration in NOD-mediated innate immunity is critical to inflammatory and infection-related diseases. The data reported here show that both NOD1 and NOD2 are expressed in human atherosclerosis both at the mRNA and protein level, suggesting involvement of previously unnoticed PRRs in the disease. NOD1 and NOD2 are intracellular PRRs specialized in sensing PG from both Gram-negative and Gram-positive bacteria [32]. They constitute an important part of the innate immune response and activation of the receptors leads to the activation and translocation of the transcription factor NF-κB [10-12,16]. NF-κB subsequently induces the expression of pro-inflammatory mediators and represents an important pathway in inflammation and atherosclerosis [17]. Expression of NOD1 and NOD2 in lesions might reflect a novel activation route for this transcription factor in disease-affected vessels.

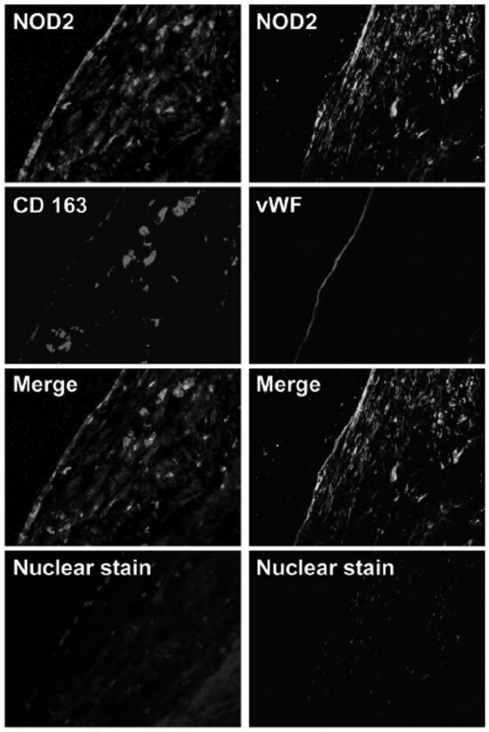

Figure 6. NOD2 is expressed by macrophages and endothelial cells in atherosclerotic plaques
Double immunofluorescent staining for NOD2 (green) and cell markers (red) on atherosclerotic lesions. Cells were stained for CD163 (macrophages) and von Willebrand Factor (endothelial cells). Double-positive cells are yellow in the overlay panel. Cell nuclei are stained blue with DAPI. Original magnification: left panel 400x, right panel 200x. *

NOD2 is expressed primarily in immune cells and epithelial cells [12-15], while the expression of NOD1 is ubiquitous [10,11]. Our findings demonstrate that the normal artery expresses both NOD1 and NOD2. We have identified marked enhancement of NOD2 transcript in human atherosclerotic lesions compared to normal control vessels. Furthermore, both NOD1 and NOD2 protein expression was increased in

* *see color supplement*

the atherosclerotic lesions. NOD2 is primarily expressed in macrophages within inflammatory areas inside the atherosclerotic lesions. This is consistent with the lower levels of NOD2 expression observed in internal mammary arteries. Also endothelial cells of the diseased artery are stained positive for NOD2 protein.

To elucidate the regulation of NOD2 expression we investigated NOD2 expression in cultured human endothelial cells and found that HUVEC constitutively express a basal level of NOD2. Additionally, NOD2 expression in endothelial cells is induced both at the transcript and protein level by the pro-inflammatory cytokines TNF-α and IL-1β and by TNF-α together with IFN-γ. This is in accordance with previous data in intestinal epithelial cells in which the NOD2 transcript is upregulated by TNF-α via activation of NF-κB [30,33]. As TNF-α strongly induces NOD2 expression in endothelial cells, it is likely that NF-κB signaling also is involved in cytokine-mediated NOD2 upregulation in endothelial cells. Pro-inflammatory cytokines and chemokines are elevated in patients with atherosclerosis and expressed locally in atherosclerotic lesions [34]. It is conceivable that NOD2 expression might be induced by these cytokines and chemokines present in the lesions.

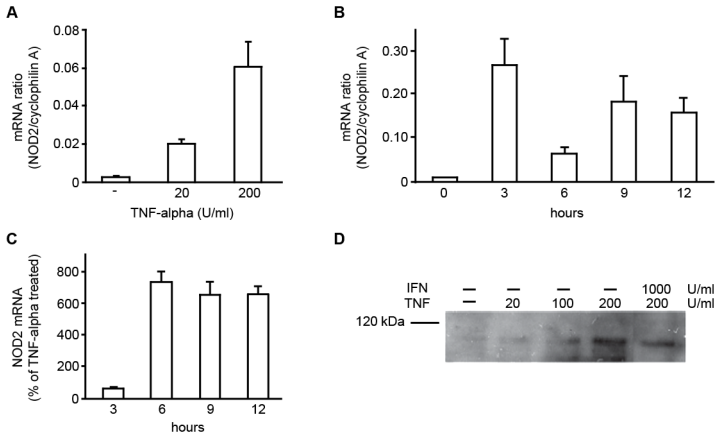

Figure 7. Pro-inflammatory cytokines induce NOD2 expression in HUVEC
Quantitative real-time RT-PCR for NOD2 transcript in HUVEC stimulated with A) different doses of TNF-α for 6 h and B) TNF-α 200 U/ml for 3, 6, 9 and 12 h. Data are presented as NOD2/cyclophilin A. C) HUVEC stimulated with TNF-α 200 U/ml together with IFN-γ 1000 U/ml for 3, 6, 9 and 12 h. Data are presented as NOD2/cyclophilin A as percentage of TNF-α treatment alone (200 U/ml). All figures presented as mean ± SEM of three different experiments. D) Western blot analysis with mouse NOD2 antibody on protein extracts from HUVEC stimulated with different doses of TNF-α or TNF-α + IFN-γ for 24 h. A representative from three different experiments is shown.

Furthermore, endothelial cells were stimulated with the NOD2 specific ligand MDP to address the functional relevance of NOD2 in these cells. Preliminary data indicate that normal endothelial cells respond to rather low doses (1 μg/ml) of MDP treatment characterized by upregulation of transcripts of the chemokine MCP-1 and the adhesion molecule ICAM-1. MCP-1 and ICAM-1 both have been shown to be important players in recruitment of immune cells into and progression of atherosclerotic lesions [35-37]. However, MDP apparently lacks effect on induction of COX-2. These results imply two things. First, endothelial cells are capable of responding to low dose of NOD2

stimulatory MDP indicating the existence of an active innate immunity in cells lining the blood vessels. Second, extracellular MDP is allowed entry into endothelial cells by a yet unknown mechanism. MDP can be released from bacteria as part of their natural synthesis of cell wall components, as well as by actions from host cells mediating the breakdown of the bacterial cell wall [32]. NOD proteins are intracellular and therefore, the ligands must localise in the cytosol in order to activate the receptors. How NOD ligands gain access to the cytosol, however, remains unclear.

Previously, we demonstrated that unstable plaque characteristics are associated with clinical symptoms [38] and that MMP-2, MMP-8 and MMP-9 activities differ among carotid plaque phenotype [29]. In the current study we found a positive relation between NOD1 expression levels and collagen staining and the presence of SMC, suggesting that NOD1 expression is associated with a stable plaque phenotype. Since SMC are abundantly present in atherosclerotic plaques and NOD1 is highly correlated with SMC, we hypothesized that the presence of SMC in those plaques might outshine any relation present between NOD1 expression and the less abundantly present macrophages. Indeed, we found that in the absence of SMC there was a significant relation between NOD1 expression and the macrophage content, suggesting that NOD1 is also expressed within inflammatory areas inside atherosclerotic lesions. Furthermore, we showed that in human atherosclerotic plaques both NOD1 and NOD2 expression is inversely correlated with EMMPRIN 45kD and MMP-2 activity and positively associated with MMP-8 and MMP-9. This is somewhat surprising since Sluijter *et al* showed that high MMP-2 levels are correlated with a stable SMC-rich carotid plaque phenotype and high MMP-8 and MMP-9 levels with a macrophage-rich vulnerable plaque phenotype [29]. They also showed that MMP-9 levels co-localize with macrophages and MMP-2 with SMC. Previous studies also showed that MMP-8 and MMP-9 staining co-localize with CD68-positive macrophages [39-41]. Both NOD1 and NOD2 protein expression are related to or expressed in macrophages and thereby explain a positive association between NOD1/NOD2 and MMP-9 and macrophage content. Furthermore, expression of NOD1 and NOD2 by macrophages and SMC in the stable atherosclerotic plaque increases production of pro-inflammatory cytokines, like IL-1β and IL-6, and thereby recruits more macrophages to the atherosclerotic plaque. This will alter the phenotype of the plaque towards a more inflammatory, vulnerable plaque, which in turn results in downregulation of the MMP-2 levels and explaining the negative association we found between NOD1 and NOD2 protein expression and MMP-2 levels.
In the present study, we also found that NOD1 expression levels are higher in asymptomatic patients as compared to symptomatic patients. In combination with the positive correlation between NOD1 expression levels and collagen content and number of SMC these results are in line with results demonstrated previously by other groups. Symptomatic patients typically have a large necrotic core that is rich in lipids [42] and lacks SMC and collagen [43]. Furthermore, the percentage of macrophage-rich areas is larger in symptomatic compared with asymptomatic patients [44].

Infection has been implicated in the pathogenesis of atherosclerosis [1-4]. However, direct evidence for pathogen involvement in cardiovascular disease is still missing.

Taken together, our data demonstrate a potential pathway for microbial detection in atherosclerotic lesions. Previous studies have identified PG in macrophage-rich areas in atherosclerotic lesions [20]. We extend these findings by demonstrating a mechanism whereby PG, or rather its minimal breakdown products, could be detected by NOD1 and NOD2 in macrophages and endothelial cells in atherosclerotic lesions. We speculate that this represents a new innate immune recognition pathway and that NOD2 activation in endothelial cells might promote or enhance the inflammatory phenotype of the atherosclerotic lesion. Further studies are needed to elucidate the functional relevance of induced NOD2 in endothelial cells.

In conclusion, we show that both NOD1 and NOD2 are expressed in human atherosclerotic lesions. NOD1 expression is associated with a stable plaque phenotype, containing increased SMC and collagen content, and is more abundant in asymptomatic patients rather than symptomatic patients. Furthermore, in the absence of SMC NOD1 expression is also associated with macrophage staining. Both NOD1 and NOD2 expression levels are associated with MMP-8 and MMP-9 levels and inversely associated with MMP-2 levels. In addition, immunohistochemistry revealed that NOD2 is expressed in macrophages within atherosclerotic lesions and in endothelial cells lining those lesions, where it might act as an innate immune pathway promoting inflammation.

ACKNOWLEDGEMENTS

The authors would like to thank Ingrid Törnberg for excellent technical assistance. This work was supported by grants from the Swedish Research Council, the Swedish Heart-Lung Foundation, the AFA Foundation and the Dutch Heart Foundation (grant 2001-077). Jon D. Laman received support from the Dutch MS Research Foundation.

REFERENCES

1. Epstein SE, Zhou YF, Zhu J. Infection and atherosclerosis: emerging mechanistic paradigms. Circulation 1999;100:e20-e28.
2. Leinonen M, Saikku P. Evidence for infectious agents in cardiovascular disease and atherosclerosis. Lancet Infect Dis 2002;2:11-7.
3. Campbell LA, Kuo CC. *Chlamydia pneumoniae*--an infectious risk factor for atherosclerosis? Nat Rev Microbiol 2004;2:23-32.
4. Paoletti R, Gotto AM, Jr., Hajjar DP. Inflammation in atherosclerosis and implications for therapy. Circulation 2004;109:III20-III26.
5. Harton JA, Linhoff MW, Zhang J, Ting JP. Cutting edge: CATERPILLER: a large family of mammalian genes containing CARD, pyrin, nucleotide-binding, and leucine-rich repeat domains. J Immunol 2002;169:4088-93.
6. Chamaillard M, Hashimoto M, Horie Y, Masumoto J, Qiu S, Saab L, et al. An essential role for NOD1 in host recognition of bacterial peptidoglycan containing diaminopimelic acid. Nat Immunol 2003;4:702-7.
7. Girardin SE, Boneca IG, Carneiro LA, Antignac A, Jehanno M, Viala J, et al. Nod1 detects a unique muropeptide from gram-negative bacterial peptidoglycan. Science 2003;300:1584-7.
8. Girardin SE, Boneca IG, Viala J, Chamaillard M, Labigne A, Thomas G, et al. Nod2 is a general sensor of peptidoglycan through muramyl dipeptide (MDP) detection. J Biol Chem 2003;278:8869-72.
9. Inohara N, Ogura Y, Fontalba A, Gutierrez O, Pons F, Crespo J, et al. Host recognition of bacterial muramyl dipeptide mediated through NOD2. Implications for Crohn's disease. J Biol Chem 2003;278:5509-12.
10. Bertin J, Nir WJ, Fischer CM, Tayber OV, Errada PR, Grant JR, et al. Human CARD4 protein is a novel CED-4/Apaf-1 cell death family member that activates NF-kappaB. J Biol Chem 1999;274:12955-8.
11. Inohara N, Koseki T, del Peso L, Hu Y, Yee C, Chen S, et al. Nod1, an Apaf-1-like activator of caspase-9 and nuclear factor-kappaB. J Biol Chem 1999;274:14560-7.
12. Ogura Y, Inohara N, Benito A, Chen FF, Yamaoka S, Nunez G. Nod2, a Nod1/Apaf-1 family member that is restricted to monocytes and activates NF-kappaB. J Biol Chem 2001;276:4812-8.
13. Gutierrez O, Pipaon C, Inohara N, Fontalba A, Ogura Y, Prosper F, et al. Induction of Nod2 in myelomonocytic and intestinal epithelial cells via nuclear factor-kappa B activation. J Biol Chem 2002;277:41701-5.
14. Ogura Y, Lala S, Xin W, Smith E, Dowds TA, Chen FF, et al. Expression of NOD2 in Paneth cells: a possible link to Crohn's ileitis. Gut 2003;52:1591-7.
15. Lala S, Ogura Y, Osborne C, Hor SY, Bromfield A, Davies S, et al. Crohn's disease and the NOD2 gene: a role for paneth cells. Gastroenterology 2003;125:47-57.
16. Inohara N, Koseki T, Lin J, del Peso L, Lucas PC, Chen FF, et al. An induced proximity model for NF-kappa B activation in the Nod1/RICK and RIP signaling pathways. J Biol Chem 2000;275:27823-31.
17. Collins T, Cybulsky MI. NF-kappaB: pivotal mediator or innocent bystander in atherogenesis? J Clin Invest 2001;107:255-64.
18. Wang JE, Jørgensen PF, Almlöf M, Thiemermann C, Foster SJ, Aasen AO, et al. Peptidoglycan and lipoteichoic acid from Staphylococcus aureus induce tumor necrosis factor alpha, interleukin 6 (IL-6), and IL-10 production in both T cells and monocytes in a human whole blood model. Infect Immun 2000;68:3965-70.
19. Yoshimura A, Lien E, Ingalls RR, Tuomanen E, Dziarski R, Golenbock D. Cutting edge: recognition of Gram-positive bacterial cell wall components by the innate immune system occurs via Toll-like receptor 2. J Immunol 1999;163:1-5.
20. Laman JD, Schoneveld AH, Moll FL, van Meurs M, Pasterkamp G. Significance of peptidoglycan, a proinflammatory bacterial antigen in atherosclerotic arteries and its association with vulnerable plaques. Am J Cardiol 2002;90:119-23.
21. Oude Nijhuis MM, van der Graaf Y, Melief MJ, Schoneveld AH, de Kleijn DP, Laman JD, et al. IgM antibody level against proinflammatory bacterial peptidoglycan is inversely correlated with extent of atherosclerotic disease. Atherosclerosis 2004;173:245-51.
22. Hugot JP, Chamaillard M, Zouali H, Lesage S, Cezard JP, Belaiche J, et al. Association of NOD2 leucine-rich repeat variants with susceptibility to Crohn's disease. Nature 2001;411:599-603.
23. Ogura Y, Bonen DK, Inohara N, Nicolae DL, Chen FF, Ramos R, et al. A frameshift mutation in NOD2 associated with susceptibility to Crohn's disease. Nature 2001;411:603-6.

24. Fiocchi C. Inflammatory bowel disease: etiology and pathogenesis. Gastroenterology 1998;115:182-205.

25. Girardin SE, Hugot JP, Sansonetti PJ. Lessons from Nod2 studies: towards a link between Crohn's disease and bacterial sensing. Trends Immunol 2003;24:652-8.

26. Economou M, Trikalinos TA, Loizou KT, Tsianos EV, Ioannidis JP. Differential effects of NOD2 variants on Crohn's disease risk and phenotype in diverse populations: a metaanalysis. Am J Gastroenterol 2004;99:2393-404.

27. Miceli-Richard C, Lesage S, Rybojad M, Prieur AM, Manouvrier-Hanu S, Hafner R, et al. CARD15 mutations in Blau syndrome. Nat Genet 2001;29:19-20.

28. Verhoeven BA, Velema E, Schoneveld AH, de Vries JP, de Bruin P, Seldenrijk CA, et al. Athero-express: differential atherosclerotic plaque expression of mRNA and protein in relation to cardiovascular events and patient characteristics. Rationale and design. Eur J Epidemiol 2004;19:1127-33.

29. Sluijter JP, Pulskens WP, Schoneveld AH, Velema E, Strijder CF, Moll F, et al. Matrix metalloproteinase 2 is associated with stable and matrix metalloproteinases 8 and 9 with vulnerable carotid atherosclerotic lesions: a study in human endarterectomy specimen pointing to a role for different extracellular matrix metalloproteinase inducer glycosylation forms. Stroke 2006;37:235-9.

30. Rosenstiel P, Fantini M, Brautigam K, Kuhbacher T, Waetzig GH, Seegert D, et al. TNF-alpha and IFN-gamma regulate the expression of the NOD2 (CARD15) gene in human intestinal epithelial cells. Gastroenterology 2003;124:1001-9.

31. Ahrens P, Kattner E, Kohler B, Hartel C, Seidenberg J, Segerer H, et al. Mutations of genes involved in the innate immune system as predictors of sepsis in very low birth weight infants. Pediatr Res 2004;55:652-6.

32. Inohara N, Nunez G. NODs: intracellular proteins involved in inflammation and apoptosis. Nat Rev Immunol 2003;3:371-82.

33. Hisamatsu T, Suzuki M, Reinecker HC, Nadeau WJ, McCormick BA, Podolsky DK. CARD15/NOD2 functions as an antibacterial factor in human intestinal epithelial cells. Gastroenterology 2003;124:993-1000.

34. Binder CJ, Chang MK, Shaw PX, Miller YI, Hartvigsen K, Dewan A, et al. Innate and acquired immunity in atherogenesis. Nat Med 2002;8:1218-26.

35. Aiello RJ, Bourassa PA, Lindsey S, Weng W, Natoli E, Rollins BJ, et al. Monocyte chemoattractant protein-1 accelerates atherosclerosis in apolipoprotein E-deficient mice. Arterioscler Thromb Vasc Biol 1999;19:1518-25.

36. Dawson TC, Kuziel WA, Osahar TA, Maeda N. Absence of CC chemokine receptor-2 reduces atherosclerosis in apolipoprotein E-deficient mice. Atherosclerosis 1999;143:205-11.

37. Bourdillon MC, Poston RN, Covacho C, Chignier E, Bricca G, McGregor JL. ICAM-1 deficiency reduces atherosclerotic lesions in double-knockout mice (ApoE(-/-)/ICAM-1(-/-)) fed a fat or a chow diet. Arterioscler Thromb Vasc Biol 2000;20:2630-5.

38. Verhoeven B, Hellings WE, Moll FL, de Vries JP, de Kleijn DP, de Bruin P, et al. Carotid atherosclerotic plaques in patients with transient ischemic attacks and stroke have unstable characteristics compared with plaques in asymptomatic and amaurosis fugax patients. J Vasc Surg 2005;42:1075-81.

39. Galis ZS, Sukhova GK, Lark MW, Libby P. Increased expression of matrix metalloproteinases and matrix degrading activity in vulnerable regions of human atherosclerotic plaques. J Clin Invest 1994;94:2493-503.

40. Pasterkamp G, Schoneveld AH, Hijnen DJ, de Kleijn DP, Teepen H, van der Wal AC, et al. Atherosclerotic arterial remodeling and the localization of macrophages and matrix metalloproteases 1, 2 and 9 in the human coronary artery. Atherosclerosis 2000;150:245-53.

41. Molloy KJ, Thompson MM, Jones JL, Schwalbe EC, Bell PR, Naylor AR, et al. Unstable carotid plaques exhibit raised matrix metalloproteinase-8 activity. Circulation 2004;110:337-43.

42. Virmani R, Narula J, Farb A. When neoangiogenesis ricochets. Am Heart J 1998;136:937-9.

43. Davies MJ. Stability and instability: two faces of coronary atherosclerosis. The Paul Dudley White Lecture 1995. Circulation 1996;94:2013-20.

44. Cipollone F, Mezzetti A, Fazia ML, Cuccurullo C, Iezzi A, Ucchino S, et al. Association between 5-lipoxygenase expression and plaque instability in humans. Arterioscler Thromb Vasc Biol 2005;25:1665-70.

CHAPTER 9

General discussion

DISCUSSION

Atherosclerosis is a chronic inflammatory disease of the vascular wall that leads to myocardial infarction (MI), heart failure and stroke. Atherosclerosis develops over decades and progresses from intimal thickening and formation of fatty streaks, the earliest lesions in atherosclerosis, to more advanced atherosclerotic lesions, called fibro-fatty plaques. Fatty streaks consist of T-lymphocytes and monocyte-derived macrophages that are able to ingest oxidized LDL (OxLDL) particles and thereby change in lipid-laden foam cells. The fibro-fatty plaques are the result of continued monocyte, macrophage and T-lymphocyte recruitment, together with smooth muscle cell (SMC) migration and proliferation. The presence of a thick fibrous cap containing SMC and collagen stabilizes the lesion and walls off an atheromatous lipid core. The macrophage-derived foam cells accumulate in this lipid core and as the lesion progresses these lipid-laden foam cells undergo apoptosis. In combination with necrosis, increased proteolytic activity and more lipid accumulation inside the lipid core this will lead to the formation of a necrotic core. The concept of stable and unstable/vulnerable atherosclerotic plaques is clearly stated during the last few years. Stable plaques are characterized by a thick fibrous cap overlying a plaque that does not contain a lipid-rich necrotic core. Unstable plaques, however, have a thin fibrous cap, contain more macrophages than SMC and have a lipid-filled necrotic core. Unstable plaques are more prone to rupture, which exposes the thrombogenic material of the lipid-rich core to the blood, resulting in platelet aggregation and formation of an arterial thrombus. Small pieces of thrombus material can break away from the wall and, as an embolism, can be transported to other sites of the arterial tree where it may lead to blockage of smaller arteries, thereby causing heart attacks or strokes.

This discussion aims to describe the role of bacterial peptidoglycan (PG) derived from the intestinal mucosa in systemic immune responses in severe atherosclerotic disease, in inflammatory mechanisms promoting atherosclerotic plaque initiation and in progression of already existing atherosclerotic lesions. Thereto, we first compared antibody levels against PG in patients with severe atherosclerosis and healthy controls and investigated whether these antibody levels are predictive factors for the development of adverse clinical events after coronary angioplasty. Second, we determined whether TLR2 stimulation of vascular cells *in vitro* induces an inflammatory response and *in vivo* promotes atherosclerotic plaque development and plaque characteristics involved in plaque vulnerability. Third, we assessed whether stimulation of monocytes with PG promotes the ability of monocytes to adhere to endothelial cells and subsequently migrate into sites of inflammation and whether stimulation of macrophages with PG increases homing into atherosclerotic plaques. And Forth, involvement of NOD1 and NOD2 in atherosclerosis was determined in a biobank study. The general premise of this thesis is that PG acts as an inflammatory cofactor influencing plaque development, as well as vulnerability.

Peptidoglycan and inflammatory diseases

Involvement of PG in atherosclerosis has been suggested since PG was identified as a potential trigger for inflammatory responses in another inflammatory disease, rheumatoid arthritis. PG is present in large amounts in the Gram-positive bacterial cell wall and in small amounts in the Gram-negative bacterial wall. PG is composed of alternating N-acetyl glucosamine (GlcNAc) and N-acetyl muramic acid (MurNAc) residues thereby forming sugar chains that are interlinked by peptide bridges resulting in a large, complex macromolecule [1]. PG is present in healthy and chronically inflamed tissues and is able to activate T-lymphocytes, B-lymphocytes and macrophages resulting in the induction of proliferation, the synthesis of antibodies and the production of cytokines and matrix metalloproteinases (MMPs) [2-4]. PG isolated from bacteria from the intestinal flora induces arthritis in a rat model [5]. In the central nervous system of multiple sclerosis patients, accumulation of PG can be detected [6,7] and in mice, *Staphylococcus aureus* PG facilitates an autoantigen-specific inflammatory immune response in the central nervous system, which results in EAE development [7]. These results suggest that PG plays an important role in the pathogenesis of chronic inflammatory diseases, such as multiple sclerosis and rheumatoid arthritis. Like atherosclerosis, these diseases are characterized by infiltration of T-lymphocytes, antigen presenting cells (APC) and blood cells. The inflammatory process in these diseases is the result of interactions between antigens, cells of the immune system and cells of the diseased tissue. Cytokines, chemokines and MMPs are crucial mediators in these inflammatory processes.

A requirement for our hypothesis that PG is involved in atherosclerosis is that this bacterial cell wall antigen is present in the arterial wall. Several groups reported that PG can be detected in leukocytes [8,9] and human blood serum [10] using a monoclonal antibody that recognizes peptidoglycan-polysaccharides (PG-PS). Our group previously demonstrated that PG can be found in atherosclerotic plaques [11]. In addition, the presence of PG is associates with characteristics of plaque vulnerability, notably, a large lipid core, a thin fibrous cap, decreased collagen and SMC content and increased macrophage content. In addition, in the atherosclerotic plaques PG can mainly be found in the macrophage-rich areas.

Clearly it can rightly be argued that other microbial compounds could act as cofactors, but in this thesis we focused on PG since 1) PG has been shown to play an important role in other inflammatory diseases, 2) some pro-inflammatory responses of PG have already been shown, 3) PG is ubiquitously present at al mucosa and especially in the intestinal mucosa, and 4) PG is resistant to intracellular degradation (except some degrading enzymes) and therefore is consequently persistent in APC.

Systemic antibody levels against PG

To investigate whether the atherosclerotic process influences immune responses induced by PG, we compared antibody levels specific for PG between atherosclerotic patients and healthy subjects (Chapter 3). Some studies have already reported that PG is able to induce antibody formation [12,13] and circulating IgM, IgG and IgA antibodies against PG isolated from human faeces can be detected in all healthy humans [14,15].

Patients with severe atherosclerotic disease have significantly lower IgM antibody levels against PG compared to control patients with increased cardiovascular risk but without clinically manifest disease. In addition, the level of IgM antibodies against PG negatively correlates with common carotid intima-media thickness and a risk score for atherosclerotic disease. Based on these results, we hypothesized that low IgM antibody titers against PG would be predictive for the occurrence of adverse cardiovascular events after elective coronary angioplasty (Chapter 4). Adverse cardiovascular events are defined as cardiovascular death, non-fatal MI, non-fatal stroke or any cardiovascular intervention. The risk of a first cardiovascular event after angioplasty is not associated with IgM, IgG and IgA antibody levels against PG. The presence of antibodies against PG has already been described in relation to rheumatoid arthritis. Systemic IgG antibody levels against PG are significantly lower in rheumatoid arthritis patients compared to healthy controls. However, to our knowledge the relation between antibodies against PG and atherosclerosis has not been studied before. Several studies suggest that an association exists between antibody responses against bacterial infections and atherosclerosis. High-titre IgG antibodies against *Chlamydia pneumoniae* are associated with an increased risk of death from coronary heart disease and MI [16,17] and in patients with end-stage renal disease seropositivity for *C. pneumoniae* IgA strongly associates with the presence of coronary stenosis [18]. In contrast, a strong body of evidence suggests that such an association does not exist [19-21]. These results demonstrate that defining markers or immune responses that are involved in development of atherosclerotic disease will always remain a problem, since different study populations and therefore other research results might be difficult, if not impossible, to interpret and compare between studies. Although we do not find PG to be a prognostic factor for cardiovascular events in patients undergoing angioplasty, this does not preclude a prognostic application of anti-PG antibodies in other patient groups. Furthermore, several lines of evidence from both human material and from animal studies do convincingly support a role for PG in atherosclerosis, as will be discussed in this chapter.

Autoantibodies are part of the adaptive immune system. The immune system is divided into two layers; the innate and the adaptive immune systems. The innate immune system is the first line of defense and provides an immediate response against invading pathogens [22]. Pathogens carry microbial-specific pattern-associated molecular patterns (PAMPs) that can be recognized by pattern recognition receptors (PRRs). Toll-like receptors (TLRs) play an important role in innate and adaptive immune responses against invading pathogens and therefore could even cause disease by inappropriate activation. The adaptive immune system provides the generation of different kinds of T-lymphocyte and B-lymphocyte receptors that determines specificity and high affinity against pathogens. The T-lymphocytes play a role in cellular immunity while B-lymphocytes secrete immunoglobulins upon activation. The adaptive immune response is a slow response taking in general days or weeks.
Animal studies showed that immunization with OxLDL is correlated with inhibition of the progression of atherosclerosis. Immunization of hypercholesterolemic and low-density lipoprotein receptor-deficient (LDLr-/-) rabbits with homologous MDA-LDL and

OxLDL leads to the induction of high-titered IgM and IgG antibodies to both MDA-LDL and OxLDL and strongly reduces atherosclerosis [23,24]. In addition, in a mice model the atheroprotective effect of immunization is confirmed [25]. In general, immunization results in the production of high antibody levels which prevent development of atherosclerosis, probably because the pathogens or antigens that are the mediators of the atherosclerotic process are removed via antibody-antigen complexes that can be formed within the body. The low antibody levels against PG that we found in atherosclerotic patients might either suggest that many antibody-antigen complexes are formed and that PG is efficiently removed from the circulation. It can also imply that an efficient immune response is absent, resulting in accumulation of PG in the circulation. An increase in accumulation of PG in the circulation suggests that PG can still exert its pro-inflammatory effects, for example in the vascular wall.

Peptidoglycan and atherosclerosis

Because our group previously showed that PG is present in atherosclerotic plaques, it is important to know whether PG present in the human vascular wall is able to induce or contribute to inflammation. The role of inflammation in atherogenesis has been increasingly recognized [26,27]. For the development of therapies and identification of patients-at-risk understanding of the mechanisms that link inflammation to atherosclerosis and plaque instability is crucial.

Inflammatory capacities of peptidoglycan

In Chapter 5 we showed that the TLR2 is present in non-stimulated vascular adventitial fibroblasts and human coronary arteries. However, TLR2 protein expression is not homogenously expressed over the whole atherosclerotic artery. TLR2 stimulation of the adventitial fibroblasts results in increased mRNA and protein expression of the pro-inflammatory cytokine interleukin (IL)-6 and the chemokines IL-8 and MCP-1. Previous studies showed that stimulation of TLR2, TLR4 and TLR5 on uterine epithelial cells results in the secretion of IL-6, IL-8 and MCP-1 [28].

IL-6 is an acute inflammatory cytokine that can be produced by macrophages, lymphocytes, fibroblasts, endothelial cells and SMC. IL-6 levels are related to traditional risk factors; they increase with age and are associated with high blood pressure and smoking [29,30]. High IL-6 levels are associated with an increased risk of MI [31], death [32] and cardiovascular disease events. Many studies showed that IL-6 plasma levels are increased in patients with unstable angina compared to those with stable angina or healthy subjects, and that it could be useful as a prognostic marker of cardiovascular disease outcome [33,34]. IL-6 mRNA is present within human atherosclerotic arteries [35]. IL-6 is even able to stimulate MCP-1 expression by macrophages [36] and it promotes expression of adhesion molecules and secretion of other cytokines by endothelial cells [37]. Taken together, IL-6 may exert an important direct pathogenic role in atherosclerosis development and progression.

Many important human diseases, including asthma, rheumatoid arthritis and atherosclerosis are characterized by acute or chronic recruitment of leukocytes from

the blood into affected tissues. Some chemokines have been implicated to play an important role in leukocyte recruitment, inflammation and immunity. Chemokines are a family of potent chemotactic cytokines that are rapidly upregulated at sites of vascular inflammation [38,39]. Chemokines can be divided into three major families; CC chemokines, CXC chemokines and CX3C chemokines.

As the name implies, monocyte chemoattractant protein (MCP)-1 (or CCL2) is a potent agonist for monocytes and has been implicated as the key player in the recruitment of these cells from the blood into early atherosclerotic lesions, the development of intimal hyperplasia after angioplasty and in development of thrombosis. Other chemokine-members of this family include RANTES (CCL5) and macrophage inflammatory protein-1α (MIP-1α, CCL3). MCP-1 can be produced by multiple cell types, including endothelial cells, SMC and macrophages [40] and is present in macrophage-rich human atherosclerotic plaques [38,41]. OxLDL present in the atherosclerotic plaque induces MCP-1 production by endothelial cells and SMC [42], thereby recruiting more monocytes/macrophages towards the atherosclerotic plaque. The receptor of MCP-1, CCR2, is expressed on circulating monocytes and is upregulated in hypercholesterolemia [43]. Monocytes from CCR2-/- mice have an abnormality in their migratory ability [44] and significantly reduced intimal hyperplasia after femoral arterial injury [45]. When these mice are crossed onto the atherosclerosis-prone ApoE-/- background, they display decreased lesion areas with less macrophage infiltration in the intimal lesions [46,47] as has also been shown for LDLr-/- mice [48]. Addition of MCP-1 and IL-8 can cause rapid arrest of human monocytes rolling on E-selectin-expressing endothelial cells under flow conditions [49]. All these *in vitro* and *in vivo* experiments suggest that MCP-1 could play an important role in monocyte recruitment by mediating monocyte chemoattraction and firm adhesion of rolling monocytes to activated endothelial cells, thereby participating in the development of atherosclerotic plaques.

IL-8 (CXCL8) is a member of the CXC family of chemokines and is a potent chemoattractant for polymorphonuclear leukocytes [50], SMC [51], T-lymphocytes and natural killer cells [52]. IL-8 activates monocytes and may direct their recruitment to vascular lesions [49,53]. During adhesion of monocytes to endothelial cells *in vitro* IL-8 expression is induced [54]. OxLDL increases IL-8 expression in monocytes and macrophages [55,56] and IL-8 can be found in macrophage-rich atherosclerotic plaques [56-58]. Furthermore, IL-8 is able to promote growth of endothelial cells [59], but is also able to induce migration and proliferation of vascular SMC [51]. The chemokine receptor CXCR2 signals in response to several CXC chemokines, including IL-8. In mice whose blood contains CXCR2-/- monocytes, the size of atherosclerotic lesions is reduced. Repopulation with CXCR2+/+ monocytes results in increased atherosclerotic lesions associated with an accumulation of macrophages [60]. The above studies clearly establish a role for IL-8 in the trafficking of monocytes into the intima and thereby pointing to this chemokine as an important participant in the pathogenesis of atherosclerosis.

The third family, the CX3C family, has only one member, fractalkine (FK, CXC3CL1). FK is a transmembrane protein that can be cleaved resulting in a soluble protein [61]. When cleaved from the cell surface, soluble forms of FK are chemoattractants for monocytes, T-lymphocytes and natural killer cells [62]. FK is expressed in atherosclerotic lesions

and FK-dependent firm adhesion of monocytes and T-lymphocytes can occur under flow conditions [63,64].

Peptidoglycan and monocyte/macrophage recruitment

Recruitment of leukocytes (e.g. neutrophils, eosinophils, monocytes) is involved in the early phase of atherosclerosis [65] and is mediated by cellular adhesion molecules expressed by the vascular endothelial cells and the circulating leukocytes. The adhesion molecules can be upregulated by inflammatory stimuli [66] and adhesion molecule expression has been observed in human atherosclerotic lesions [67-69]. The mechanisms by which PG could enhance initiation or progression of atherosclerotic plaque formation are unknown. Since PG is present in macrophage-rich regions within human atherosclerotic lesions and macrophages in atherosclerotic plaques are mainly monocyte-derived, we hypothesized that *in vitro* stimulation of monocytes by PG affects the adhesive and migratory properties of these cells contributing to lesion growth (Chapter 6).

Selectins participate in the early steps of leukocyte recruitment. The selectin family consists of three members; L-selectin (CD62L) expressed by leukocytes, E-selectin (CD62E) expressed by endothelial cells and P-selectin (CD62P) that can be found on platelets and endothelial cells. Under shear forces selectins are involved in leukocyte rolling over and tethering to endothelial cells. Rolling is observed only under flow conditions and is the consequence of shear forces acting on the leukocyte and adhesive interactions between the leukocytes and the endothelial cells. The major ligand for all selectins is P-selectin glycoprotein ligand (PSGL-1, CD162), that is expressed by leukocytes [70]. It is the main ligand for P-selectin [71], but can also bind to L- and E-selectins though with lower affinity [72,73]. P-selectin/PSGL-1 binding triggers leukocyte activation, integrin mobilization and induces inflammation and thrombosis. Firm adhesion of the tethering and rolling leukocytes is mediated by integrins on the leukocyte surface and immunoglobin-like molecules on the endothelial cells. Intercellular adhesion molecule-1 (ICAM-1, CD54) and vascular cell adhesion molecule-1 (VCAM-1, CD106) are widely expressed by endothelial cells and can be upregulated by pro-inflammatory cytokines. Stimulation of monocytes isolated from human peripheral blood with PG results in significantly increased expression of adhesion molecules CD11b and CD18, whereas CD62L expression is significantly decreased (Chapter 6). CD11b (α_m-integrin) forms a complex with CD18 (β_2-integrin) resulting in the heterodimer MAC-1 ($\alpha_m\beta_2$-integrin). These molecules mediate adhesion of leukocytes to the activated endothelium and thereby participate in leukocyte extravasation. Integrins are transmembrane proteins comprised of non-covalently associated α and β subunits. In resting cells integrins are non-functional and non-adhesive. They are present on the cell surface but in a conformation that provides low affinity for ligands. Activation of the cell triggers signals that modify their conformation to develop a higher affinity. This is called “inside-out” signaling, whereas “outside-in” signals are delivered within the cell after ligation between an integrin and its ligand [74]. The integrins on tethering and rolling leukocytes are activated by endothelial membrane-bound chemokines, locally secreted chemoattractants [75] or bacterial products. Stimulation of monocytes with PG not only increases the expression but

also the function of β_2-integrins. Binding of the stimulated monocytes to ICAM-1 coated beads is significantly increased compared to control monocytes. This monocyte binding to the fluorescent beads can be abrogated by monocyte incubation with a β_2-integrin blocking antibody. VCAM-1 interacts with integrin $\alpha_4\beta_1$, also known as very late antigen (VLA)-4, and then induces signals in endothelial cells that trigger changes in shape and allow leukocyte migration [76]. Firm adhesion of the monocytes to the endothelium is significantly increased after PG stimulation due to the increased β_2-integrin expression and function on the monocytes. PG stimulation induces activation of the monocytes resulting in cell spreading over the endothelial surface, suggesting that monocytes are able to migrate to sites of inflammation [77]. In a Boyden-chamber migration assay we demonstrated that chemotaxis of monocytes stimulated with PG towards C5a is significantly increased compared to control monocytes. Furthermore, the effects of PG on monocyte migration are TLR2-specific and totally β_2-integrin-dependent. The process of transendothelial migration involves several junctional proteins, such as platelet endothelial cellular adhesion molecule (PECAM)-1 that can be found on leukocytes, platelets and endothelial cells [78].
In Chapter 7 we demonstrated that isolated peritoneal macrophages stimulated with PG and $Pam_3Cys\text{-}SK_4$ *in vitro* are able to home to atherosclerotic plaques in ApoE-/- mice. Homing of macrophages towards atherosclerotic plaques in ApoE-/- mice has already been described by Patel *et al* [79]. Homing of macrophages was significantly decreased when ApoE-/- mice were pretreated with blocking monoclonal antibodies against $\alpha_4\beta_1$-integrin and ICAM-1, implying that these specific cell adhesion molecules play an important role in macrophage adhesion to endothelial cells overlying atherosclerotic lesions *in vivo*, the same as we described *in vitro*. Our data support the hypothesis that TLR2-mediated activation of monocytes or macrophages with PG via the bloodstream contributes to enhanced accumulation of monocytes/macrophages at sites of inflammation like the atherosclerotic arterial wall.

Peptidoglycan promotes atherosclerotic plaque progression

Animal models of arterial injury are characterized by the development of intimal hyperplasia caused by migration and proliferation of SMC [80,81]. Intimal hyperplasia is an important component of the atherosclerotic plaque and is thought to be critical for the development of restenosis after coronary artery angioplasty and stenting [82]. SMC chemoattractants are key mediators of intimal hyperplasia. The source of these molecules is likely to include the major cells of the arterial wall, such as endothelial cells, SMC and adventitial fibroblasts. In addition, leukocytes accumulating at the surface or migrating into injured or atherosclerotic vessels are rich in growth factors and cytokines that can activate SMC. A number of studies have raised the possibility that MCP-1 and other chemokines, like IL-8, play a direct role in proliferation of SMC [51,83]. Based on these studies and our results a new hypothesis can be formed; if TLR2 is able to increase the expression and secretion of pro-inflammatory cytokines and chemokines that are known to be involved in the atherosclerotic process, and is able to recruit monocytes and macrophages to the site of inflammation, stimulation of TLR2 *in vivo* should promote development of atherosclerotic lesions.

Peptidoglycan and atherosclerotic plaque development

The most widely used small animal model of atherosclerosis is the ApoE-/- mouse [84]. The apolipoprotein ApoE is the main protein ligand on the surface of the murine LDL particle, which is recognized by the LDL receptor, and mediates the clearance of very low density lipoprotein (VLDL), LDL and chylomicrons from plasma. Mice that lack a functional gene encoding ApoE have elevated levels of plasma cholesterol (hypercholesterolaemia) [85] and spontaneously develop atherosclerotic lesions throughout the arterial tree from 10 weeks of age [86]. Plasma cholesterol levels can be further elevated by feeding mice a high-fat high-cholesterol 'western-type diet'. Feeding ApoE-/- mice such a high-fat diet, results in the development of atherosclerotic lesions in their major arteries, including the aorta and coronary arteries. Another mouse model that has been developed is the ApoE*3Leiden transgenic mouse. Compared to the normal ApoE gene, the ApoE*3Leiden gene contains a tandem duplication of codons 120-126 in the ApoE gene. The presence of a single allele for this mutation results in the development of familial dysbetalipoproteinemia in patients [87]. The transgenic mouse model, carrying this human ApoE*3Leiden gene, exhibits high levels of cholesterol and triglycerides [88,89]. These mice develop initial and advanced atherosclerosis, but only after being fed an atherogenic diet [90]. Both ApoE-/- and ApoE*3Leiden transgenic mice develop atherosclerotic lesions that share many features of early human atherosclerosis, containing a fibrous cap and a lipid core. As in human atherosclerosis these lesions contain macrophages, SMC, T-lymphocytes, endothelial cells and fibroblasts. However, unlike the situation in human arterial disease, the murine lesions show little or no evidence of atherosclerotic plaque rupture with the resultant thrombo-embolism that characterizes human cardiovascular disease.

In both mice and humans the normal blood vessel wall comprises an inner layer of endothelial cells (the intima) in contact with the lumen of the vessel, in which the blood circulates, a middle layer of SMC and elastic extracellular matrix fibres (the tunica media) and an outer layer of connective tissue (the adventitia) in contact with the tissues. In an atherosclerotic plaque, a cholesterol-rich lipid core forms within the intimal layer and this is infiltrated with cell types such as macrophages, macrophage-derived foam cells, SMC and CD4+ T-lymphocytes. If the plaque ruptures, the thrombogenic core of the lesion is exposed to blood in the lumen of the artery. Platelet adhesion and activation initiates the formation of an arterial thrombus.

Having demonstrated that TLR2 can be activated in vascular cells *in vitro*, we performed *in vivo* TLR2 stimulation studies in mice to investigate neointima or atherosclerotic plaque formation (Chapter 5). Adventitial $Pam_3Cys\text{-}SK_4$ application augments intimal lesion formation in C57BL6 wild type mice induced by a cuff. We showed that these *in vivo* effects are mediated through TLR2 since TLR2-/- mice showed no significant increase in intimal hyperplasia after $Pam_3Cys\text{-}SK_4$ application. In ApoE-/- mice that are put on a high cholesterol diet for 3 weeks prior to operation, TLR2 stimulation results in dramatically increased atherosclerotic plaque formation. The role of TLR2 in atherosclerosis has not been extensively studied. The survival rate after MI is significantly higher in TLR2-/- mice as compared to wild type mice [91]. Furthermore, myocardial fibrosis in the non-infarcted area is significantly lower in the TLR2-/- mice,

suggesting that TLR2 plays an important role in MI. Chapter 7 shows a further role for TLR2 in atherosclerotic plaque formation. After placement of a non-constrictive cuff containing either PG or $Pam_3Cys\text{-}SK_4$ around the femoral arteries of ApoE*3Leiden transgenic mice that were on a high-cholesterol and high-fat diet for 4 weeks, atherosclerotic plaque formation is significantly increased compared to untreated arteries. Furthermore, arterial size is increased after application of the ligands, thereby preventing a decrease in luminal area. This indicates that TLR2 ligation by PG and $Pam_3Cys\text{-}SK_4$ might promote outward arterial remodeling, which is an important determinant for lumen loss because it can compensate for plaque accumulation in the arterial lumen [92]. However, although the luminal area is preserved, the plaque beneath the surface of the lumen often has a vulnerable plaque phenotype [93]. We have previously shown that TLR4 is involved in both plaque formation and outward arterial remodeling [94].

Peptidoglycan and atherosclerotic plaque characteristics

Chapter 7 also describes that atherosclerotic lesions in ApoE*3Leiden transgenic mice are not only increased after PG stimulation, the phenotype of these plaques is significantly different from the atherosclerotic plaques at the contralateral control side. After stimulation the plaques are enriched in SMC and collagen, whereas macrophage content is not changed. Interestingly, in the media a switch in cellular content is evident; the number of α-SMC actin-positive cells is strongly reduced whereas the number of macrophages is clearly increased. Since outward arterial remodeling often is accompanied by formation of a vulnerable plaque and since we found that PG is present in human atherosclerotic plaques that reveal an inflammatory unstable phenotype, we expected that application of PG after cuff placement around the femoral artery of an atherosclerotic mouse model would induce the formation of vulnerable atherosclerotic plaques. Our results show that stimulation of the arterial wall with PG induces a more stable plaque phenotype on the luminal side of the arterial wall (at least based on macrophage/SMC ratio in the plaque). Ligands that are present on the outside of the arterial wall probably do not have the ability to pass the external elastic lamina and thereby to enter the media and atherosclerotic plaque. In contrast, ligands like LPS and are able to activate adventitial fibroblasts [95]. These fibroblasts in turn act as innate immune cells and increase the production and secretion of pro-inflammatory cytokines and chemokines, like IL-1α, IL-1β, IL-6, IL-8 and MCP-1 (also shown in Chapter 5). These cytokines and chemokines are able to activate MMP-2 and MMP-9 [96,97]. Pro-inflammatory cytokines and chemokines as well as MMPs facilitate SMC migration and proliferation and are associated with development of atherosclerosis [98]. In our experiments it is likely that PG stimulated the adventitial fibroblasts, thereby increasing the production and secretion of pro-inflammatory cytokines, chemokines and MMPs, followed by the induction of migration and proliferation of medial SMC and resulting in increased atherosclerotic plaque development. Activation of cells present in the adventitia and the subsequent pro-inflammatory response might be a reasonable explanation by which PG present at the outside of the vessel wall is able to induce an increase in migration of SMC from the media to the atherosclerotic plaque. This increase in migration of SMC is supported by increased fragmentation

of the internal elastic lamina after PG application. Disruption of the internal elastic lamina might indicate increased elastase activity, thereby facilitating migration of SMC from the media into the atherosclerotic plaques at these fragmentation sites. It is likely that the PG at the outside of the arterial wall does not trigger cells that are present in the blood stream to migrate into the arterial wall. Therefore macrophage content in the atherosclerotic plaque is comparable between the PG-treated arteries and the control arteries and the change in plaque phenotype is contributable solely to increased SMC migration and proliferation.

Innate immune receptors recognizing peptidoglycan

PG stimulates the immune system by binding to innate receptors on and within APC. Innate immune receptors that recognize PG are TLR2, nucleotide-binding oligomerization domain (NOD) receptors and Nalp/cryopyrin (for details see general introduction).

NOD1 and NOD2 receptors in atherosclerosis

We already discussed the involvement of TLR2 in atherosclerosis. Pathophysiologic functions of NOD are only beginning to be understood and the role of NOD proteins in atherosclerosis is as yet unexplored. NOD1 and NOD2 are expressed in human tissue both at the mRNA and protein level (Chapter 8). NOD1 and NOD2 protein expression is significantly increased in atherosclerotic lesions. NOD2 is primarily expressed in macrophages within inflammatory areas inside the atherosclerotic plaques and in endothelial cells lining the vessel wall. NOD1 is ubiquitously expressed in a variety of adult tissues [99,100], as opposed to NOD2 which has been found mainly in macrophages, dendritic cells, granulocytes and epithelial cells of the intestine [101,102]. NOD2 expression in endothelial cells is induced by the pro-inflammatory cytokines TNF-α, IFN-γ and Il-1β (Chapter 8). This has already been shown before; stimulation of human umbilical vascular endothelial cells with LPS, IL-1β and TNF-α significantly upregulates NOD2 protein expression. NOD2 is localized in the cytoplasm and induces NF-κB-dependent transcriptional activity in endothelial cells upon MDP stimulation. NOD2 is also expressed in human ocular tissue and endothelial cells from choroid demonstrate enhanced release of IL-6 in response to MDP and synergy is observed following treatment with MDP and either $Pam_3Cys\text{-}SK_4$ or LPS. In Chapter 8 we also describe a positive relation between NOD1 expression levels and collagen staining and the presence of SMC in human atherosclerotic lesions, suggesting that NOD1 expression is associated with a stable plaque phenotype. Furthermore, in the absence of SMC there is also a significant relation between NOD1 expression and macrophage content, indicating that NOD1 protein is also expressed within inflammatory areas inside atherosclerotic lesions, as we already demonstrated with immunohistochemistry for NOD2. Both NOD1 and NOD2 protein expression is inversely correlated with MMP-2 activity and positively associated with MMP-8 and MMP-9 levels. Both NOD1 and NOD2 protein expression are related to or expressed in macrophages and thereby explain a positive association between NOD1/NOD2 and MMP-9 and a positive relation between NOD1 and macrophage content. Furthermore, expression of NOD1 and NOD2

by macrophages and SMC in the stable atherosclerotic plaque increases production of pro-inflammatory cytokines, like IL-6, and thereby recruits more macrophages to the atherosclerotic plaque. This will change the phenotype of the plaque towards a more inflammatory, vulnerable plaque, which in turn results in downregulation of the MMP-2 levels and explaining the negative association we found between NOD1 and NOD2 protein expression and MMP-2 levels.
We demonstrated that PG is present in macrophage-rich areas in atherosclerotic lesions [103]. In this thesis we extend these findings by demonstrating a mechanism whereby PG, or rather its minimal breakdown products, can be detected by NOD1 and NOD2 in macrophages and endothelial cells in atherosclerotic lesions and thereby promote the inflammatory phenotype of the atherosclerotic lesion.

Peptidoglycan as active player in atherosclerosis

We have previously shown that PG can be found in atherosclerotic plaques and that the presence of PG is associated with characteristics of plaque vulnerability. In this thesis we assessed whether pro-inflammatory PG is involved in atherosclerotic plaque initiation and progression. *Figure 1* illustrates how PG might be distributed and contributes to atherosclerotic plaque progression.
PG is present in the bacterial flora at all mucosal sites and abundantly in the intestinal flora. Bacteria are able to pass the intestinal mucosal epithelium by M cells and APC. M cells are specialized epithelial cells that have abundant vesicles with lysosomal enzymes [104]. The M cell basolateral membrane is deeply invaginated to form an intraepithelial pocket that contains B- and T-lymphocytes and macrophages [105]. This invagination brings the basolateral cell surface to within a few microns of the apical membrane and thereby shortens the distance that transcytotic vesicles must travel to cross the epithelial barrier. After M cell transport, the bacteria are processed by macrophages, dendritic cells, and B- or T-lymphocytes. In this way bacteria are transported into the circulation and lymphoid organs. Another mechanism which might lead to increased distribution of bacteria or bacterial products, and thereby higher levels of PG, into the circulation is intestinal injury, which can be caused by infections. Whether released PG can pass the mucosal epithelial barrier or translocates only within the whole bacterium is not known. PG can also be derived from bacterial infections at non-mucosal sites or by release of PG fragments during bacterial replication. Transport of PG to atherosclerotic plaques can occur via monocytes that are present in the bloodstream. The presence of PG in leukocytes has been confirmed by the detection of muramic acid in these cells. Up till now, free circulating PG has not been detected in the bloodstream. Leukocytes are able to pass the lymph nodes and the spleen where they might activate B-lymphocytes. These B-lymphocytes in turn, produce and secrete IgM, IgG and IgA antibodies specific for PG, which can be found in serum of healthy and atherosclerotic patients. When IgM antibody levels against PG decrease, less antigen-antibody complexes can be formed and tissue may be increasingly exposed to PG. The monocytes that carry PG can adhere to activated endothelial cells lining the vessel wall at sites of inflammation (also shown in *figure 2*).

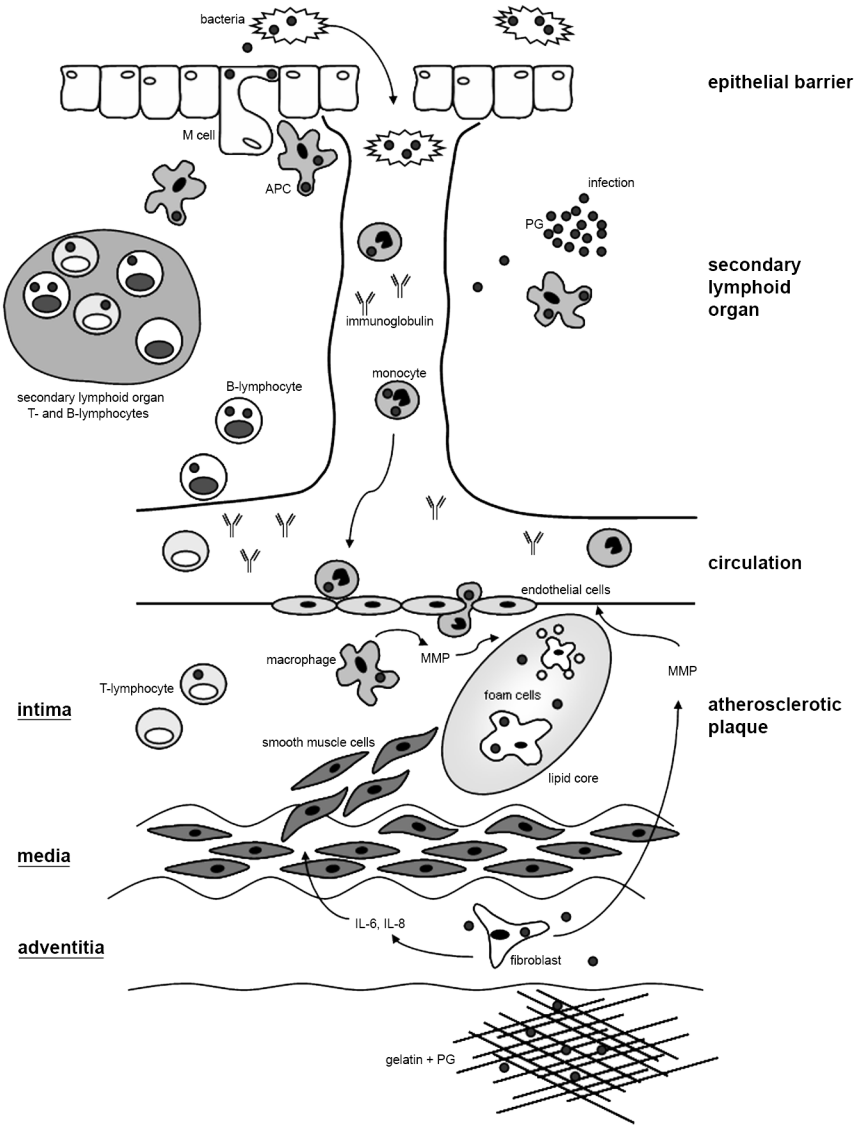

Figure 1. Pro-inflammatory PG promotes atherosclerotic plaque progression
(This illustration is explained in the text)

Monocytes express L-selectin (CD62L) and MAC-1 (CD18/CD11b, β_2-integrin) on their surface. L-selectin is involved in monocyte tethering to and rolling over the endothelial cells. Firm adhesion of the tethering and rolling monocyte is mediated by integrins on the monocyte surface and ICAM-1 and VCAM-1 expressed by the endothelial cells. The monocytes that carry PG and thus are activated by this ligand shed L-selectin from their surface and increase expression and functionality of the β_2-integrins thereby promoting firm adhesion of the monocytes to the endothelial cells (shown in Chapter 6). ICAM-1 on the endothelial surface binds to MAC-1 expressed by the monocyte and VCAM-1 interacts with VLA-4. Once firmly adhered the monocyte changes in

shape, triggered by the endothelial cell, and migrates over and through/between the endothelial surface. The migratory process involves proteins like PECAM-1 present on monocytes, platelets and endothelial cells and β_2-integrins (Chapter 6).
Once present in the arterial wall the monocytes differentiate into macrophages, which in turn digest OxLDL that is present within the atherosclerotic plaque to become foam cells. The activated macrophages increase production and secretion of pro-inflammatory cytokines and chemokines and subsequently increase SMC migration from the media and proliferation and recruitment of more macrophages and T-lymphocytes to the atherosclerotic plaque. In this way the activated macrophages promote progression of the atherosclerotic lesion and formation of a vulnerable plaque phenotype. The lipid-laden foam cells accumulate in large lipid cores and finally will undergo apoptosis thereby releasing their lipid content and promoting formation of a necrotic core that is prone to rupture. A thick fibrous cap containing SMC and collagen prevents rupture of the atherosclerotic plaque. However, activated macrophages secrete besides cytokines and chemokines also proteases, protease activators and protease inhibitors that facilitate remodeling of the extracellular matrix. The MMPs and other proteolytic enzymes cause degradation of the matrix and as a result thinning of the fibrous cap covering the lipid core and thereby increasing the risk of rupture of the atherosclerotic plaque. When applied on the outside of the arterial wall PG is able to activate fibroblasts, which in turn, secrete pro-inflammatory cytokines and MMPs and thereby contribute to progression of the atherosclerotic plaque and might even induce rupture.

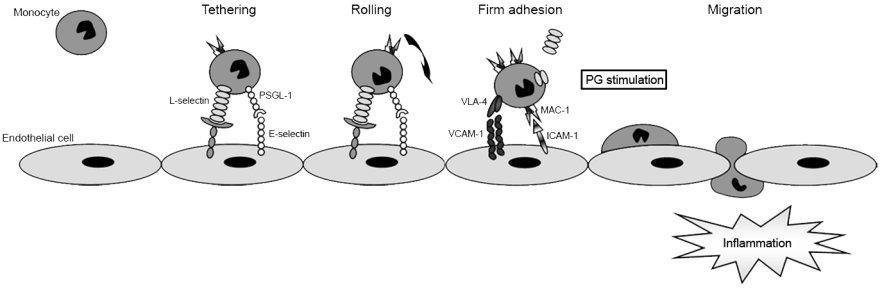

Figure 2. Monocyte migration into the arterial wall after PG stimulation
Monocyte migration from the bloodstream into the arterial wall is a multistep process, involving tethering, rolling, firm adhesion and subsequently monocyte migration. PG sheds L-selectin from the monocyte surface and increases expression of MAC-1, thereby promoting firm adhesion of the monocyte. Furthermore, PG stimulates TLR2- and β_2-integrin-dependent monocyte migration, suggesting that TLR2 stimulation of monocytes by PG might enhance accumulation of these cells at sites of inflammation.

Future perspectives

Although, as demonstrated in this thesis, bacterial PG promotes inflammation and is involved in monocyte/macrophage recruitment to and progression of atherosclerotic plaques, the exact mechanism whereby PG exerts its action is not entirely known. PG stimulates the innate immune system by binding to TLR2/6, NOD1, NOD2 and Nalp3/cryopyrin. The role of TLRs in atherosclerosis has extensively been studied the last 10 years, but little is known about the role NOD1, NOD2 and Nalp3 play in the atherosclerotic process. In this thesis we already described that NOD1 and NOD2

protein is expressed in atherosclerotic plaques. Furthermore, we demonstrated a mechanism whereby PG, or rather its minimal breakdown products, could be detected by NOD1 and NOD2 in macrophages and endothelial cells. However, further studies need to elucidate the functional relevance of induced NOD1 and NOD2 expression in these cells. In addition, involvement of Nalp in atherosclerotic disease has not been studied either. Another possibility might be that PG promotes an inflammatory response by a combination of these receptors. Perhaps experiments in TLR2/ApoE double knockout mice could give an answer to this question.
Stimulation of monocytes and macrophages with PG results in increased monocyte adhesion to endothelial cells, followed by migration, and macrophage homing into atherosclerotic plaques, thereby stimulating formation of a more vulnerable plaque. However, the involvement of PG in degradation of the fibrous cap and plaque rupture has not been studied yet. Atherosclerotic mice do not develop plaques that are prone to rupture and therefore this is difficult to study in a mouse model.
Recent studies already showed that NOD1 and NOD2 are involved in the apoptosis-pathway. This suggests that PG, or its degradation products play a role in this process as well. This has to be studied in more detail.
As described in the introduction, PG can be degraded by different enzymes; lysozyme, NAMLAA and N-acetylglucosaminidase. PG is able to escape degradation when it is expressed in cells that do not express the enzymes. It is interesting to investigate which cells in the atherosclerotic plaque express these enzymes and whether PG is present in those cells as well.
In this thesis we focused on the role of monocytes and macrophages as PG-"carrying" cells into the atherosclerotic plaque. After passage of the intestinal epithelial barrier, other APC, such as dendritic cells and T-lymphocytes, are also able to take up PG and transport it through the circulation. Not only the monocytes and macrophages but also these other APC might be involved in the relation that exists between atherosclerotic plaque formation and the presence of PG in those plaques.

Concluding remarks

Taken together, the studies described in this thesis suggest that PG is involved in initiation and progression of atherosclerosis. IgM, IgG and IgA antibodies against PG are present in human serum and decreased IgM antibody levels against PG are associated with more pronounced human atherosclerotic disease in a cross-sectional study. These IgM antibody levels against PG are not prognostic markers for development of adverse cardiovascular events after elective coronary angioplasty. We also showed that adventitial fibroblasts and human coronary arteries express TLR2 and that TLR2 stimulation of adventitial fibroblasts increases expression of pro-inflammatory cytokines and chemokines, like IL-1β, IL-6, IL-8 and MCP-1. In addition, in wild type mice $Pam_3Cys\text{-}SK_4$ stimulation promotes intimal lesion formation, which is clearly TLR2-mediated since TLR2-/- mice show no significant increase in intimal hyperplasia. In ApoE-/- mice TLR2 stimulation increases development of atherosclerotic plaques. In addition to recognition by TLR2, degradation products of PG can also be recognized by NOD and Nalp proteins. NOD1 and NOD2 protein expression is significantly

increased in human atherosclerotic lesions and in those lesions NOD2 protein is present in macrophages within inflammatory areas and in endothelial cells lining the vessel wall. NOD1 protein expression is associated with the presence of SMC and collagen, but also with macrophages when SMC are absent. Furthermore, both NOD1 and NOD2 protein levels are inversely associated with MMP2 levels and positively with MMP-8 and MMP-9 levels. Previous studies from our group showed that PG is present in human atherosclerotic plaques that reveal an inflammatory, vulnerable phenotype and that PG is present in the macrophage-rich regions of those plaques. In this thesis we extend this finding by showing that peripheral blood monocytes that are stimulated with PG increase expression and functionality of integrins on their surface, thereby promoting firm adhesion of the monocytes to endothelial cells and migratory properties. Furthermore, peritoneal macrophages stimulated with PG *in vitro* show increased homing into atherosclerotic plaques *in vivo*. These studies not only suggest that PG promotes atherosclerotic lesion development and progression; they also show that PG enhances accumulation of monocytes and/or macrophages at sites of inflammation thereby promoting the formation of vulnerable plaques.

REFERENCES

1. Schleifer KH, Kandler O. Peptidoglycan types of bacterial cell walls and their taxonomic implications. Bacteriol Rev 1972;36:407-77.
2. Schrijver IA, Melief MJ, Eulderink F, Hazenberg MP, Laman JD. Bacterial peptidoglycan polysaccharides in sterile human spleen induce proinflammatory cytokine production by human blood cells. J Infect Dis 1999;179:1459-68.
3. Schrijver IA, Melief MJ, Tak PP, Hazenberg MP, Laman JD. Antigen-presenting cells containing bacterial peptidoglycan in synovial tissues of rheumatoid arthritis patients coexpress costimulatory molecules and cytokines. Arthritis Rheum 2000;43:2160-8.
4. Schrijver IA, Melief MJ, Markusse HM, Van A, I, Opdenakker G, Hazenberg MP, et al. Peptidoglycan from sterile human spleen induces T-cell proliferation and inflammatory mediators in rheumatoid arthritis patients and healthy subjects. Rheumatology (Oxford) 2001;40:438-46.
5. Kool J, Ruseler-van Embden JG, van Lieshout LM, de Visser H, Gerrits-Boeye MY, van den Berg WB, et al. Induction of arthritis in rats by soluble peptidoglycan-polysaccharide complexes produced by human intestinal flora. Arthritis Rheum 1991;34:1611-6.
6. Schrijver IA, van Meurs M, Melief MJ, Wim AC, Buljevac D, Ravid R, et al. Bacterial peptidoglycan and immune reactivity in the central nervous system in multiple sclerosis. Brain 2001;124:1544-54.
7. Visser L, Jan dH, Boven LA, van Riel D, van Meurs M, Melief MJ, et al. Proinflammatory bacterial peptidoglycan as a cofactor for the development of central nervous system autoimmune disease. J Immunol 2005;174:808-16.
8. Lehtonen L, Eerola E, Oksman P, Toivanen P. Muramic acid in peripheral blood leukocytes of healthy human subjects. J Infect Dis 1995;171:1060-4.
9. Lehtonen L, Eerola E, Toivanen P. Muramic acid in human peripheral blood leucocytes in different age groups. Eur J Clin Invest 1997;27:791-2.
10. Schrijver IA, De Man YA, Melief MJ, Van Laar JM, Markusse HM, Klasen IS, et al. Reduced systemic IgG levels against peptidoglycan in rheumatoid arthritis (RA) patients. Clin Exp Immunol 2001;123:140-6.
11. Laman JD, Schoneveld AH, Moll FL, van Meurs M, Pasterkamp G. Significance of peptidoglycan, a proinflammatory bacterial antigen in atherosclerotic arteries and its association with vulnerable plaques. Am J Cardiol 2002;90:119-23.
12. Dziarski R. Polyclonal activation of immunoglobulin secretion in B lymphocytes induced by staphylococcal peptidoglycan. J Immunol 1980;125:2478-83.
13. Rasanen L, Lehto M, Jokinen I, Leinikki P. Polyclonal antibody formation of human lymphocytes to bacterial components. Immunology 1986;58:577-81.
14. Johnson PM, Phua KK, Perkins HR, Hart CA, Bucknall RC. Antibody to streptococcal cell wall peptidoglycan-polysaccharide polymers in seropositive and seronegative rheumatic disease. Clin Exp Immunol 1984;55:115-24.
15. Hazenberg MP, de Visser H, Bras MJ, Prins ME, Van de Merwe JP. Serum antibodies to peptidoglycan-polysaccharide complexes from the anaerobic intestinal flora in patients with Crohn's disease. Digestion 1990;47:172-80.
16. Sessa R, Di Pietro M, Santino I, del Piano M, Varveri A, Dagianti A, et al. *Chlamydia pneumoniae* infection and atherosclerotic coronary disease. Am Heart J 1999;137:1116-9.
17. Siscovick DS, Schwartz SM, Corey L, Grayston JT, Ashley R, Wang SP, et al. *Chlamydia pneumoniae,* herpes simplex virus type 1, and cytomegalovirus and incident myocardial infarction and coronary heart disease death in older adults : the Cardiovascular Health Study. Circulation 2000;102:2335-40.
18. Nishimura M, Hashimoto T, Kobayashi H, Fukuda T, Okino K, Yamamoto N, et al. Close association of *Chlamydia pneumoniae* IgA seropositivity by ELISA with the presence of coronary artery stenosis in haemodialysis patients. Nephrol Dial Transplant 2005;20:1944-50.
19. Koh WP, Taylor MB, Chew SK, Phoon MC, Kang KL, Chow VT. *Chlamydia pneumoniae* IgG seropositivity and clinical history of ischemic heart disease in Singapore. J Microbiol Immunol Infect 2003;36:169-74.
20. Haim M, Tanne D, Battler A, Boyko V, Reshef T, Goldbourt U, et al. *Chlamydia pneumoniae* and future risk in patients with coronary heart disease. Int J Cardiol 2004;93:25-30.
21. Ieven MM, Hoymans VY. Involvement of *Chlamydia pneumoniae* in atherosclerosis: more evidence for lack of evidence. J Clin Microbiol 2005;43:19-24.
22. Janeway CA, Jr., Medzhitov R. Innate immune recognition. Annu Rev Immunol 2002;20:197-216.

23. Palinski W, Miller E, Witztum JL. Immunization of low density lipoprotein (LDL) receptor-deficient rabbits with homologous malondialdehyde-modified LDL reduces atherogenesis. Proc Natl Acad Sci U S A 1995;92:821-5.

24. Ameli S, Hultgardh-Nilsson A, Regnstrom J, Calara F, Yano J, Cercek B, et al. Effect of immunization with homologous LDL and oxidized LDL on early atherosclerosis in hypercholesterolemic rabbits. Arterioscler Thromb Vasc Biol 1996;16:1074-9.

25. Maron R, Sukhova G, Faria AM, Hoffmann E, Mach F, Libby P, et al. Mucosal administration of heat shock protein-65 decreases atherosclerosis and inflammation in aortic arch of low-density lipoprotein receptor-deficient mice. Circulation 2002;106:1708-15.

26. Ross R. Atherosclerosis--an inflammatory disease. N Engl J Med 1999;340:115-26.

27. Libby P. Inflammation in atherosclerosis. Nature 2002;420:868-74.

28. Schaefer TM, Desouza K, Fahey JV, Beagley KW, Wira CR. Toll-like receptor (TLR) expression and TLR-mediated cytokine/chemokine production by human uterine epithelial cells. Immunology 2004;112:428-36.

29. Fernandez-Real JM, Vayreda M, Richart C, Gutierrez C, Broch M, Vendrell J, et al. Circulating interleukin 6 levels, blood pressure, and insulin sensitivity in apparently healthy men and women. J Clin Endocrinol Metab 2001;86:1154-9.

30. Bermudez EA, Rifai N, Buring J, Manson JE, Ridker PM. Interrelationships among circulating interleukin-6, C-reactive protein, and traditional cardiovascular risk factors in women. Arterioscler Thromb Vasc Biol 2002;22:1668-73.

31. Ridker PM, Rifai N, Stampfer MJ, Hennekens CH. Plasma concentration of interleukin-6 and the risk of future myocardial infarction among apparently healthy men. Circulation 2000;101:1767-72.

32. Harris TB, Ferrucci L, Tracy RP, Corti MC, Wacholder S, Ettinger WH, Jr., et al. Associations of elevated interleukin-6 and C-reactive protein levels with mortality in the elderly. Am J Med 1999;106:506-12.

33. Biasucci LM, Vitelli A, Liuzzo G, Altamura S, Caligiuri G, Monaco C, et al. Elevated levels of interleukin-6 in unstable angina. Circulation 1996;94:874-7.

34. Lindmark E, Diderholm E, Wallentin L, Siegbahn A. Relationship between interleukin 6 and mortality in patients with unstable coronary artery disease: effects of an early invasive or noninvasive strategy. JAMA 2001;286:2107-13.

35. Seino Y, Ikeda U, Ikeda M, Yamamoto K, Misawa Y, Hasegawa T, et al. Interleukin 6 gene transcripts are expressed in human atherosclerotic lesions. Cytokine 1994;6:87-91.

36. Biswas P, Delfanti F, Bernasconi S, Mengozzi M, Cota M, Polentarutti N, et al. Interleukin-6 induces monocyte chemotactic protein-1 in peripheral blood mononuclear cells and in the U937 cell line. Blood 1998;91:258-65.

37. Romano M, Sironi M, Toniatti C, Polentarutti N, Fruscella P, Ghezzi P, et al. Role of IL-6 and its soluble receptor in induction of chemokines and leukocyte recruitment. Immunity 1997;6:315-25.

38. Terkeltaub R, Boisvert WA, Curtiss LK. Chemokines and atherosclerosis. Curr Opin Lipidol 1998;9:397-405.

39. Reape TJ, Groot PH. Chemokines and atherosclerosis. Atherosclerosis 1999;147:213-25.

40. Hansson GK. Immune mechanisms in atherosclerosis. Arterioscler Thromb Vasc Biol 2001;21:1876-90.

41. Nelken NA, Coughlin SR, Gordon D, Wilcox JN. Monocyte chemoattractant protein-1 in human atheromatous plaques. J Clin Invest 1991;88:1121-7.

42. Cushing SD, Berliner JA, Valente AJ, Territo MC, Navab M, Parhami F, et al. Minimally modified low density lipoprotein induces monocyte chemotactic protein 1 in human endothelial cells and smooth muscle cells. Proc Natl Acad Sci U S A 1990;87:5134-8.

43. Han KH, Tangirala RK, Green SR, Quehenberger O. Chemokine receptor CCR2 expression and monocyte chemoattractant protein-1-mediated chemotaxis in human monocytes. A regulatory role for plasma LDL. Arterioscler Thromb Vasc Biol 1998;18:1983-91.

44. Boring L, Gosling J, Chensue SW, Kunkel SL, Farese RV, Jr., Broxmeyer HE, et al. Impaired monocyte migration and reduced type 1 (Th1) cytokine responses in C-C chemokine receptor 2 knockout mice. J Clin Invest 1997;100:2552-61.

45. Roque M, Kim WJ, Gazdoin M, Malik A, Reis ED, Fallon JT, et al. CCR2 deficiency decreases intimal hyperplasia after arterial injury. Arterioscler Thromb Vasc Biol 2002;22:554-9.

46. Boring L, Gosling J, Cleary M, Charo IF. Decreased lesion formation in CCR2-/- mice reveals a role for chemokines in the initiation of atherosclerosis. Nature 1998;394:894-7.

47. Dawson TC, Kuziel WA, Osahar TA, Maeda N. Absence of CC chemokine receptor-2 reduces atherosclerosis in apolipoprotein E-deficient mice. Atherosclerosis 1999;143:205-11.

48. Gu L, Okada Y, Clinton SK, Gerard C, Sukhova GK, Libby P, et al. Absence of monocyte chemoattractant protein-1 reduces atherosclerosis in low density lipoprotein receptor-deficient mice. Mol Cell 1998;2:275-81.

49. Gerszten RE, Garcia-Zepeda EA, Lim YC, Yoshida M, Ding HA, Gimbrone MA, Jr., et al. MCP-1 and IL-8 trigger firm adhesion of monocytes to vascular endothelium under flow conditions. Nature 1999;398:718-23.

50. Jagels MA, Daffern PJ, Zuraw BL, Hugli TE. Mechanisms and regulation of polymorphonuclear leukocyte and eosinophil adherence to human airway epithelial cells. Am J Respir Cell Mol Biol 1999;21:418-27.

51. Yue TL, McKenna PJ, Gu JL, Feuerstein GZ. Interleukin-8 is chemotactic for vascular smooth muscle cells. Eur J Pharmacol 1993;240:81-4.

52. Xu L, Kelvin DJ, Ye GQ, Taub DD, Ben Baruch A, Oppenheim JJ, et al. Modulation of IL-8 receptor expression on purified human T lymphocytes is associated with changed chemotactic responses to IL-8. J Leukoc Biol 1995;57:335-42.

53. Gerszten RE, Lim YC, Ding HT, Snapp K, Kansas G, Dichek DA, et al. Adhesion of monocytes to vascular cell adhesion molecule-1-transduced human endothelial cells: implications for atherogenesis. Circ Res 1998;82:871-8.

54. Lukacs NW, Strieter RM, Elner V, Evanoff HL, Burdick MD, Kunkel SL. Production of chemokines, interleukin-8 and monocyte chemoattractant protein-1, during monocyte: endothelial cell interactions. Blood 1995;86:2767-73.

55. Terkeltaub R, Banka CL, Solan J, Santoro D, Brand K, Curtiss LK. Oxidized LDL induces monocytic cell expression of interleukin-8, a chemokine with T-lymphocyte chemotactic activity. Arterioscler Thromb 1994;14:47-53.

56. Wang N, Tabas I, Winchester R, Ravalli S, Rabbani LE, Tall A. Interleukin 8 is induced by cholesterol loading of macrophages and expressed by macrophage foam cells in human atheroma. J Biol Chem 1996;271:8837-42.

57. Rus HG, Vlaicu R, Niculescu F. Interleukin-6 and interleukin-8 protein and gene expression in human arterial atherosclerotic wall. Atherosclerosis 1996;127:263-71.

58. Apostolopoulos J, Davenport P, Tipping PG. Interleukin-8 production by macrophages from atheromatous plaques. Arterioscler Thromb Vasc Biol 1996;16:1007-12.

59. Koch AE, Kunkel SL, Pearce WH, Shah MR, Parikh D, Evanoff HL, et al. Enhanced production of the chemotactic cytokines interleukin-8 and monocyte chemoattractant protein-1 in human abdominal aortic aneurysms. Am J Pathol 1993;142:1423-31.

60. Boisvert WA, Santiago R, Curtiss LK, Terkeltaub RA. A leukocyte homologue of the IL-8 receptor CXCR-2 mediates the accumulation of macrophages in atherosclerotic lesions of LDL receptor-deficient mice. J Clin Invest 1998;101:353-63.

61. Bazan JF, Bacon KB, Hardiman G, Wang W, Soo K, Rossi D, et al. A new class of membrane-bound chemokine with a CX3C motif. Nature 1997;385:640-4.

62. Tsou CL, Haskell CA, Charo IF. Tumor necrosis factor-alpha-converting enzyme mediates the inducible cleavage of fractalkine. J Biol Chem 2001;276:44622-6.

63. Fong AM, Robinson LA, Steeber DA, Tedder TF, Yoshie O, Imai T, et al. Fractalkine and CX3CR1 mediate a novel mechanism of leukocyte capture, firm adhesion, and activation under physiologic flow. J Exp Med 1998;188:1413-9.

64. Haskell CA, Cleary MD, Charo IF. Molecular uncoupling of fractalkine-mediated cell adhesion and signal transduction. Rapid flow arrest of CX3CR1-expressing cells is independent of G-protein activation. J Biol Chem 1999;274:10053-8.

65. Price DT, Loscalzo J. Cellular adhesion molecules and atherogenesis. Am J Med 1999;107:85-97.

66. Springer TA. Adhesion receptors of the immune system. Nature 1990;346:425-34.

67. Johnson-Tidey RR, McGregor JL, Taylor PR, Poston RN. Increase in the adhesion molecule P-selectin in endothelium overlying atherosclerotic plaques. Coexpression with intercellular adhesion molecule-1. Am J Pathol 1994;144:952-61.

68. O'Brien KD, McDonald TO, Chait A, Allen MD, Alpers CE. Neovascular expression of E-selectin, intercellular adhesion molecule-1, and vascular cell adhesion molecule-1 in human atherosclerosis and their relation to intimal leukocyte content. Circulation 1996;93:672-82.

69. DeGraba TJ, Siren AL, Penix L, McCarron RM, Hargraves R, Sood S, et al. Increased endothelial expression of intercellular adhesion molecule-1 in symptomatic versus asymptomatic human carotid atherosclerotic plaque. Stroke 1998;29:1405-10.

70. Schumacher A, Liebers U, John M, Gerl V, Meyer M, Witt C, et al. P-selectin glycoprotein ligand-1 (PSGL-1) is up-regulated on leucocytes from patients with chronic obstructive pulmonary disease. Clin Exp Immunol 2005;142:370-6.

71. Sako D, Chang XJ, Barone KM, Vachino G, White HM, Shaw G, et al. Expression cloning of a functional glycoprotein ligand for P-selectin. Cell 1993;75:1179-86.

72. Norman KE, Katopodis AG, Thoma G, Kolbinger F, Hicks AE, Cotter MJ, et al. P-selectin glycoprotein ligand-1 supports rolling on E- and P-selectin *in vivo*. Blood 2000;96:3585-91.

73. Rinko LJ, Lawrence MB, Guilford WH. The molecular mechanics of P- and L-selectin lectin domains binding to PSGL-1. Biophys J 2004;86:544-54.

74. Hughes PE, Pfaff M. Integrin affinity modulation. Trends Cell Biol 1998;8:359-64.

75. Carman CV, Springer TA. Integrin avidity regulation: are changes in affinity and conformation underemphasized? Curr Opin Cell Biol 2003;15:547-56.

76. Chuluyan HE, Issekutz AC. VLA-4 integrin can mediate CD11/CD18-independent transendothelial migration of human monocytes. J Clin Invest 1993;92:2768-77.

77. Schenkel AR, Mamdouh Z, Muller WA. Locomotion of monocytes on endothelium is a critical step during extravasation. Nat Immunol 2004;5:393-400.

78. Muller WA, Weigl SA, Deng X, Phillips DM. PECAM-1 is required for transendothelial migration of leukocytes. J Exp Med 1993;178:449-60.

79. Patel SS, Thiagarajan R, Willerson JT, Yeh ET. Inhibition of alpha4 integrin and ICAM-1 markedly attenuate macrophage homing to atherosclerotic plaques in ApoE-deficient mice. Circulation 1998;97:75-81.

80. Schwartz SM, Heimark RL, Majesky MW. Developmental mechanisms underlying pathology of arteries. Physiol Rev 1990;70:1177-209.

81. Reidy MA, Fingerle J, Lindner V. Factors controlling the development of arterial lesions after injury. Circulation 1992;86:III43-III46.

82. Ross R. Growth regulatory mechanisms and formation of the lesions of atherosclerosis. Ann N Y Acad Sci 1995;748:1-4.

83. Viedt C, Vogel J, Athanasiou T, Shen W, Orth SR, Kubler W, et al. Monocyte chemoattractant protein-1 induces proliferation and interleukin-6 production in human smooth muscle cells by differential activation of nuclear factor-kappaB and activator protein-1. Arterioscler Thromb Vasc Biol 2002;22:914-20.

84. Plump AS, Smith JD, Hayek T, Aalto-Setala K, Walsh A, Verstuyft JG, et al. Severe hypercholesterolemia and atherosclerosis in apolipoprotein E-deficient mice created by homologous recombination in ES cells. Cell 1992;71:343-53.

85. Zhang SH, Reddick RL, Piedrahita JA, Maeda N. Spontaneous hypercholesterolemia and arterial lesions in mice lacking apolipoprotein E. Science 1992;258:468-71.

86. Nakashima Y, Plump AS, Raines EW, Breslow JL, Ross R. ApoE-deficient mice develop lesions of all phases of atherosclerosis throughout the arterial tree. Arterioscler Thromb 1994;14:133-40.

87. Wardell MR, Weisgraber KH, Havekes LM, Rall SC, Jr. Apolipoprotein E3-Leiden contains a seven-amino acid insertion that is a tandem repeat of residues 121-127. J Biol Chem 1989;264:21205-10.

88. van den Maagdenberg AM, Hofker MH, Krimpenfort PJ, de B, I, van Vlijmen B, van der BH, et al. Transgenic mice carrying the apolipoprotein E3-Leiden gene exhibit hyperlipoproteinemia. J Biol Chem 1993;268:10540-5.

89. van Vlijmen BJ, van den Maagdenberg AM, Gijbels MJ, van der BH, HogenEsch H, Frants RR, et al. Diet-induced hyperlipoproteinemia and atherosclerosis in apolipoprotein E3-Leiden transgenic mice. J Clin Invest 1994;93:1403-10.

90. Groot PH, van Vlijmen BJ, Benson GM, Hofker MH, Schiffelers R, Vidgeon-Hart M, et al. Quantitative assessment of aortic atherosclerosis in APOE*3 Leiden transgenic mice and its relationship to serum cholesterol exposure. Arterioscler Thromb Vasc Biol 1996;16:926-33.

91. Shishido T, Nozaki N, Yamaguchi S, Shibata Y, Nitobe J, Miyamoto T, et al. Toll-like receptor-2 modulates ventricular remodeling after myocardial infarction. Circulation 2003;108:2905-10.

92. Glagov S, Weisenberg E, Zarins CK, Stankunavicius R, Kolettis GJ. Compensatory enlargement of human atherosclerotic coronary arteries. N Engl J Med 1987;316:1371-5.

93. Pasterkamp G, Smits PC. Imaging of atherosclerosis. Remodelling of coronary arteries. J Cardiovasc Risk 2002;9:229-35.

94. Hollestelle SC, De Vries MR, Van Keulen JK, Schoneveld AH, Vink A, Strijder CF, et al. Toll-like receptor 4 is involved in outward arterial remodeling. Circulation 2004;109:393-8.

95. Vink A, Schoneveld AH, van der Meer JJ, van Middelaar BJ, Sluijter JP, Smeets MB, et al. *In vivo* evidence for a role of toll-like receptor 4 in the development of intimal lesions. Circulation 2002;106:1985-90.

96. Li A, Dubey S, Varney ML, Dave BJ, Singh RK. IL-8 directly enhanced endothelial cell survival, proliferation, and matrix metalloproteinases production and regulated angiogenesis. J Immunol 2003;170:3369-76.

97. Xie Z, Singh M, Singh K. Differential regulation of matrix metalloproteinase-2 and -9 expression and activity in adult rat cardiac fibroblasts in response to interleukin-1beta. J Biol Chem 2004;279:39513-9.

98. Pasterkamp G, Schoneveld AH, Hijnen DJ, de Kleijn DP, Teepen H, van der Wal AC, et al. Atherosclerotic arterial remodeling and the localization of macrophages and matrix metalloproteases 1, 2 and 9 in the human coronary artery. Atherosclerosis 2000;150:245-53.

99. Inohara N, Koseki T, del Peso L, Hu Y, Yee C, Chen S, et al. Nod1, an Apaf-1-like activator of caspase-9 and nuclear factor-kappaB. J Biol Chem 1999;274:14560-7.

100. Bertin J, Nir WJ, Fischer CM, Tayber OV, Errada PR, Grant JR, et al. Human CARD4 protein is a novel CED-4/Apaf-1 cell death family member that activates NF-kappaB. J Biol Chem 1999;274:12955-8.

101. Ogura Y, Inohara N, Benito A, Chen FF, Yamaoka S, Nunez G. Nod2, a Nod1/Apaf-1 family member that is restricted to monocytes and activates NF-kappaB. J Biol Chem 2001;276:4812-8.

102. Gutierrez O, Pipaon C, Inohara N, Fontalba A, Ogura Y, Prosper F, et al. Induction of Nod2 in myelomonocytic and intestinal epithelial cells via nuclear factor-kappa B activation. J Biol Chem 2002;277:41701-5.

103. Kwon HM, Sangiorgi G, Ritman EL, McKenna C, Holmes DR, Jr., Schwartz RS, et al. Enhanced coronary vasa vasorum neovascularization in experimental hypercholesterolemia. J Clin Invest 1998;101:1551-6.

104. Neutra MR, Mantis NJ, Frey A, Giannasca PJ. The composition and function of M cell apical membranes: implications for microbial pathogenesis. Semin Immunol 1999;11:171-81.

105. Neutra MR, Frey A, Kraehenbuhl JP. Epithelial M cells: gateways for mucosal infection and immunization. Cell 1996;86:345-8.

Summary

The immune system protects the host against invading microbial pathogens and plays an important role in development of atherosclerosis. Transmembrane Toll-like receptors (TLRs) can detect pathogen-associated molecular patterns (PAMPs) that are carried by infectious micro-organisms and therefore play an important role in optimal functioning of our immune system. The TLR2/6 heterodimer detects peptidoglycan (PG) and degraded PG fragments or PG motifs released during bacterial replication and infection are recognized by intracellular nucleotide-binding oligomerization domain (NOD) and Nalp receptors.

Chapter 1 is a general introduction about the process of atherosclerotic plaque formation and immunity and it described the immunologic properties of PG as well as the different receptors that can recognize PG in particular. Not only exogenous ligands are able to activate TLRs but they can also be stimulated by endogenous ligands that are mostly produced during stress and cell damage. ***Chapter 2*** is a review in which the role that TLRs and their exogenous and endogenous ligands play in innate immunity and in the development of atherosclerotic disease is described. Since not only inflammation but also infection has been shown to play an important role in development of cardiovascular disease, this chapter also discussed the role of infectious pathogens in atherosclerotic lesion development.
PG is able to induce antibody formation and IgM, IgG and IgA antibodies against PG can be detected in serum of healthy humans. In ***chapter 3*** we studied whether immune responses induced by PG are related to the atherosclerotic process by measuring systemic antibody levels against PG in patients with severe atherosclerotic disease. Patients with severe atherosclerosis had significantly lower IgM antibody levels against PG compared to control patients with increased cardiovascular risk but without clinically manifest disease. Furthermore, the IgM antibody level against PG was negatively associated with increased intima-media thickness and a risk score for cardiovascular disease. Based on the results of this clinical study, we postulated that systemic antibody levels against PG might be prognostic determinants for the occurrence of cardiovascular events after coronary angioplasty. In ***chapter 4*** we showed that the risk of a first cardiovascular event after angioplasty was not associated with IgM, IgG and IgA antibody levels against PG.
We demonstrated in ***chapter 5*** that the TLR2 is present on vascular adventitial fibroblasts and in human coronary arteries. TLR2 stimulation resulted in increased mRNA and protein expression of the pro-inflammatory cytokines and chemokines interleukin (IL)-1β, IL-Ra, IL-6, IL-8 and monocyte chemoattractant protein (MCP)-1 by adventitial fibroblasts. Since pro-inflammatory cytokines and chemokines play an important role in leukocyte recruitment, inflammation and immunity and above all are known to be involved in development of atherosclerosis, we also investigated whether stimulation of the TLR2 *in vivo* could promote development of atherosclerotic lesions. Adventitial application of $Pam_3Cys\text{-}SK_4$, a synthetic TLR2 ligand, increased intimal lesion formation induced by placement of a cuff around the femoral artery in wild type mice. These *in vivo* effects were TLR2-mediated since TLR2-/- mice showed

no significant increase in intimal hyperplasia formation after local $Pam_3Cys\text{-}SK_4$ application. In ApoE-/- mice that were put on a high cholesterol diet for 3 weeks prior to operation TLR2 stimulation resulted in dramatically increased atherosclerotic plaque formation.

Recruitment of leukocytes is involved in the early phase of atherosclerosis and is mediated by cellular adhesion molecules expressed by the vascular endothelial cells and the circulating leukocytes. In ***chapter 6*** we demonstrated that stimulation of monocytes isolated from human peripheral blood with both PG and $Pam_3Cys\text{-}SK_4$ resulted in significantly increased expression of the adhesion molecules CD18 and CD11b, whereas CD62L expression was significantly decreased. Since binding of the stimulated monocytes to ICAM-1 coated beads was significantly higher compared to control monocytes and since this monocyte binding to the fluorescent beads could be abrogated by monocyte incubation with a β_2-integrin blocking antibody, we concluded that stimulation of monocytes with PG and $Pam_3Cys\text{-}SK_4$ not only increased the expression but also the function of β_2-integrins. Firm adhesion of the monocytes to the endothelium was significantly increased after PG and $Pam_3Cys\text{-}SK_4$ stimulation due to the increased β_2-integrin expression on and increased L-selectin shedding from the monocyte surface. PG stimulation induced activation of the monocytes resulting in cell spreading over the endothelial surface and increased chemotaxis of the monocytes towards the chemoattractant C5a, which was TLR2-specific and totally β_2-integrin-dependent.

In ***chapter 7*** we demonstrated that isolated peritoneal macrophages stimulated with PG and $Pam_3Cys\text{-}SK_4$ *in vitro* were able to home to atherosclerotic plaques in ApoE-/- mice. In this chapter we also described that cuff-induced atherosclerotic lesions in ApoE*3Leiden transgenic mice that were put on a high fat/high cholesterol diet prior to operation were not only increased after PG stimulation, but the phenotype of these plaques was also significantly different from the atherosclerotic plaques at the contralateral control side. After stimulation the plaques were enriched in SMC and collagen, whereas macrophage content was not changed. In the media a switch in cellular content was evident; the number of α-SMC actin-positive cells was strongly reduced whereas the number of macrophages was clearly increased. Our results show that local PG application on the outside of the vascular wall induced a more stable plaque phenotype on the luminal side of the arterial wall.

The role of NOD proteins in atherosclerosis is as yet unexplored. In ***chapter 8*** we showed that NOD1 and NOD2 protein expression is significantly increased in atherosclerotic lesions. NOD1 expression levels were positively associated with collagen staining and the presence of SMC in human atherosclerotic lesions, suggesting that NOD1 expression is associated with a stable plaque phenotype. Furthermore, in the absence of SMC there is a significant correlation between NOD1 expression and macrophage content, indicating that NOD1 protein is also expressed within inflammatory areas inside atherosclerotic lesions, as we also demonstrated with immunohistochemistry for NOD2. Both NOD1 and NOD2 protein expression was inversely correlated with matrix metalloproteinase (MMP)-2 activity and positively correlated with MMP-8 and MMP-9 levels.

Chapter 9 is a general discussion of the results from the aforementioned studies.

Samenvatting

Het immuunsysteem beschermt de gastheer tegen binnendringende pathogenen en speelt een erg belangrijke rol bij het ontstaan van aderverkalking (atherosclerose). Membraangebonden Toll-like receptoren (TLR) kunnen pathogen-associated molecular patterns (PAMP) die aanwezig zijn op en in micro-organismen detecteren. TLR spelen daarom ook een belangrijke rol bij het optimaal functioneren van ons immuun systeem. De TLR2/6 heterodimeer detecteert o.a. het complete peptidoglycaan (PG) maar ook PG dat is afgebroken tot kleinere stukken of PG motieven die vrijkomen tijdens replicatie van bacteriën of bij infectie. Deze fragmenten worden herkend door intracellulaire receptoren; de nucleotide-binding oligomerization domain (NOD) en Nalp receptoren.

Hoofdstuk 1 is een algemene introductie waarin het proces van aderverkalking, immuniteit, de immunologische capaciteiten van PG en de verschillende receptoren die PG kunnen herkennen worden beschreven. Niet alleen exogene (lichaamsvreemde), maar ook endogene (lichaamseigen) liganden, die vooral geproduceerd worden tijdens celbeschadiging, kunnen TLR activeren. ***Hoofdstuk 2*** is een review waarin de rol van TLR en de exogene en endogene liganden in immuniteit en het ontstaan van aderverkalking wordt beschreven. En aangezien er is aangetoond dat niet alleen ontsteking maar ook bacteriële infecties een belangrijke rol spelen bij het ontstaan van hart- en vaatziekten, bediscussiëren we in dit hoofdstuk ook de relatie tussen infectie, ontsteking en de pathogenen die daar vaak bij betrokken zijn enerzijds en het ontstaan van atherosclerotische plaques anderzijds.
PG kan de productie van antilichamen induceren en IgM, IgG en IgA antilichamen tegen PG kunnen gemeten worden in het bloedserum van gezonde personen. In ***hoofdstuk 3*** staat beschreven dat patiënten met aderverkalking significant lagere IgM antilichaam niveaus tegen PG hadden vergeleken met controle patiënten met risico factoren, maar zonder klinische kenmerken van aderverkalking. Daarnaast was het IgM antilichaam niveau tegen PG negatief geassocieerd met een toename in intima-media dikte (een maat voor de hoeveelheid aderverkalking) en een risico score voor hart- en vaatziekten. Gebaseerd op de resultaten van deze klinische studie suggereerden we dat systemische antilichaam niveaus tegen PG prognostische (voorspellende) determinanten voor het opnieuw ontstaan van hart- en vaatziekten na coronair angioplastiek zouden kunnen zijn. In ***hoofdstuk 4*** hebben we laten zien dat het risico op een eerste nieuwe uiting van hart- en vaatziekten na angioplastiek niet geassocieerd is met IgM, IgG en IgA antilichaam niveaus tegen PG.
In ***hoofdstuk 5*** hebben we aangetoond dat de TLR2 aanwezig is op vasculaire adventitiële fibroblasten en in humane coronair arteriën. TLR2 stimulatie resulteerde in de toegenomen mRNA en eiwit expressie van cytokines en chemokines als interleukine (IL)-1β, IL-1Ra, IL-6, IL-8 en monocyte chemoattractant protein (MCP)-1 door adventitiële fibroblasten. Aangezien deze cytokines en chemokines een belangrijke rol spelen in witte bloedcel rekrutering, ontsteking en immuniteit en bovenal betrokken zijn bij het ontstaan aderverkalking, hebben we ook onderzocht of stimulatie van de TLR2 *in vivo* het ontstaan van aderverkalking zou kunnen beïnvloeden. Adventitiële toediening van $Pam_3Cys\text{-}SK_4$, een synthetisch TLR2 ligand, zorgde bij wild type muizen voor een

toename in intimale verdikking van de vaatwand, geïnduceerd door het plaatsen van een cuff om de femoraal arterie. Deze *in vivo* effecten waren TLR2-gemedieerd aangezien er in muizen met een defecte TLR2 geen significante toename in intima verdikking te zien was na lokale $Pam_3Cys\text{-}SK_4$ toediening. In ApoE knockout (atherosclerotische) muizen, die op een cholesterolrijk dieet geplaatst waren, resulteerde TLR2 stimulatie in een drastische toename in atherosclerotische plaque vorming.

Aantrekking van witte bloedcellen vindt plaats in de eerste fase van aderverkalking en cellulaire adhesie moleculen tot expressie gebracht door vasculaire endotheelcellen en circulerende witte bloedcellen zijn hierbij van groot belang. In ***hoofdstuk 6*** hebben we aangetoond dat stimulatie van monocyten, geïsoleerd uit humaan perifeer bloed, resulteerde in een toename in expressie van de adhesie moleculen CD18 en CD11b, terwijl CD62L expressie juist drastisch was afgenomen. Aangezien binding van de geïsoleerde monocyten aan ICAM-1 gecoate beads significant hoger was dan van controle monocyten en aangezien deze binding teniet gedaan kon worden met een β_2-integrine blokkerend antilichaam, concludeerden we dat stimulatie van monocyten met PG en $Pam_3Cys\text{-}SK_4$ niet alleen zorgde voor een toename in expressie maar ook in de functie van β_2-integrines. Door de toegenomen β_2-integrine expressie op en L-selektine shedding van het monocyt oppervlak, was de stevige binding van de monocyten aan endotheel significant toegenomen na PG stimulatie. Stimulatie met PG induceerde activatie van de monocyten, resulterende in celspreiding over het endotheeloppervlak en toegenomen chemotaxie (migratie) van de monocyten naar de chemoattractant C5a. De migratie van de monocyten was TLR2-specifiek en totaal β_2-integrine afhankelijk.

In ***hoofdstuk 7*** hebben we aangetoond dat geïsoleerde peritoneale macrofagen, *in vitro* gestimuleerd met PG en $Pam_3Cys\text{-}SK_4$, *in vivo* de capaciteit hadden om naar atherosclerotische plaques te homen. In dit hoofdstuk hebben we ook beschreven dat in ApoE*3Leiden transgene muizen de omvang van de atherosclerotische plaques niet alleen toegenomen was, maar ook dat het fenotype van de plaques significant verschillend was vergeleken met de plaques aan de contralaterale controle kant. Na stimulatie waren de plaques verrijkt met gladde spiercellen en collageen, terwijl de macrofaag hoeveelheid niet veranderd was. In de media was zelfs een duidelijke omschakeling in samenstelling van het weefsel te zien; het aantal gladde spiercellen was sterk gedaald terwijl het aantal macrofagen juist duidelijk was toegenomen. Onze resultaten tonen aan dat lokale PG toediening aan de buitenkant van de vaatwand een stabieler plaque fenotype aan de luminale kant van de arterie induceert.

De rol die NOD eiwitten spelen in hart- en vaatziekten en aderverkalking is tot nu toe nog niet onderzocht. In ***hoofdstuk 8*** hebben we laten zien dat NOD1 en NOD2 eiwit expressie significant was toegenomen in atherosclerotische plaques. De NOD1 eiwit expressie niveaus waren positief geassocieerd met een collageen-kleuring en met de aanwezigheid van gladde spiercellen in de humane plaques. Dit suggereert dat NOD1 expressie geassocieerd is met een stabiel plaque fenotype. Daarnaast bleek echter dat er in de afwezigheid van gladde spiercellen een significante correlatie is tussen NOD1 expressie en macrofaag hoeveelheid, aangevende dat NOD1 eiwit ook tot expressie komt in de ontstekingsgebieden in de plaques, zoals we ook aangetoond hadden voor NOD2 expressie door middel van immunohistochemie. Zowel NOD1 als NOD2 eiwit expressie was negatief gerelateerd met matrix metalloproteinase (MMP)-2 activiteit en

positief geassocieerd met MMP-8 en MMP-9 niveaus.

Hoofdstuk 9 is een algemene discussie over de resultaten van de voorgaande studies.

Color Supplement

Chapter 7

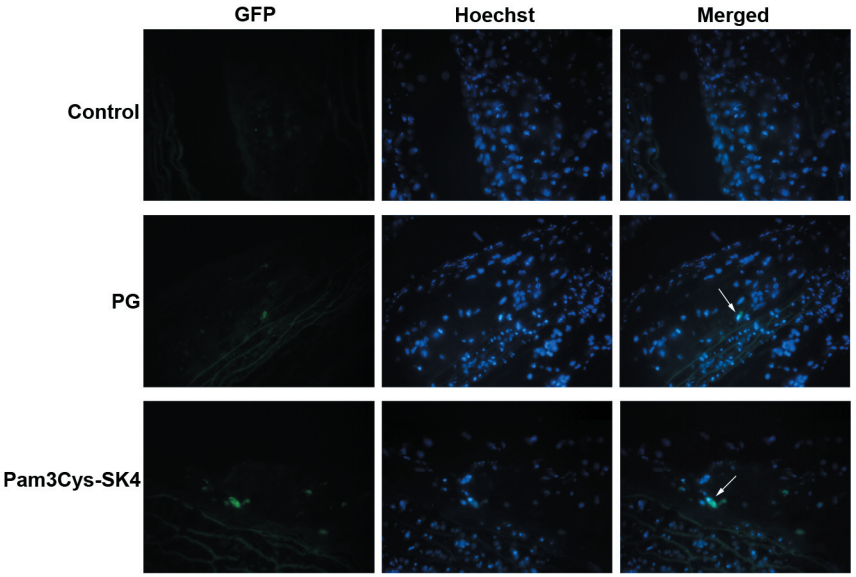

Figure 7. PG and Pam_3Cys-SK_4 increase macrophage homing into atherosclerotic plaques
The ability of macrophages to migrate into the atherosclerotic plaque upon PG and Pam_3Cys-SK_4 stimulation was investigated in ApoE-/- mice. Control GFP-macrophages could hardly be found in the atherosclerotic plaques (only a few cells in the whole aortic arch), whereas macrophages that were stimulated with either PG or Pam_3Cys-SK_4 were found easier (a few macrophages in each atherosclerotic plaque within the aortic arch). The left panel shows GFP cells in the arterial wall, the middle panel shows Hoechst nuclear staining and the right panel shows the merged picture.

Chapter 8

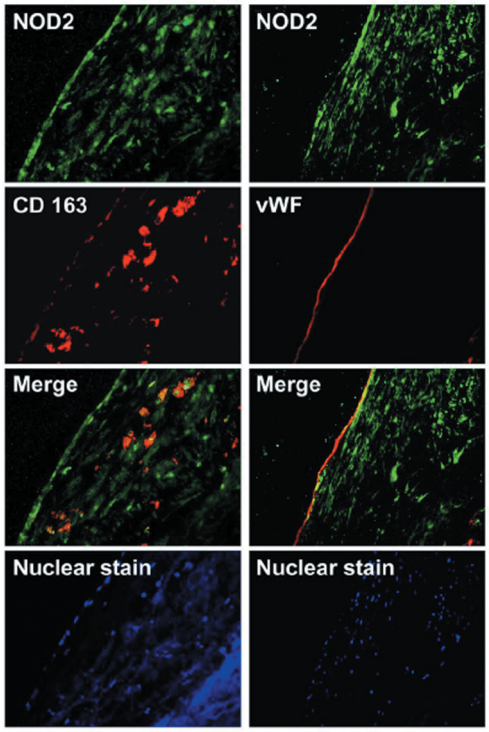

Figure 6. NOD2 is expressed by macrophages and endothelial cells in atherosclerotic plaques
Double immunofluorescent staining for NOD2 (green) and cell markers (red) on atherosclerotic lesions. Cells were stained for CD163 (macrophages) and von Willebrand Factor (endothelial cells). Double-positive cells are yellow in the overlay panel. Cell nuclei are stained blue with DAPI. Original magnification: left panel 400x, right panel 200x.

Dankwoord

Mijn proefschrift is klaar! Vier jaren zwoegen in het lab en een aantal maanden ploeteren met schrijven, verbeteren en lay-outen van dit proefschrift liggen nu achter me. Natuurlijk hebben veel mensen in de afgelopen jaren een grote bijdrage geleverd aan de totstandkoming van dit proefschrift. Zonder jullie enthousiasme, betrokkenheid en vaak goede adviezen was dit boekje er nu niet geweest; BEDANKT!! Een aantal mensen wil ik echter nog even in het bijzonder bedanken.

Ten eerste gaat mijn dank natuurlijk uit naar mijn promotoren;
Gerard, bedankt voor alle steun en vertrouwen de afgelopen jaren. Je bent altijd enthousiast en positief ingesteld waardoor je er vaak in slaagde me te stimuleren toch door te gaan als het weer eens tegenzat met bepaalde experimenten of met een artikel. Wanneer ik ook binnenviel, je nam altijd de tijd om mijn werk door te spreken. Ook wil ik je heel erg bedanken voor de mogelijkheid die je me geboden hebt om naast mijn promotie met de master-opleiding “Clinical Epidemiology” te beginnen. Die opleiding heb ik bijna afgerond en het heeft me doen inzien dat, hoezeer ik ook genoten heb van de afgelopen 4 jaren in het lab, ik toch meer betrokken wil zijn bij de uitvoering van patiëntenstudies. Daarnaast ben ik er natuurlijk trots op je eerste promovenda te zijn!!
Jon, hoewel wat meer op afstand, was jij ook steeds bereikbaar voor hulp. Jij hebt altijd oog voor zowel de grote lijnen als details. Bedankt voor je kritische kijk op mijn artikelen en dit proefschrift; daardoor is het zeker beter geworden! Daarnaast ben je gewoon een lopende encyclopedie, of hoe je het ook maar moet noemen, op het gebied van de wetenschap. Als we het over artikelen hebben weet je zo auteur, tijdschrift, jaar van publicatie en inhoud op te ratelen… elke keer verbaas ik me er weer over! Meerdere keren heeft dat me een aantal interessante artikelen opgeleverd, dus bedankt daarvoor.

Natuurlijk wil ik ook mijn co-promotor bedanken. Dominique, jij bent in mijn ogen een “echte” onderzoeker. Bedankt voor je ideeën en je kritische kijk op de vasculaire biologie. De laatste maanden hebben we elkaar weinig gesproken omdat je in Singapore zit, maar ik ben je erg dankbaar voor je belangrijke input in mijn studies en voor je snelle reacties als ik je per email weer een manuscript ter beoordeling toestuurde.

Werk is niet alleen leuk vanwege de werkzaamheden op het lab, maar zeker ook vanwege de geweldige collega´s. Ik wil bij deze dan ook al mijn collega´s bedanken voor hun interesse in mijn onderzoek, de gezellige lunches en de praatjes op de gang en in het lab.

Mijn kamergenoten. Ik heb genoten van de levendige en gezellige sfeer (soms bijna iets te gezellig??). Dik en Karlijn, succes met het afronden van jullie promoties en ook al zagen jullie op tegen de stapel papier die ik uitprintte voor de beoordelings-commissie… toch weet ik zeker dat ook jullie achteraf zullen zeggen dat het niet zo erg is als het lijkt. Karlijn, jammer dat je niet bij mijn promotie kunt zijn. Ik wens je veel

plezier en succes toe in Singapore (niet alleen werken hè, je moet ook genieten).
Juan, good luck with finalising your experiments and papers. Thank you for your ideas and all the discussions we had during the last four years. I hope your return to Spain will be fruitful.

Arjan, bedankt dat je altijd weer klaar stond als ik een vraag had over experimenten of als er weer iets uitgezocht moest worden over chemicaliën, antilichamen of andere materialen voor in het lab. Met je ervaringen en deskundigheid ben je een erg belangrijke spil in het lab. Zorg je ervoor dat het lab een prettige en overzichtelijke plek blijft om in te werken?

Ben, ik ben erg blij met je hulp bij het uitvoeren van de muizenoperaties. Jij bent als "muizen-dokter" van groot belang geweest bij een aantal van de geweldige resultaten die ik in de afgelopen vier jaren behaald heb. Miranda wil ik bij deze ook even bedanken voor haar hulp bij een aantal experimenten toen jij op werkbezoek was in Amerika.

Astrid en Ineke, heel erg bedankt voor de gezelligheid op het secretariaat. Ik heb bewondering voor de manier waarop jullie de organisatie draaiende weten te houden en er altijd weer in slagen om jullie door de chaos heen te werken die op het bureau gestort wordt. Astrid, we blijven contact houden hoor... ik blijf gewoon naar de spelletjesavonden komen.

Imo, Sebastian en Stephan, sinds jullie komst wordt er naast Nederlands en Engels ook af en toe Duits gesproken op het lab. Daarnaast hebben jullie je ook de Nederlandse taal ontzettend snel eigen gemaakt. Ik weet niet of alle Duitsers zo gemakkelijk Nederlands kunnen leren, maar ik heb toch bewondering voor jullie bijna vlekkeloze aanpassing aan onze taal. Succes met jullie onderzoeken.

Marie-José, Anke, Corina, Joost, Piet, Sander en Simone; de stamcelgroep. Ik heb de afgelopen vier jaren veel veranderingen meegemaakt in het lab. Uitbreiding van het lab met de komst van een hele nieuwe stamcel-onderzoekslijn is zeker één van de positieve veranderingen. Naast een heleboel kennis brachten jullie ook een hoop gezelligheid met jullie mee. Bedankt daarvoor.

Chaylendra, Els, Evelyn, Leo, Pieter en Willem, bedankt voor de gezelligheid op het lab en voor alle hulp bij het snijden van coupes, het bij elkaar zoeken van eiwit en RNA van Athero-Express, het opzetten van protocollen, etc, etc.

Dennis, hoe gaat het nu in Nijmegen? Het was fijn je als "lab-buurman" te hebben en lekker met je te kunnen ouwehoeren tijdens het werk. Gelukkig vertrok je pas naar Nijmegen toen mijn praktische werk erop zat en ik al aan het schrijven was. Af en toe mis ik het schaap en het vrolijke roodkapje-liedje eigenlijk wel…

De MICABG groep wil ik graag bedanken voor de gezellige uitjes, borrels, etentjes en labstapdagen.

Nienke, bedankt voor de enthousiaste bijdrage die je als studente aan mijn onderzoek hebt geleverd; vooral bij het opzetten van de rol-, adhesie-, en migratie-experimenten. Ik ben erg benieuwd of je besloten hebt om AIO te worden of dat je toch iets heel anders gaat doen.

Laurien, jou wil ik natuurlijk bedanken voor je hulp bij hoofdstuk 6 van dit proefschrift. Het rollen en migreren bleef altijd leuk om te doen… Ik ben trots op "ons" hoofdstuk. Nu wordt het echter eindelijk eens tijd dat de tijdschriften het daarmee eens zijn en het artikel accepteren. Longziekten, jullie wil ik bij deze natuurlijk bedanken voor de mogelijkheid om bij jullie de rol- en migratie-experimenten uit te voeren en voor jullie interesse in mijn onderzoek, bereidheid te helpen bij experimenten en het afstaan van wat bloed, zo nu en dan.

Ik wil ook de vakgroep Immunologie uit het Erasmus MC bedanken voor hun gastvrijheid. Marie-José Melief, bedankt voor je ervaring met het PG onderzoek en dan vooral met de ELISA´s. Je efficiënte manier van werken en bereidheid experimenten voor mij voor te bereiden hebben me veel tijd bespaard.

Yolanda van der Graaf, bedankt voor je hulp bij de twee patiënten-studies die ik heb gedaan binnen SMART en natuurlijk ook voor je kritische reacties op deze studies. Verder vond ik het erg fijn dat ik altijd even bij je langs kon komen als ik een vraag had over klinische studies of als er weer eens iets geregeld moest worden voor de master Clinical Epidemiology.

Kristina Edfeldt and Zhong-Qun Yan, thank you for the collaboration on the NOD-part of my thesis. Our paper is not finished yet and some more experiments need to be done, but let's try to make it a good paper suitable for publication.

Margreet de Vries en Paul Quax, bedankt dat jullie de operaties en terminaties van de ApoE*3Leiden transgene muizen voor me gedaan hebben.

Vasculaire geneeskunde en Nefrologie, bedankt voor jullie opbouwende kritiek tijdens de gezamenlijke werkbesprekingen en voor de gezelligheid op het lab.

Natuurlijk wil ik het ICIN en dan in het bijzonder Manja Helmers bedanken voor het supersnel regelen van allerlei zaken.

Monique, al heel snel wist ik dat ik jou als paranimf wilde vragen en ik vind het geweldig dat je daar volmondig ja op zei. Ik ben heel blij jou al bijna 12 jaar als vriendin te mogen hebben. Bedankt voor je steun en interesse in alles wat ik doe en voor de goede gesprekken die we in al die jaren vaak gevoerd hebben. Ik heb je de laatste maanden veel te weinig gezien; ik vind dat daar weer verandering in moet komen! Succes met je eigen carrière en ik hoop dat ik ooit eens een apotheek binnen zal lopen waar jij de beherend apotheker bent.

Mijn vrienden dank ik voor wat ze zijn, vrienden. Beste Arjan en Anke, Christiaan en Marieke, Giel en Marja, Dick en natuurlijk Joyce, bedankt voor jullie steun, gezelschap en vooral het slappe geouwehoer waar we met z'n allen heel goed in zijn. Ik ken jullie nu al heel lang en ik ben erg blij dat we er in geslaagd zijn contact te blijven houden sinds de middelbare school ook al wonen we verspreid over (sinds kort weer) heel Nederland. De afgelopen tijd heb ik het ontzettend druk gehad en hebben we elkaar niet zo veel gezien, maar nu heb ik weer tijd om een weekendje met z´n allen weg te gaan, dus wanneer gaan we?

Verder wil ik nog alle familie en andere vrienden bedanken voor jullie interesse en steun. Ik weet niet of ik altijd goed heb kunnen uitleggen waar ik mee bezig was, maar dit is in elk geval het eindresultaat. Ik heb nog wel een tip voor jullie; lees alleen de Nederlandse samenvatting en dit dankwoord en leg dan vooral het proefschrift snel weg…

Jo en Marian, Robert en Erna, jullie wil ik bedanken voor het warme welkom in jullie gezin en natuurlijk ook voor jullie steun en interesse in mijn onderzoek.

Lieve pap en mam, ik ben jullie zeer dankbaar voor jullie onvoorwaardelijke steun. En ook nu ik heb besloten niet als postdoc in het onderzoekswereldje verder te gaan maar een andere weg in te slaan, weet ik dat jullie toch weer achter mijn beslissing staan en alle vertrouwen in me hebben. Ik hoop dat jullie beseffen hoezeer ik dat weet te waarderen. Dit is het resultaat van vier jaar hard werken… ik hoop dat jullie net zo trots zijn als ikzelf ben, want dit boekje heb ik zeker ook aan jullie te danken!

Lieve Niels, ik ben heel blij dat je als paranimf mij terzijde wil staan bij dit voor mij belangrijke moment. Bedankt voor je hulp bij het verzorgen van de lay-out van dit proefschrift; het ziet er echt super uit!! Bij deze wil ik je dus even duidelijk maken dat ik je een geweldige broer vind.

Lieve Bram, jouw pad kruiste het mijne en daar ben ik echt ontzettend blij mee. Ik wil jou heel erg bedanken voor je liefde en steun en voor het vele geduld dat je opbracht gedurende de afgelopen drukke, en zeker niet altijd eenvoudige, periode. Jij weet nu wat je te wachten staat en over 6 maanden staan we gewoon weer in het Academiegebouw om jouw promotie te vieren. Een dikke knuffel!

Manon

Curriculum Vitae

Ik ben geboren op 31 maart 1979 te Almelo en behaalde in 1997 het VWO diploma aan het Florens Radewijns College te Raalte. In oktober van datzelfde jaar begon ik met de studie Medische Biologie aan de Universiteit Utrecht. Tijdens de studie heb ik twee onderzoeksstages gelopen. De bijvakstage werd uitgevoerd bij de afdeling Medische Farmacologie van het Rudolf Magnus Instituut voor Neurowetenschappen in het Universitair Medisch Centrum Utrecht onder begeleiding van Dr. Patrick J. Kamphuis en Prof. Dr. Victor M. Wiegant. De hoofdvakstage heb ik gelopen bij de vakgroep Experimentele Cardiologie in het Universitair Medisch Centrum Utrecht onder begeleiding van Dr. Marion J. Sierevogel en Prof. Dr. Gerard Pasterkamp. Het doctoraalexamen Medische Biologie heb ik behaald in februari 2002. In maart 2002 begon ik aan mijn onderzoeksproject als onderzoeker in opleiding bij de afdeling Experimentele Cardiologie van het Universitair Medisch Centrum Utrecht onder begeleiding van Prof. Dr. Gerard Pasterkamp en Prof. Dr. Jon D. Laman. Dit onderzoek heeft uiteindelijk geresulteerd in dit proefschrift. Daarnaast ben ik in 2004 ook nog begonnen met de master-opleiding Clinical Epidemiology die in juni 2006 afgerond zal worden.

Publications

Matrix metalloproteinase inhibition reduces adventitial thickening and collagen accumulation following balloon dilation.
M.J. Sierevogel, E. Velema, F.J. van der Meer, **M.M. Oude Nijhuis**, M. Smeets, D.P.V. de Kleijn, C. Borst, and G. Pasterkamp.
Cardiovascular Research 2002;55(4):864-869

IgM antibody level against pro-inflammatory bacterial peptidoglycan is inversely correlated with extent of atherosclerotic disease
M.M. Oude Nijhuis, Y. van der Graaf, M.J. Melief, AH SChoneveld, D.P.V. de Kleijn, J.D. Laman and G. Pasterkamp
Atherosclerosis, 2004; 173(2): 245-251

Toll-like receptor 2 stimulation induces intimal hyperplasia and atherosclerotic lesion development
A.H. Schoneveld, **M.M. Oude Nijhuis**, B. van Middelaar, J.D. Laman, D.P.V. de Kleijn, and G. Pasterkamp
Cardiovascular Research, 2005; 66(1): 162-169

Exogenous and endogenous ligands triggering the innate immune system and atherosclerotic disease
M.M. Oude Nijhuis, J.K. van Keulen, G. Pasterkamp, P. Quax and D.P.V. de Kleijn
Current Pharmaceutical Design, accepted for publication

Peptidoglycan increases firm adhesion of monocytes to endothelial cells under flow and primes monocyte chemotaxis
M.M. Oude Nijhuis, G. Pasterkamp, N.I. Sluis, D.P.V. de Kleijn, J.D. Laman and L.H. Ulfman
Submitted

Assessment of the prognostic value of immunoglobulin levels against bacterial peptidoglycan in patients undergoing coronary angioplasty
M.M. Oude Nijhuis, Y. van der Graaf, J.D. Laman, P.R. Stella, D.P.V. de Kleijn, D.E. Grobbee and G. Pasterkamp
Submitted