Medical Faculty of the Martin-Luther-Universität Halle-Wittenberg

Statin retains the capacity of monocyte-derived macrophages to respond to endotoxin via the geranylgeranylation-pathway

Dissertation
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Medical Physiology and Pathophysiology

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Born on the 19.01.1987 in Chongqing, PR China

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Medizinische Fakultät der Martin-Luther-Universität Halle-Wittenberg

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Summary report

Atherosclerosis, which causes high mortality in the western world, has been identified as a chronic inflammatory disease. Interaction of smooth muscle cells (SMC) with monocytes or macrophages may contribute to the progression of atherosclerosis. Previous findings demonstrated that IL-1-mediated interaction of SMC and monocytes in a coculture model resulted in synergistic IL-6-production in LPS-stimulated cocultures. In the present study, we found that IL-1 also contributed to the synergistic effect in 25-hydroxycholesterol-(Chol)-stimulated cocultures. Trans-well insert experiments suggested that SMC are the major source of IL-6, whereas monocytes are the major source of IL-1ß. This suggestion was further supported by time-course and Western blot analyses. After the invasion of monocytes into the vessel wall, monocytes may differentiate into macrophages, which may importantly contribute to atherogenesis. Thus, we also evaluated the capacity of macrophages to produce IL-1. Since monocytes and macrophages only produced little IL-6 or IL-1ß upon Chol-stimulation in our system, we mainly focused on the capacity of these cells to respond to endotoxin. In contrast to the monocytes, macrophages did not respond to endotoxin. However, statin-pretreatment retained the capacity of macrophages to respond to endotoxin. Addition of intermediates of the isoprenoid pathway, but not of the cholesterol-synthesis pathway, blocked the retainment. Similar to statin, geranylgeranyl transferase-inhibitor provided the retainment, whereas farnesyl transferase-inhibitor and zaragozic acid-A did not. The retainment-effect of statin was blocked by geranylgeranyl pyrophosphates and this blockade was reversed by geranylgeranyl transferase-inhibitor. Inhibition of Rac1-activation or its downstream activators PI3K- and p38-MAPK-activation blocked the cytokine expression in the retainment. In line with the above, statin-preincubation enhanced the activation of Rac1. Taken together, the data suggest that in SMC-monocyte interaction, monocytes may produce IL-1ß and that IL-1ß further activates SMC to produce IL-6. Macrophages do not have this capacity, since they do not produce much IL-1. However, statin-treatment retains the capacity of the macrophages to respond to endotoxin. This appears to be regulated by the activation of Rac1. Since statin has been widely used in the treatment for cardiovascular diseases, this finding may be of potential relevance for the comprehension of the role of statins in cardiovascular diseases.

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Referat

Arteriosklerose, wurde als Erkrankung mit chronischer Inflammation identifiziert, welche eine hohe Mortalität in der westlichen Welt verursacht. Die Interaktion von Gefäßmuskelzellen (SMC) mit Monozyten oder Makrophagen könnte zum Fortschreiten der Erkrankung beitragen. Bisherige Ergebnisse zeigten, dass die IL-1-vermittelte Interaktion von SMC und Monozyten in einem Kokulturmodel eine synergetische IL-6 Produktion in LPS-stimulierten Kokulturen hervorruft. In der vorliegenden Studie fanden wir heraus, dass IL-1 auch zu den synergetischen Effekten in 25-Hydroxycholesterol-(Chol)-stimulierten Kokulturen beiträgt. Transwell insert-Experimente deuteten darauf hin, dass SMC die Produzenten von IL-6 sind, wohingegen Monozyten für die IL-1β Produktion verantwortlich sind. Dieses Ergebnis konnte durch Zeitverlaufs- und Western-Blot Analysen bestätigt werden. Nach der Invasion von Monozyten in Blutgefäßwände können diese zu Makrophagen differenzieren, welche zur Atherogenese beitragen könnten. Zudem bewerteten wir das Potential von Makrophagen zur Produktion von IL-1. Da Monozyten und Makrophagen durch die Chol-Stimulation nur geringe Mengen von IL-6 oder IL-1β produzierten, fokussierten wir uns auf die Fähigkeit dieser Zellen auf Endotoxine zu reagieren. So reagierten Makrophagen, im Gegensatz zu Monozyten, nicht auf Endotoxine. Eine Vorbehandlung mit Statin hielt die Fähigkeit von Magrophagen, auf Endotoxine zu reagieren, aufrecht. Die Zugabe von Bestandteilen des Isoprenoid-Signalweges, aber nicht des Cholesterol-Synthese-Signalwegs, konnte die Aufrechterhaltung blockieren. Ähnlich wie Statin, führte auch die Inhibition von Geranylgeranlytransferase zur Aufrechterhaltung der Fähigkeit, während der Farnesyltransferase-Inhibitor und Saragosinsäure-A dies nicht taten. Der aufrechterhaltende Effekt von Statin wurde durch Geranylgeranylpyrophosphate blockiert und diese Blockade durch den Geranylgeranyltransferase-Inhibitor aufgehoben. Die Hemmung der Rac1-Aktivierung oder ihrer nachgeschalteten Aktivatoren PI3K- und p38-MAPK-Aktivierung blockierte die Zytokin-Expression in der Aufrechterhaltung. Damit im Einklang steht die verbesserte Aktivierung von Rac1 durch eine Statin-Vorinkubation. Zusammenfassend deuten die Daten darauf hin, dass Monozyten in der SMC-Monozyten-Interaktion IL-1ß produzieren können und dass IL-1ß SMCs weiter aktiviert, um IL-6 zu produzieren. Makrophagen haben diese Fähigkeit nicht, da sie nicht viel IL-1 produzieren. Die Statin-Behandlung hält die Fähigkeit der Makrophagen auf Endotoxin zu reagieren, jedoch aufrecht. Dies scheint durch die Aktivierung von Rac1 reguliert zu sein. Da Statin in der Behandlung von Herz-Kreislauf-Erkrankungen weit verbreitet ist, kann dieses Ergebnis für das Verständnis der Rolle von Statinen bei Herz-Kreislauf-Erkrankungen von potenzieller Bedeutung sein.

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Contents:

Abbreviations

ApoE	Apolipoprotein-E
ASC	Apoptosis-associated speck-like protein containing a CARD
Chol	25-Hydroxycholesterol
Co	Coculture
Co_{Mo}	Monocyte-compartment of the coculture
Co_{SMC}	SMC-compartment of the coculture
EC	Endothelial cell
FPP	Farnesyl pyrophosphate
FTI	Farnesyl transferase-inhibitor
GAP	GTPase-activating protein
GDI	Guanine nucleotide dissociation-inhibitor
GEF	Guanine nucleotide exchange factor
GGPP	Geranylgeranyl pyrophosphate
GGTI	Geranylgeranyl transferase-inhibitor
HMG-CoA	3-Hydroxy-3-methylglutaryl-coenzyme-A
Mac	Macrophage
Mev	Mevalonic acid
MNC	Mononuclear cells
Mo	Monocyte
NLRP3	NACHT, LRR and PYD domains containing protein-3
SMC	Smooth muscle cell
ZAA	Zaragozic acid-A

1 Introduction

1.1 Atherosclerosis

Atherosclerosis is considered to be the most common cause of cardiovascular diseases which have the highest mortality in Western countries. The pathogenesis of atherosclerosis is not fully understood, however, inflammatory pathways are suggested to play a central role in the initiation and progression of atherosclerosis (Hansson and Libby, 2006; Ross, 1999). Atherosclerosis may initiate with the activation of endothelial cells (Figure 1). Activated endothelial cells express chemokines and adhesion molecules. The chemokines promote invasion of leukocytes, such as monocytes, into the arterial wall by directional induction. The adhesion molecules trigger the adhesion of leukocytes to the endothelial cells. Thereafter, the leukocytes migrate into the vessel wall intima. Meanwhile, the endothelial activation induces the migration of smooth muscle cells (SMC) from media to intima. There, the inflammatory response of leukocytes and local vessel wall cells may be induced by various activators, such as endotoxin, cholesterol and cytokines. Interaction between these cells, which may be regulated by direct cell-cell contact or by soluble mediators, may importantly contribute to the development of atherosclerosis.

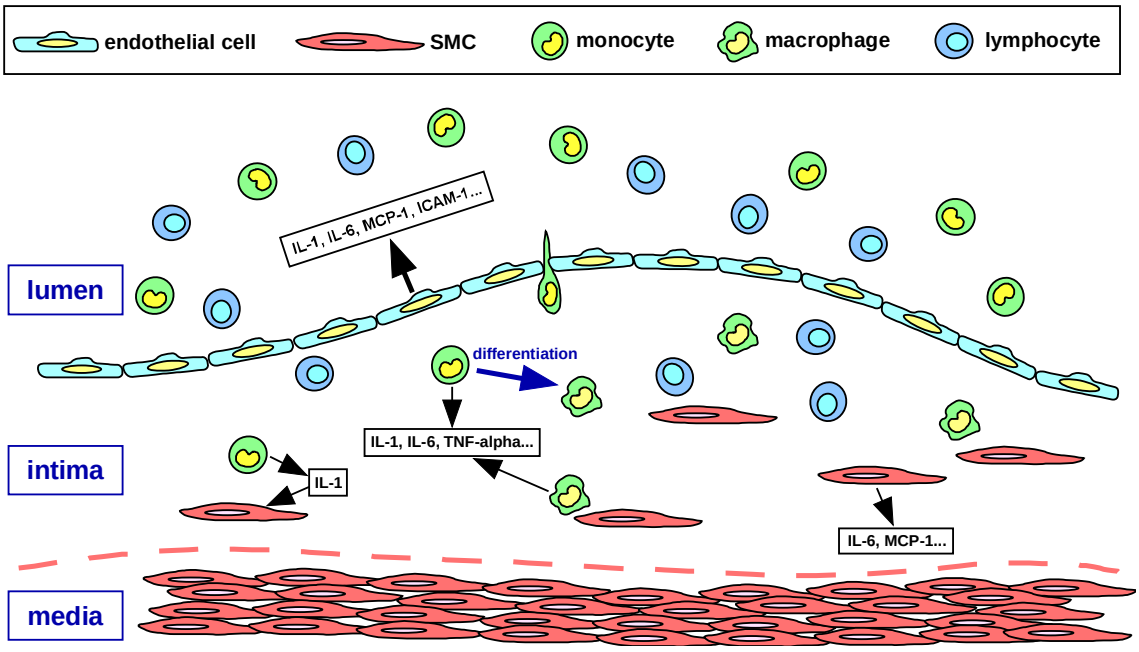

Figure 1. The early phase of atherosclerosis. Various cell types are involved in atherogenesis, including endothelial cells, smooth muscle cells (SMC), monocytes, macrophages and lymphocytes. Cytokines expressed by these cells contribute to the initiation and progression of atherosclerosis.

1.2 The inflammatory mediators IL-6 and IL-1

In the inflammatory pathways involved in atherosclerosis, cytokines are considered to be central regulators (Loppnow et al., 2008; Loppnow et al., 2011a; Tedgui and Mallat, 2006). Cytokines are small signaling protein molecules, which are produced by various cells and have specific effects on the behavior of cells or communication between cells. In the process of atherogenesis, many cytokines such as IL-6 and IL-1 are thought to regulate inflammatory pathways. Many cells which are involved in atherosclerosis may produce such cytokines, including endothelial cells (Loppnow and Libby, 1989b; Miossec et al., 1986; Sironi et al., 1989), SMC (Loppnow and Libby, 1990) and monocytes (Boutten et al., 1992; Navarro et al., 1989).

IL-6 is a pleiotropic cytokine which regulates various aspects of the immune response. The molecular weight of human IL-6 is 21 – 28 KDa depending on post-translational modification, such as glycosylation and phosphorylation (Keller et al., 1996). The expression of IL-6 has been detected in atherosclerotic lesions in human tissues (Rus et al., 1996; Schieffer et al., 2000), as well as in animal tissues (Ikeda et al., 1992; Sukovich et al., 1998). Increasing IL-6-levels have been found to be associated with an increasing risk of coronary heart disease (Danesh et al., 2008). Further evidence for a contribution of IL-6 to atherogenesis was suggested by experiments with male mice, fed normal or high fat diets, which were additionally treated with recombinant IL-6 for 6 - 21 weeks (Huber et al., 1999). In these experiments, the lesion size in ApoE-deficient mice was significantly enhanced by the IL-6-treatment, as compared to lesions in saline-treated mice. In other mice studies, however, the IL-6 was proposed to have an atheroprotecive role in atherosclerotic lesion development (Madan et al., 2008). In *in vitro* experiments, it has been identified that IL-6 is produced by various cell types, including endothelial cells (Loppnow and Libby, 1989a), SMC (Loppnow and Libby, 1990), monocytes (Tosato and Jones, 1990) and macrophages (Jian et al., 1995). IL-6 may impact the development of atherosclerosis by various aspects. IL-6 may upregulate the expression of chemokines and adhesion molecules, which may promote the recruitment of leukocytes from blood to vessel wall, in endothelial cells or SMC (Klouche et al., 1999; Romano et al., 1997). Moreover, IL-6 can induce monocyte differentiation into macrophages (Chomarat et al., 2000). Furthermore, it has been shown that IL-6 induces the proliferation and migration of SMCs (Ikeda et al., 1991; Wang and Newman, 2003). However, IL-6 does not only show pro-inflammatory effects, some of the research have revealed that IL-6 has anti-inflammatory capacities through its inhibitory effects on TNF-α and IL-1, and activation of IL-1ra and IL-10 (Steensberg et al., 2003; Tilg et al., 1994; Xing et al., 1998). Taken together, IL-6 contributes progression of atherosclerosis in different stages with pro- or anti-inflammatory effects.

IL-1 is a central mediator in the cytokine network mediating various immune responses. IL-1 has two isoforms, IL-1α and IL-1ß. Both isoforms are first synthesized as 31-kDa

precursor protein, but only the IL-1α-precursor is active (March et al., 1985) whereas the IL-1ß-precursor lacks biologic activity (Black et al., 1988). The mature IL-1α-protein is processed from its precursor by calpain cleavage (Watanabe and Kobayashi, 1994), whereas mature IL-1ß-protein is produced from its precursor by caspase-1 (Thornberry et al., 1992). However, besides caspase-1, other proteases have been found to have the capacity to cleave the IL-1ß-precursor, such as trypsin, plasmin (Matsushima et al., 1986) and caspase-8 (Maelfait et al., 2008). Both IL-1α and IL-1ß bind to same membrane receptor (IL-1RI) to trigger signal pathways. Interestingly, besides these two isoforms, there is a third ligand – the IL-1 receptor antagonist (IL-1Ra), which binds to the IL-1RI, but does not activate downstream signaling and is, therefore, a natural inhibitor of IL-1 activity (Weber et al., 2010). IL-1 is expressed by many cells which are involved in atherosclerosis, such as endothelial cells, monocytes or SMCs (Moyer et al., 1991; Tipping and Hancock, 1993), but in SMCs, no active IL-1ß is processed (Schönbeck et al., 1997). In atherosclerosis, the pro-atherogenic effect of IL-1 is attributed to its ability to modulate various important events involved in the complex inflammatory process. In early steps of atherosclerosis, IL-1 may activate the expression of adhesion molecules on vascular cells, such as intercellular adhesion molecule-1, endothelial leukocyte adhesion molecule-1 and vascular cell adhesion molecule-1 (Bochner et al., 1991; Braun et al., 1995). Furthermore, production of chemokines which induce leukocyte recruitment into the vessel wall is also regulated by IL-1 (Wang et al., 1991). IL-1 also induces different cytokines in various cell types, such as tumor necrosis factor-alpha (TNF-α) (Ikejima et al., 1990), granulocyte colony-stimulating factor (G-CSF) (Zoellner et al., 1992) and IL-6 (Loppnow et al., 1998). More direct evidence for the pro-atherogenic effect of IL-1 was provided by animal studies, in which significant reduction of the lesion size in IL-1ß / ApoE double-deficient mice was observed, as compared with IL-1ß expressing ApoE deficient mice (Kirii et al., 2003). Another strong evidence for the role of IL-1 in atherogenesis comes from the investigation of IL-1RI. IL-1 must bind IL-1RI to activate the signal pathway, absence of IL-1RI markedly reduced the lesion size in ApoE+/- mice, both in uninfected mice and in *P. gingivalis*-infected mice (Chi et al., 2004). On the other hand, some groups focused on IL-1Ra studies to investigate the role of IL-1 in atherosclerosis. IL-1Ra+/- / ApoE-/- mice presented a significantly increased lesion size, as compared with IL-1Ra+/+ / ApoE-/- mice (Isoda et al., 2004).

1.3 Activators: lipopolysaccharides and 25-hydroxycholesterol

Various activators could induce immune responses of the cells involved in atherosclerosis. Endotoxin lipopolysaccharides (LPS), which may represent an infectious agent, is widely used in atherosclerosis research. LPS are large molecules consisting of a lipid and a polysaccharide linked by a covalent bond. They are found in the outer membrane of Gram-negative bacteria. Several studies have suggested a relation between bacterial pathogens such as *Chlamydia* and atherosclerosis (Danesh

et al., 1997; Epstein et al., 1996). *Chlamydia pneumoniae* is a gram-negative bacterium which can survive intracellularly in macrophages (Gieffers et al., 2001). In some investigations, *Chlamydia pneumoniae* has been detected in atherosclerotic lesions (Kuo et al., 1993). Moreover, animal experiments have also suggested that *Chlamydia pneumoniae* plays a role in atherosclerosis (Moazed et al., 1997). In *in vitro* experiments, LPS can activate various cell types such as SMC, EC, and monocytes to release cytokines and other mediators (Anand et al., 2009; Bauermeister et al., 1998; Eggesbo et al., 1994; Loppnow et al., 1994). This activation requires recognition of LPS at the cell surface. The recognition of LPS and the subsequent activation is mediated by a complex receptor system and a signal transduction system including lipopolysaccharide-binding-protein (Jack et al., 1997), CD14 (Wright et al., 1990), Toll-like receptor (TLR) (Beutler, 2003), MD2 (Shimazu et al., 1999) and others. Infections have been suggested to have a role in atherosclerosis, and some investigations showed that infections can induce vascular inflammation (Laitinen et al., 1997), however, later investigations suggested that infections are not essential for atherogenesis (Wright et al., 2000).

In recent years, cholesterols, in particular oxysterols, have been suspected to be potent activators in atherogenesis. Oxysterols are products resulting from non-enzymatic or enzymatic oxidation of cholesterol. They can be produced *in vivo* from endogenous cholesterol (Johnson et al., 1994). Some clinical studies have reported that oxysterols are elevated in patients with atherosclerosis (Olkkonen and Lehto, 2004; Vaya et al., 2001). Oxysterols promote EC dysfunction which may initiate atherogenesis (Shentu et al., 2012). Oxysterols also induce differentiation of monocytes into macrophages and foam cell formation (Hayden et al., 2002), as well as SMC apoptosis (Perales et al., 2009). Several studies have shown that oxysterols are able to induce the expression of various key inflammatory cytokines, such as IL-1ß (Palozza et al., 2011), IL-6 (Rosklint et al., 2002), IL-8 (Rydberg et al., 2003) and MCP-1 (Leonarduzzi et al., 2005; Prunet et al., 2006). More recently, cholesterol crystals have been shown to induce IL-1ß-expression in macrophages by activating NACHT, LRR and PYD domains containing protein 3 (NLRP3) inflammasome, which promotes caspase-1-activation (Duewell et al., 2010). Another group has shown that oxidized low-density lipoprotein induced inflammatory signaling through a receptor complex containing TLR-4, TLR-6 and CD36 (Stewart et al., 2010). In our experiments, we used both LPS and 25-hydroxycholesterol as activators.

1.4 Interaction of SMC with monocytes or macrophages

In the early steps of atherogenesis, local vessel wall cells such as SMC may interact with invading monocytes. This interaction may importantly contribute to the progression of atherosclerosis. Several studies have reported that interaction of SMC and monocytes induced matrix metalloproteinase-1 (Zhu et al., 2000), vascular endothelial

growth factor (Hojo et al., 2000) and hepatocyte growth factor (Okada et al., 2000), which may be involved in atherogenesis. In our previous work, in order to investigate this interaction, we have introduced a coculture (Co) model containing both SMC and monocytes. We have shown that in the LPS-stimulated cocultures, the IL-6- and MCP-1-expression was synergistically enhanced, as compared to the monocultures, and that the IL-1 in the cocultures importantly contributes to this synergistic IL-6-expression (Chen et al., 2009). Statin reduced the synergistic IL-6-expression in the LPS-stimulated cocultures (Loppnow et al., 2011b).

1.5 Statin and the isoprenoid-pathway

Statins, as lipid lowering drugs, are widely used in the treatment of patients with cardiovascular diseases. In atherosclerosis, statins have been shown to provide pleiotropic effects, which are independent of its lipid lowering effect. Statin attenuates dysfunction of endothelial cells by increasing the endothelial NO production (Laufs et al., 1998; Liao and Laufs, 2005). Statin reduces SMC proliferation through the inhibition of the isoprenylation pathway, but not the cholesterol-synthesis-pathway (Laufs et al., 1999). Statin inhibits release of adhesion molecules and MCP-1, which in turn reduces the recruitment of the circulating leukocytes into the vessel wall (Weber et al., 1997). In a coculture system, we have shown previously that statin reduces the synergistic IL-6-production in LPS-stimulated cocultures (Loppnow et al., 2011b). However, other groups have shown that statin potently enhanced the IL-1ß-expression in LPS-stimulated macrophages (Kiener et al., 2001; Kuijk et al., 2008a; Mandey et al., 2006).

Statins inhibit the isoprenoid pathway by blocking the synthesis of mevalonic acid from 3-hydroxy-3-methylglutaryl-coenzyme-A (HMG-CoA) through inhibition of HMG-CoA reductase (Figure 2). In the isoprenoid pathway, mevalonic acid (Mev) undergoes serial modifications including phosphorylation and decaboxylation. Finally, these modifications lead to the synthesis of farnesyl pyrophosphates (FPP). FPP is the branch point in the isoprenoid pathway and there are several pathways downstream of FPP, including cholesterol-synthesis- and isoprenylation-pathways (Goldstein and Brown, 1990). In the cholesterol-synthesis-pathway, squalene can be condensed from two FPP by squalene synthase. The synthesis of squalene can be inhibited by squalene-synthase-inhibitors, such as zaragozic acid-A (ZAA). Subsequently, squalene leads to the synthesis of cholesterol. In the isoprenylation pathway, FPP can be converted to geranylgeranyl pyrophosphate (GGPP). Both FPP and GGPP are important in isoprenylation of Ras and Rho proteins. Isoprenylation is a posttranslational modification of proteins and this modification is essential for membrane association and activation of certain proteins, including the small GTPases (Csepanyi-Komi et al., 2012). The process of isoprenylation by FPP (farnesylation) and

GGPP (geranylgeranylation) can be inhibited by farnesyl transferase-inhibitor (FTI) and geranylgeranyl transferase-inhibitor (GGTI), respectively.

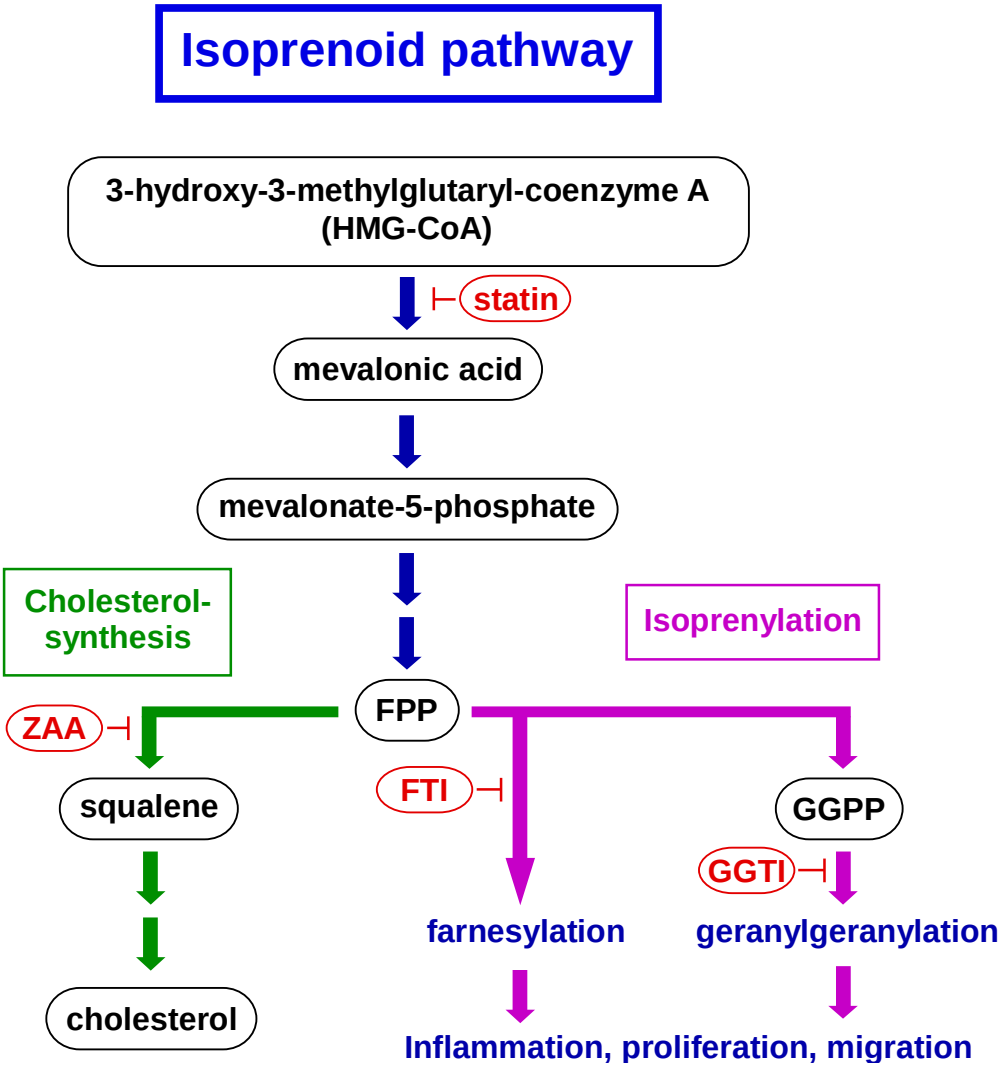

Figure 2. Statins inhibit the isoprenoid pathway. Synthesis of mevalonic acid is inhibited by statin, and subsequently the cholesterol-synthesis and isoprenylation pathways are blocked. On the other hand, synthesis of cholesterol is inhibited by ZAA. Farnesylation is inhibited by FTI and geranylgeranylation is inhibited by GGTI. FPP, farnesyl pyrophosphate; FTI, farnesyl transferase-inhibitor; GGPP, geranylgeranyl pyrophosphate; GGTI, geranylgeranyl transferase-inhibitor; ZAA, zaragozic acid A.

1.6 Rac1 regulation and its downstream signaling pathway

Statin inhibits the synthesis of FPP and GGPP, which serve as "lipid tails" in the posttranslational modification of large numbers of proteins, such as small GTP-binding proteins including Ras, Rho, Rab and Rap small GTPases (Van Aelst and D'Souza-Schorey, 1997). Basically, the modification with FPP or GGPP enables attachment of small GTPases to cell membranes. After isoprenylation, the small GTPases are translocated from cytosol to membrane and where they can be activated. Rho

GTPases are an important subgroup of the Ras superfamily and have been shown to be involved in a wide spectrum of cellular functions. Several studies focused on the Rho family members RhoA, Rac1 and Cdc42 (Lawson and Ridley, 2018; Wang and Beier, 2005; Yamao et al., 2015). These proteins are implicated in the regulation of different biological processes, such as cell migration, adhesion and differentiation (Jaffe and Hall, 2005). In the inflammatory process, the Rho proteins participate in the activation of NFκB, therefore, play a critical role in the regulation of cytokines (Montaner et al., 1998; Perona et al., 1997). Similar to other small GTPases, most of Rho GTPases triger downstream pathways when bound to GTP, while the GDP-associated form has to be considered inactive. Three classes of proteins have been found to regulate the shift between the active GTP-bound state and inactive GDP-bound state: guanine nucleotide-exchange factors (GEF), GTPase-activating proteins (GAP) and guanine nucleotide dissociation-inhibitors (GDI) (Cherfils and Zeghouf, 2013). GEF promote exchange of GDP by GTP, whereas GAP stimulates the intrinsic GTP-hydrolysis and promotes the return to the GDP-bound state. GDI binds to the inactive form of Rho GTPases and maintains these proteins in the cytosol. Statin may exert its pleiotropic effects by influencing the activity of the small GTPases. Literature has revealed that in statin-preincubated THP-1 cells, Rac1-activation has been shown to be involved in the hypersecretion of IL-1ß after LPS stimulation (Kuijk et al., 2008a). Rac1-activation regulates cellular functions through differential signaling pathways, including PI3K (Murga et al., 2002; Zheng et al., 1994), p38-MAPK (Veluthakal et al., 2015; Zhang et al., 1995), ERK1/2 (Laboureau et al., 2004; Smith et al., 2008) and JNK (Crespo et al., 1996; Kanazawa et al., 2010).

2 Aim of the study

The aim of the study was to investigate the mechanism causing the synergistic IL-6-expression in the Chol-stimulated cocultures and on the other hand compare the inflammatory response in the freshly isolated monocytes and overnight-differentiated macrophages. For this purpose, we proposed the following questions:

1) Does IL-1 contribute to the synergistic IL-6-expression in Chol-stimulated cocultures?
2) Which cells of the coculture produce IL-6 or IL-1?
3) Do monocytes and macrophages produce cytokines differentially in response to endotoxin?
4) Do statins exert different effects in the inflammatory response of monocytes and macrophages?
5) Is the isoprenoid pathway differentially involved in the inflammatory response of monocytes or macrophages?
6) Are small GTPases involved in the inflammatory response of monocytes or macrophages?

3 Material and Methods

3.1 Material

3.1.1 Cell culture material and media

Tissue culture flasks (75 cm^2 and 175 cm^2) were obtained from Greiner Bio-One GmbH (Frickenhausen, Germany). Culture plates (6- and 24-well) and transwell inserts were purchased from Nunc (Roskilde, Denmark). Dulbecco's MEM (DMEM, 1 g/L D-glucose), RPMI-1640 (VLE; very low endotoxin (≤ 0.01 EU/ml)), Biocoll separating solution, phosphate buffered saline (PBS, without Ca^{2+}/Mg^{2+}), penicillin/streptomycin (100 ×), L-glutamine (200 mM), trypsin and EDTA were obtained from Biochrom AG (Berlin, Germany). Fetal calf serum (FCS) was purchased from Thermo Fisher Scientific (Bonn, Germany). Bovine serum albumin (BSA) was obtained from Roth (fraction V; Karlsruhe, Germany).

3.1.2 Reagents

In the experiments, we used lipopolysaccharide of *Salmonella enterica* serovar Friedenau (LPS) and 25-hydroxycholesterol (Chol) as stimuli. LPS was kindly provided by Prof. Dr. H. Brade (Forschungszentrum Borstel, Germany). 25-Hydroxycholesterol was obtained from Sigma Aldrich (Taufkirchen, Germany). Fluvastatin was purchased from Calbiochem (Darmstadt, Germany). Recombinant IL-1 receptor antagonist (IL-1Ra) was obtained from Cell Concepts (Umkirch, Germany). Mevalonic acid (Mev), geranylgeranyl transferase-inhibitor-298 (GGTI), farnesyl transferase-inhibitor-277 (FTI), squalene and zaragozic acid-A (ZAA) were purchased from Sigma Aldrich (Taufkirchen, Germany), geranylgeranyl pyrophosphate (GGPP) and farnesyl pyrophosphate (FPP) were obtained from Echelon Biosciences (Salt Lake City, USA). The Rac1-inhibitor NSC23766 and the PI3K-inhibitor LY294002 were obtained from Tocris (Bristol, United Kingdom). The p38-MAPK-inhibitor SB203580, the ERK1/2-inhibitor PD98059 and the JNK-inhibitor SP600125 were purchased from Invivogen (Toulouse, France).

3.2 Methods

3.2.1 Cell isolation and culture

3.2.1.1 Isolation of human vascular smooth muscle cells

Human vascular smooth muscle cells (SMC) were isolated from unused portions of saphenous veins, which were obtained after bypass surgery as previously described (Chen et al., 2009; Loppnow and Libby, 1990). The specimens were transported in heparinized blood (4°C). After washing and removal of the endothelial layer and the connective tissue, the vessel wall tissue was cut into small pieces and planted into

Petri dishes with SMC-culture-medium. When the layer of outgrowing SMC became confluent in the Petri dishes, the cultures were treated with trypsin-solution and the cells were transferred into cell culture flasks. The cells were used in the experiments within the third and seventh passages.

SMC-culture-medium		DMEM
	1 %	Penicillin/streptomycin
	1 %	L-glutamine
	10 %	FCS

Trypsin-solution		PBS
	0.05 %	Trypsin
	0.02 %	EDTA

3.2.1.2 Isolation of mononuclear cells and preparation of monocytes

Mononuclear cells (MNC) were isolated from heparinized buffy coat using the Biocoll gradient isolation. The usage of the vascular tissues and the blood samples was approved by the local ethical committee. Briefly, heparinized buffy coat from healthy donors was mixed with one volume of PBS. This mixture was layered on top of 25 ml Biocoll separating solution and centrifuged (30 minutes, 20°C, 400 x g, no acceleration, no deceleration). The interphase, containing the mononuclear cells, was collected. The MNC were washed two times (10 minutes, 20°C, 200 x g) with Mo-wash-medium. The cell number and viability were detected following Türk's and Trypan blue stain.

Mo-wash-medium		RPMI-1640
	1 %	Penicillin/streptomycin
	1 %	L-glutamine

Monocytes (Mo) were prepared using CD14-microbeads according to the manufacturer's instruction (Miltenyi Biotech, Bergisch Gladbach, Germany). The MNC were first centrifuged (10 minutes, 20°C, 300 x g), the cell pellets were then resuspended in 5 ml MACS-buffer and centrifuged (10 minutes, 20°C, 300 x g). The cells were again resuspended in MACS-buffer (60 µl per 10^7 cells) and incubated with FcR blocking reagent and CD14-microbeads (20 µl per 10^7 cells) for 15 minutes at 4°C. After the incubation, the cells were washed with MACS-buffer and centrifuged (10 minutes, 20°C, 300 x g). The cells were resuspended in MACS-buffer (10^8 cells in 500 µl). The cell suspension was loaded onto a MACS-Column which was placed in the magnetic field of the MACS-separator. The cells restrained in the column were collected and finally resuspended in Mo-wash-medium.

MACS-buffer		PBS
	2 mM	EDTA
	2 %	FCS

3.2.1.3 Cocultures of SMC with monocytes

The monocytes were then used in coculture experiments with SMC, in monoculture experiments with the freshly isolated monocytes, or in experiments where the monocytes were differentiated in parallel by overnight incubation (Figure 3 and 4). The SMC-monocyte coculture was prepared as previously described (Chen et al., 2009; Fu et al., 2014). At day 1, SMC-culture-medium was incubated in 6- or 24-well plates (10,000 cells/cm^2; 37ºC, 7.5% CO_2) with or without SMC. After 24 hours the SMC-culture-medium was removed and Mo-culture-medium (SMC monoculture, Figure 3-condition 1) or monocytes in Mo-culture-medium (coculture and monocyte monoculture, Figure 3-conditions 2 and 3, respectively) were added. The monocyte to SMC ratio was adjusted to ratios of 1 to 20 Mo per SMC, respectively, as mentioned in the respective legends. Subsequently, control medum, LPS (100 ng/ml) or Chol (20 µg/ml) were added. After 24 hours incubation the supernatants were collected and stored at -20 °C for ELISA and the cells were harvested for qPCR or Western Blot.

Mo-culture-medium		RPMI-1640
	1 %	Penicillin/streptomycin
	1 %	L-glutamine
	10 %	FCS

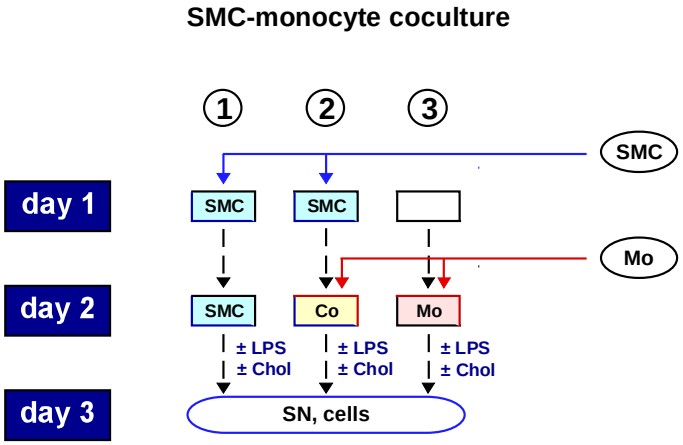

Figure 3. Experimental design of the SMC-monocyte coculture. In order to obtain the SMC-monocyte coculture, SMC were incubated for 24 hours (condition 1 and 2). Then, freshly isolated monocytes were added to the cell culture (condition 2 and 3) with or without stimulus. After 24 hours incubation, the supernatants and cell lysates were collected.

3.2.1.4 Cocultures of SMC with monocytes with insert

In some experiments parallel cocultures were performed with transwell inserts in order to avoid direct cell contact between the SMC and the monocytes. In this case, after 24 hours incubation of SMC, the SMC-culture-medium was removed, fresh Mo-culture-medium was added (SMC-compartment), and inserts were placed into the respective cell cultures. Subsequently, monocytes were added into the inserts (monocyte-compartment). Thereafter, LPS (100 ng/ml) and Chol (20 µg/ml) were added into both compartments of the insert-cocultures. After 24 hours incubation the supernatants of both compartments were separately collected and stored at -20 °C for ELISA and the cells were separately harvested for qPCR or Western Blot.

3.2.1.5 Monocultures of monocytes or overnight differentiated macrophages

In order to obtain monocyte-monocultures (Figure 4-conditions 1), freshly isolated monocytes were incubated in Mo-culture-medium at a cell number of 10,000 – 200,000 cells/cm^2, as described in the respective legends. These cell numbers would reflect ratios of 1 to 20 monocytes per SMC in coculture experiments. Subsequently, LPS (100 ng/ml) and statin (10 µg/ml) were added. Thereafter, the monocyte cultures were incubated at 37ºC, 7.5% CO_2 for 24 hours. Subsequently, the supernatants were collected and stored at -20 °C for ELISA and the cells were harvested for Western Blot.

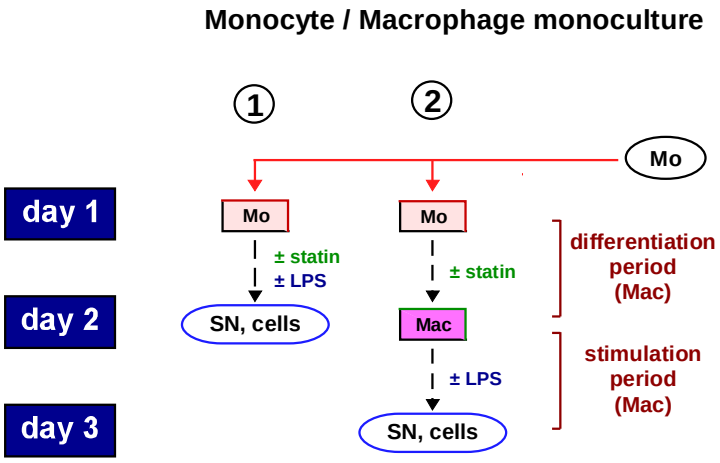

Figure 4. Experimental design of the monocyte / macrophage monoculture. In monocyte monoculture (condition 1), freshly isolated monocytes were incubated with LPS for 24 hours in the presence or absence of statin. Then, the supernatants and cell lysates were collected. In order to obtain the macrophage monoculture (condition 2), freshly isolated monocytes were incubated for 24 hours in the presence or absence of statin. Then the LPS were added to the cell cultures. After 24 hours incubation, the supernatants and cell lysates were collected.

In order to obtain macrophage-monocultures (Mac; Figure 4-condition 2), freshly isolated monocytes were incubated for 24 hours in the presence or absence of statin (10 µg/ml; differentiation period). After this incubation, LPS or medium without LPS was

added and the cultures were incubated for further 24 hours (stimulation period). Subsequently, the supernatants were collected and stored at -20 °C for ELISA and the cells were harvested for Western Blot.

3.2.2 Measurement of cytokines by ‘‘Enzyme-Linked Immunosorbent Assay’’ (ELISA)

The cytokines in the different culture supernatants were determined by ELISA (BD, Heidelberg, Germany). Every kit contained the capture antibody (anti-human IL-6 or IL-1ß; monoclonal antibodies), the detection antibody (biotinylated anti-human IL-6 or IL-1ß; monoclonal antibodies), the enzyme reagent (avidin-horseradish peroxidase conjugate) and standards (recombinant human IL-6 or IL-1ß; lyophilized).

Coating-buffer (pH 9.5)	8.40 g/L	$NaHCO_3$
	3.56 g/L	Na_2CO_3
Elisa-PBS	8.00 g/L	NaCl
(EPBS, pH 7.0)	1.16 g/L	Na_2HPO_4
	0.20 g/L	KH_2PO_4
	0.20 g/L	KCl
Blocking-solution		EPBS
	10 %	FCS (v/v)
Washing-buffer		EPBS
	0.05 %	Tween 20 (v/v)
Substrate-solution	50 %	TMB (3,3’,5,5’-tetramethylbenzidine)
	50 %	Hydrogen peroxide
Stop-solution	2 N	H_2SO_4

For the ELISA measurements, 96-well flat bottom plates were coated (100 µl/well) with the capture antibody diluted in coating-buffer (1:1000 for IL-6, 1:500 for IL-1ß) and incubated overnight at 4°C. The buffer was removed and the wells were washed 3 times with washing-buffer. The wells were then incubated with blocking-solution (200 µl/well) for 1 hour. The blocking-solution was removed and the wells were washed 3 times with washing-buffer. Different standard concentrations or cell culture supernatants were added to the wells (100 µl/well) in various dilution steps in blocking-solution and incubated for 2 hours. The wells were then washed 5 times. Then, in the IL-6 ELISA, 100 µl/well detection antibody(1:1000 diluted in blocking-solution) and enzyme reagent (1:1000) were added and incubated for 1 hour. In the IL-1ß ELISA,

detection antibody and enzyme reagent were added separately. First, 100 µl/well detection antibody (1:2000) was added and incubated for 1 hour. The wells were washed for 5 times. Then, 100 µl/well enzyme reagent (1:500) was added and incubated 30 minutes. After the detection antibody and enzyme incubation step, the wells were washed 7 times. Substrate-solution was added into each well (100 µl/well) and incubated for 5 - 30 minutes in the dark. Finally, 50 µl/well stop-solution was added into each well and the absorption of the solution in the wells was read in an ELISA-reader (450 nm; reference wave length: 620 nm) (Infinite M200 Pro, Tecan, Männedorf, Switzerland).

3.2.3 Total RNA isolation, reverse transcription and real-time PCR

Total RNA was extracted with the "RNeasy Plus Mini Kit" or "RNeasy Micro Kit" (Qiagen, Hilden, Germany) according to the manufacturer's instructions and then stored at -20°C. Before reverse transcription, the RNA-concentration was measured with a NanoDrop 1000 spectrophotometer (Thermo scientific, Bonn, Germany).

We used the "Omniscript Reverse Transcription Kit" (Qiagen) for reverse transcription. "Rnasin Plus Rnase Inhibitor" (Promega, Mannheim, Germany) and oligo(dT)18 primer (Fermentas, St. Leon-Rot, Germany) were also used for the reaction. RNA of SMC, MNC or cocultured cells was adjusted to 100 ng per reaction and reverse-transcribed at 37 °C for 60 minutes. Each reaction (20 µl) contained the compounds listed in Table 1. After the reverse transcription, the cDNA was stored at -20 °C until real time PCR.

Table 1. Reverse transcription reaction components

Component	Volume	Final concentration
10× buffer RT	2 µl	1×
dNTP mix (5 mM each dNTP)	2 µl	0.5 mM each dNTP
Oligo-dT primer(10 µM)	2 µl	1 µM
RNase inhibitor(10 units/µl)	1 µl	0.5 units/µl
Omniscript reverse transcriptase	1 µl	0.2 units/µl
RNA sample and DEPC-treated water	12 µl	

The real time PCR was set up in quadruplicate in a volume of 25 µl consisting of 2.5 µl cDNA, 12.5 µl "GoTaq qPCR Master Mix" (Promega; including the double-strand-DNA stain), 0.5 µl sense primer (Table 2), 0.5 µl antisense primer (0.25 µM each; MWG-Biotech, Ebersberg, Germany), and 9 µl DNase-free water. The reaction was performed using the Bio-Rad "iCycler iQ PCR detection system" (Bio-Rad Laboratories, Munich, Germany). PCR amplification was started by an initial denaturation step (95 °C, 5 min). This was followed by 35 cycles of denaturation (95 °C, 30 sec), annealing

(57 °C, 20 sec) and extension (72 °C, 30 sec). Finally, another extension step was performed (72 °C, 2 min). For quantification, the mean of each IL-6 or IL-1ß mRNA expression derived from the quadruplicate measurement of each sample, was normalized to the mean of the respective GAPDH mRNA measurement resulting one final value.

Table 2. Primer sequence for real-time PCR

GAPDH	sense	5'- AGG GCT GCT TTT AAC TCT GGT -3'
	antisense	5'- CCC CAC TTG ATT TTG GAG GGA -3'
IL-6	sense	5'-TCG GTA CAT CCT CGA CGG CA-3'
	antisense	5'-TCA CCA GGC AAG TCT CCT CA-3'
IL-1ß	sense	5'-ACA AGG CAC AAC AGG CTG CTC-3'
	antisense	5'-GGT CCT GGA AGG AGC ACT TCA T-3'

3.2.4 Western blot

Briefly, after removal of the supernatants, the cell layers from 6-well plates were lysed with 500 µl 2% SDS in PBS and the cell lysates were ultrasonicated for 5 minutes. Then, the cell lysates were mixed with 167 µl sample-buffer. The samples were then heated at 95 °C for 5 minutes. After the heating, the samples were ready to use or stored in -20 °C.

Sample-buffer (4×)	252 mM	Tris-HCl (1M, PH 6.8)
	40 %	Glycerol
	8 %	SDS (w/v)
	0.04 %	Bromophenol blue (w/v)
	2 %	2-Mercaptoethanol (1.12 g/ml, v/v)
Running-buffer (pH 9.5)	3.03 g/L	Tris
	14.4 g/L	Glycin
	1 g/L	SDS
Tris-Buffered -Saline (TBS)	2.42 g/L	Tris
	8.77 g/L	NaCl
Washing-buffer	1 ×	TBS
	0.1 %	Tween 20 (v/v)
Blocking-solution	1 ×	Washing-buffer
	50 g/L	BSA

Blotting-buffer	3.03 g/L	Tris
	14.4 g/L	Glycin
	20 %	Methanol (v/v)

The proteins of the samples were separated by SDS-PAGE (15%). Then, the proteins were transferred from the gel to a nitrocellulose membrane (GE Healthcare, Freiburg, Germany) by semidry electroblotting (1 mA/cm^2, 2 h; Multiphor II Electrophoresis system, Thermo Fisher Scientific, Schwerte). After the electroblotting, the membrane was quickly washed in TBS and incubated with blocking-solution for 1 hour at 4 °C. After this blocking step, the membrane was washed in washing-buffer for 5 minutes and incubated with anti-GAPDH (AF5718, R&D systems, Wiesbaden-Nordenstadt, Germany), anti-caspase-1 (sc-515, Santa Cruz Biotechnology, Heidelberg, Germany), anti-IL-1ß (Herzbeck et al., 1989) or anti-Rac1 (610650, BD, Heidelberg, Germany) antibodies (diluted in blocking-solution), respectively, for 2 hours at room temperature or overnight at 4 °C. After the incubation with first antibody, the membrane was washed 3 times with washing-buffer for 5 minutes. The membrane then was incubated with the respective second antibody for 1 hour at room temperature. Then, the membrane was washed 3 times with washing-buffer for 5 minutes and covered with "SuperSignal West Dura Extended Duration Substrate" (Thermo Scientific, Bonn, Germany) according to the manufacturer’s instructions. Chemiluminescent exposures were captured on an ECL system (Thermo Scientific, Bonn, Germany) and Image Quant LAS 4000 (GE Healthcare Life Sciences, Freiburg, Germany). The band density was analysed in "Totalab" (TotalLab limited; Newcastle; GB) and normalized to GAPDH.

3.2.5 Rac1 pulldown assay

The Rac1 pulldown assay was performed with the "Rac1 Activation Assay Biochem Kit" (Cytoskeleton, Denver, USA) according to the manufacturer's instruction. Briefly, after removal of the culture supernatants, the cell layers were lysed in 800 µl "pulldown-cell-lysis-buffer" provided with the kit. From the cell lysates 80 µl was used for the quantification of the protein concentration (Pierce BCA Protein Assay Kit, Thermo Scientific, Bonn, Germany). The rest of the cell lysates (720 µl) was snap frozen in liquid nitrogen and stored at -80°C. Thereafter, the cell lysates were thawed at room temperature in a water bath and then 100 µg of the cell lysates was incubated at 4°C with 10 µl "PAK-PBD beads" on a rotator for 1 hour. After the incubation, the "PAK-PBD beads" were pelleted by centrifugation (1 minute, 4°C, 5,000 x g). The supernatants were carefully removed and the beads were resuspended with 500 µl wash buffer provided with the Kit. Subsequently, the "PAK-PBD beads" were pelleted by centrifugation (3 minutes, 4°C, 5,000 x g). Thereafter, the supernatants were carefully removed and 10 µl 4× sample buffer was added. The samples were heated at 95 °C for 2 minutes and analysed in Western blot.

3.2.6 Statistical analysis

ELISA measurements were performed in quadruplicates. These multiple values were used to calculate the mean or the standard deviation, as outlined in the respective figure legends. Significances were calculated using SPSS (One Way ANOVA, LSD post hoc analysis), as described in the respective figure legends. P values of less than 0.05 were regarded as statistically significant.

4 Results

4.1 In 25-hydroxycholesterol-(Chol)-stimulated SMC-monocyte cocultures, monocytes produce IL-1ß, which induces IL-6-production of SMC

4.1.1 IL-1 is the major activator of the synergistic IL-6-production in 25-hydroxycholesterol-stimulated cocultures

Previous studies have shown that smooth muscle cells (SMC) and monocytes (Mo) synergistically enhance the IL-6 protein expression in endotoxin-(LPS)-stimulated cocultures. In these cocultures, IL-1 has been proven to be the major cause of the synergistic IL-6-production. However, the role of IL-1 in 25-hydroxycholesterol-(Chol)-stimulated cocultures has not been investigated yet. Thus, we used the IL-1 receptor antagonist (IL-1Ra) to inhibit the IL-1-mediated cell activation in LPS- and Chol-stimulated cocultures. As expected, in the LPS- and Chol-stimulated cocultures the interaction of SMC and monocytes synergistically enhanced the IL-6 protein expression (Figure 5). In line with previous results, in the LPS-stimulated cocultures, IL-1Ra significantly reduced the IL-6 protein expression (57% reduction), as compared to the cocultures without IL-1Ra. In the Chol-stimulated cocultures the IL-1Ra reduced the IL-6 protein expression even more potently (80% reduction).

We also investigated the influence of IL-1Ra on IL-1-expression, since IL-1 can regulate its own production. As expected, LPS-stimulated monocytes and cocultures, as well as Chol-stimulated cocultures, contained IL-1. In accordance with the IL-6 inhibition-data shown above, IL-1Ra significantly reduced 42% and 86% of the IL-1-protein expression in the LPS- and Chol-stimulated cocultures, respectively.

Taken together, these results indicate that, similar to the LPS-stimulated cocultures, IL-1 is a major activator of the synergistic IL-6-production in the Chol-stimulated cocultures.

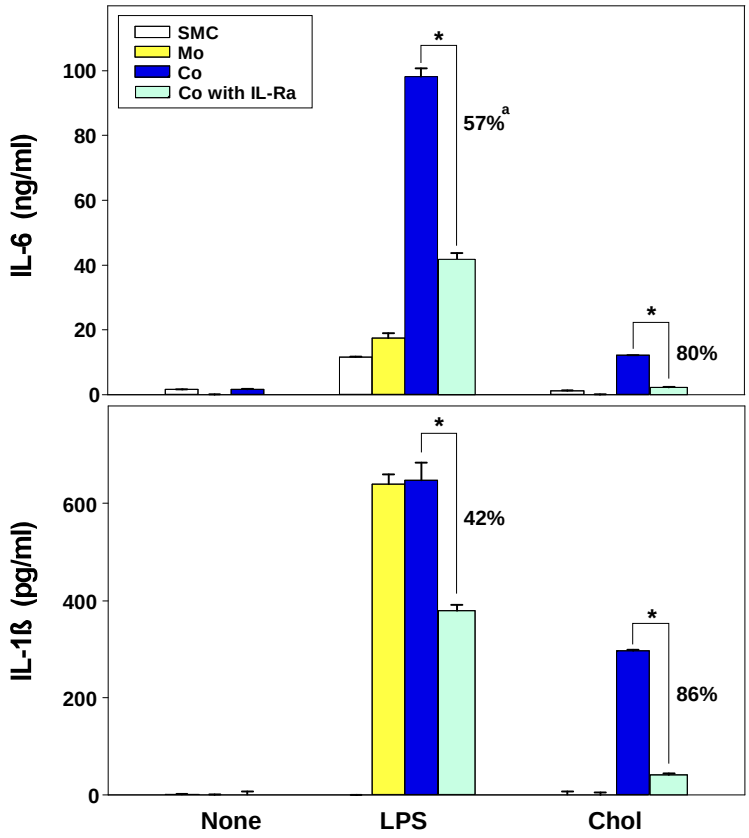

Figure 5. IL-1Ra reduces the IL-6- and IL-1ß-expression after LPS- and Chol-stimulation in SMC-monocyte cocultures. Vascular smooth muscle cells (10,000/cm^2) were incubated overnight in 24-well plates. SMC-culture-medium was replaced by fresh Mo-culture-medium (SMC) or by freshly isolated monocytes (Co) (10 Mo per 1 SMC) in Mo-culture-medium. To additional wells only the freshly isolated monocytes were added (Mo). Finally, the stimuli (100 ng/ml LPS; 20 µg/ml Chol) in Mo-culture-medium, both were added. Into parallel cocultures, IL-1Ra (300 ng/ml) was added. The supernatants were harvested after 24 hours and the IL-6- or IL-1ß-concentration was determined in ELISA. The cytokine concentrations are presented as mean ± SD. Significances were calculated by One Way ANOVA with subsequent post hoc analysis (LSD) (without IL-1Ra vs. with IL-1Ra; *, $p < 0.05$). The data show one of two experiments with similar results.

a - The percentages in Figure 5 show the reduction of cytokine expression by IL-1Ra, calculated by the following equation: reduction = ($Cytokine_{without\ IL\text{-}1Ra}$ − $Cytokine_{with\ IL\text{-}1Ra}$) / $Cytokine_{without\ IL\text{-}1Ra}$ × 100%.

4.1.2 In Chol-stimulated cocultures, the synergistic effect is present in various monocyte-SMC ratios

In cocultures, both, SMC and monocytes may contribute to the synergistic cytokine expression. Thus, the ratio of monocytes to SMC may influence the cytokine expression. Therefore, we performed a ratio analysis, including the monocyte-SMC ratios 0, 1, 2, 5, 10 and 20 in LPS- and Chol-stimulated cocultures. As expected, the IL-6-production in the LPS-stimulated cocultures (Figure 6, round symbols) increased depending on the monocyte-SMC ratio. The same was true for the IL-6-production in the Chol-stimulated cocultures (triangle symbols), as well as for the IL-1ß-production in the LPS- and Chol-stimulated cocultures. This result indicates that the IL-6- and IL-1ß-

expression in the cocultures is influenced by the Mo-SMC ratio. In addition, these ratios may also alter the synergistic effect in the cocultures. It was not clear, whether the synergistic effect in the Chol-stimulated cocultures was present only in one ratio. Table 3 and 4 present the synergisms of IL-6- and IL-1ß-production at different monocyte-SMC ratios in various cocultures. Synergistic IL-6-production was present at all ratios in the LPS-stimulated cocultures (Table 3). The same was true for the synergistic IL-6-production in the Chol-stimulated cocultures. However, different from the LPS-stimulated cocultures, the synergisms in the Chol-stimulated cocultures appeared to be more potently increased at high monocyte-SMC ratios. In contrast to the IL-6-data, no synergistic IL-1ß-production was present at all ratios in the LPS-stimulated cocultures (Table 4). Synergistic IL-1ß-production in the Chol-stimulated cocultures was obtained starting with the ratio 2 and was further enhanced at the ratios 5 to 20.

Taken together, these results indicate that in the LPS- and Chol-stimulated cocultures, the monocyte-SMC ratio influences the IL-6- and IL-1ß-expression. The synergistic effect is higher in Chol-stimulated cocultures. Furthermore, the synergistic effect is obtained at all monocyte-SMC ratios and not restricted to a particular monocyte-SMC ratio.

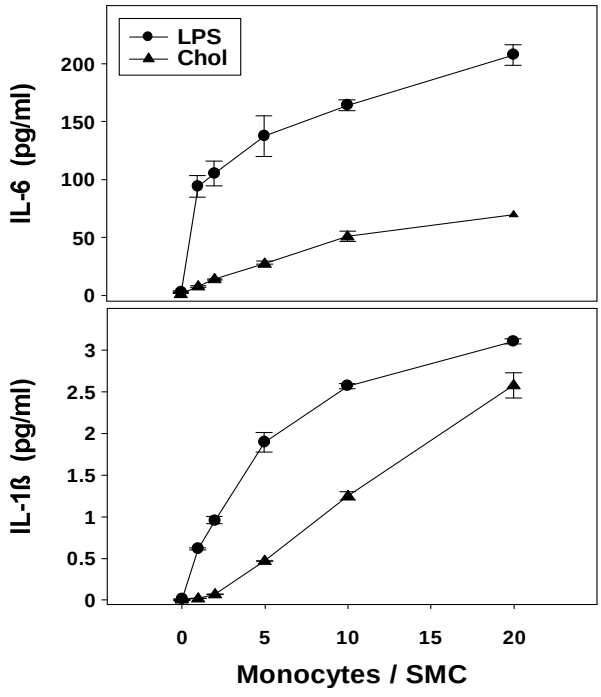

Figure 6. The IL-6- and IL-1-expression in the cocultures is monocyte-SMC ratio dependent. SMC (10,000/cm^2) were incubated overnight in 24-well plates. SMC-culture-medium was replaced by Mo-culture-medium containing the stimuli (100 ng/ml LPS; 20 µg/ml Chol) after 24 hours. Freshly isolated monocytes were added at different ratios (0, 1, 2, 5, 10, and 20 monocytes per 1 SMC) to the wells. The supernatants were harvested and the IL-6- and IL-1ß-concentration was determined in ELISA and presented as mean ± SD. The data represent one of five experiments with similar results.

Table 3. In the Chol-stimulated cocultures, synergistic IL-6- and IL-1-production is enhanced depending on the Mo-SMC ratio[a]

		None[b]	LPS[c]					Chol[c]				
	Exp.	**5**	**1**	**2**	**5**	**10**	**20**	**1**	**2**	**5**	**10**	**20**
IL-6	**1**	6	7.2	7.7	8.2	10.2	7.6	6.6	10.8	19.9	44.7	59.2
	2	1.2	11.6	14.2	14.8	9.6	7.6	4.8	18.4	37.2	120.0	151.7
	3	1.4	9.8	14.2	10.2	6.3	6.7	6.7	18.1	25.1	45.8	72.3
	4	1.3	4.8	6.2	5.1	3.4	3.2	1.3	2.3	5.8	10.3	13.0
	5	1.5	14.7	11.6	6.7	4.2	3.6	5.3	9.5	18.4	34.2	42.8
	Mean ± sd[d]	2.3 ± 2.1	9.6 ±3.8	10.8 ±3.7	9.0 ±3.8	6.7 ±3.1	5.7 ±2.2	5.0 ± 2.2	11.8 ± 6.7	21.3 ± 11.4	51.0 ± 41.1	67.8 ± 51.9
IL-1ß	**1**	0.9	1.1	0.8	0.4	0.5	0.6	2.6	11.2	27.2	39.9	16.8
	2	0.1	1.2	0.9	0.7	0.7	0.7	0.6	19.5	48.4	31.5	21.1
	3	0.5	1.2	0.6	0.4	0.4	0.3	0.5	5.1	58.8	25.5	41.1
	4	0.5	2.4	1.7	1.4	1.0	0.7	0.1	2.4	47.4	160.4	63.8
	5	0.3	2.4	1.9	1.2	1.0	1.0	1.6	13.2	46.8	46.2	64.6
	Mean ± sd[d]	0.5 ± 0.3	1.7 ±0.7	1.2 ±0.6	0.8 ±0.5	0.7 ±0.3	0.6 ±0.3	1.1 ± 1.0	10.3 ± 6.8	45.7 ± 11.5	60.7 ± 56.3	41.5 ± 22.7

a - The synergism was calculated (Syn = $cytokine_{coculture}/(cytokine_{SMC} + cytokine_{Mo})$) for unstimulated (None), LPS- and Chol-stimulated conditions, respectively.

b - In unstimulated cocultures, only the monocyte-SMC ratio 5 was used.

c - In LPS- and Chol-stimulated cocultures, the synergisms at all monocyte-SMC ratios were calculated.

d - Mean and standard deviation of five experiments were calculated, respectively.

4.1.3 In the LPS- and Chol-stimulated cocultures, SMC preferentially produced IL-6 and monocytes preferentially produced IL-1ß

The results described above suggest that IL-1 is a central mediator for the synergistic IL-6-production in cocultures. However, the cell source of the IL-6 and IL-1 has not been determined. In order to address this question, we used trans-well inserts in the

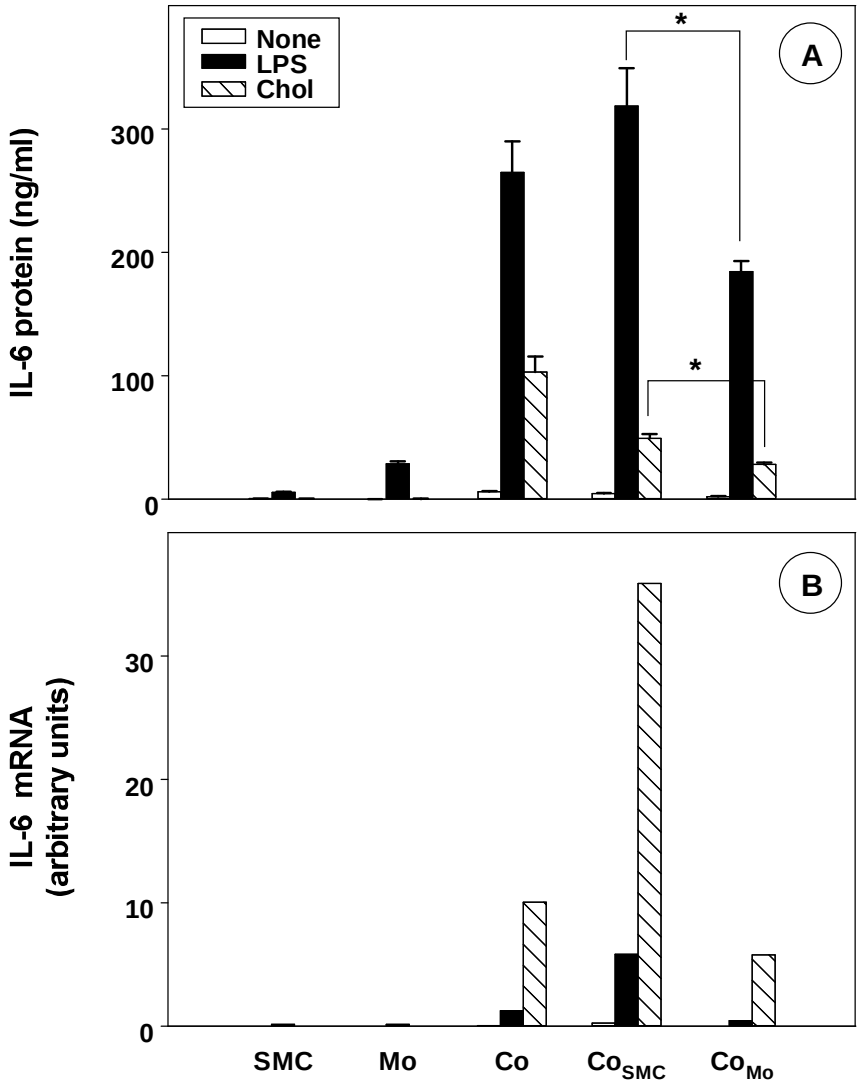

Figure 7. The SMC-compartment in the cocultures expresses more IL-6-mRNA than the monocyte-compartment. The experimental design for SMC, monocytes (Mo) and cocultures (Co) is similar to that described in Figure 5, except that 6-well-plates were used. In the cocultures with insert, SMC were incubated in cell culture plate (Co_{SMC}) and freshly isolated monocytes were placed into the trans-well insert (Co_{Mo}). The supernatants were harvested after 24 hours of incubation. The supernatants of the SMC- and the monocyte-compartments were harvested separately. After removal of the supernatants, the cell layers were washed and used for mRNA isolation, the mRNAs of the compartment cells were harvested separately (Co_{SMC}; Co_{Mo}). A) The IL-6-concentration was determined in ELISA and presented as mean ± SD. B) Total RNA was isolated and reverse-transcribed. The cDNA was used for real time PCR with IL-6 and GAPDH primers. The values obtained for IL-6 were normalized to GAPDH. Significances were calculated by One Way ANOVA with subsequent post hoc analysis (LSD) (Co_{SMC} vs. Co_{Mo}; *, $p < 0.05$). The figure shows one of two experiments with similar results.

cocultures to separate the cells of the cocultures. The monocytes inside the inserts were determined as monocyte-compartment and the SMC cultured outside the inserts were determined as SMC-compartment. Analysis of protein and mRNA expression in the SMC- and monocyte-compartments might prove which cells preferentially produce IL-6 and IL-1. As expected, LPS-stimulated SMC (SMC) and monocytes (Mo) produced some IL-6 protein. Synergistic IL-6-production was detected in the LPS- and Chol-stimulated cocultures (Co). Also, in the SMC-compartment (Co_{SMC}) and the monocyte-compartment (Co_{Mo}) of the cocultures with insert, high levels of IL-6 were detected (Figure 7A). However, the IL-6 protein expression in the SMC-compartment (Co_{SMC}) was significantly higher than in the monocyte-compartment (Co_{Mo}) of the LPS- and the Chol-stimulated cocultures. These data indicated that the SMC may be a more potent source of the IL-6 in the cocultures than the monocytes. However, the IL-6 probably has moved from SMC-compartment to the other compartment of the insert because of diffusion. In order to avoid this, we also measured the IL-6 mRNA expression in the cells (Figure 7B), since the cells do not move between the compartments, mRNA investigation should show a much clearer situation. The results show that in the monocultures both SMC and monocytes produced only little IL-6 mRNA after LPS- and Chol-stimulation. The cells in the LPS- and Chol-stimulated cocultures, as well as in the SMC-compartment (Co_{SMC}) and the monocyte-compartment (Co_{Mo}) produced IL-6 mRNA. However, the IL-6 mRNA expression in the SMC-compartment was much higher than in the monocyte-compartment. Taken together, the mRNA results in Figure 7 suggest that SMC are the major source of IL-6 in the LPS- and Chol-stimulated cocultures.

Also, IL-1ß was measured in the compartments. In the SMC-monocultures, as well as in the unstimulated and Chol-stimulated monocyte monocultures, no or only little IL-1ß-protein has been detected, whereas LPS-stimulated monocytes produced a high level of IL-1ß (Figure 8A). Similar to the IL-6-data shown above, large amounts of IL-1ß were detected in the LPS- and Chol-stimulated cocultures, as well as in the SMC-compartment (Co_{SMC}) and the monocyte-compartment (Co_{Mo}). In contrast to the IL-6-data, the IL-1ß-protein expression in the SMC-compartment was lower than in the monocyte-compartments of both, LPS- and Chol-stimulated cocultures, although only slightly. The IL-1ß mRNA analysis, similar to the protein-data, showed that SMC, as well as unstimulated and Chol-stimulated monocytes produced only little IL-1ß mRNA. In contrast, the LPS-stimulated monocytes produced larger amounts of IL-1ß mRNA (Figure 8B). In the LPS- and Chol-stimulated cocultures, as well as in the SMC-compartment and the monocyte-compartment, the cells also expressed IL-1ß mRNA. However, the IL-1ß mRNA expression in the SMC-compartment was much lower than in the monocyte-compartment of both, the LPS- and Chol-stimulated cocultures.

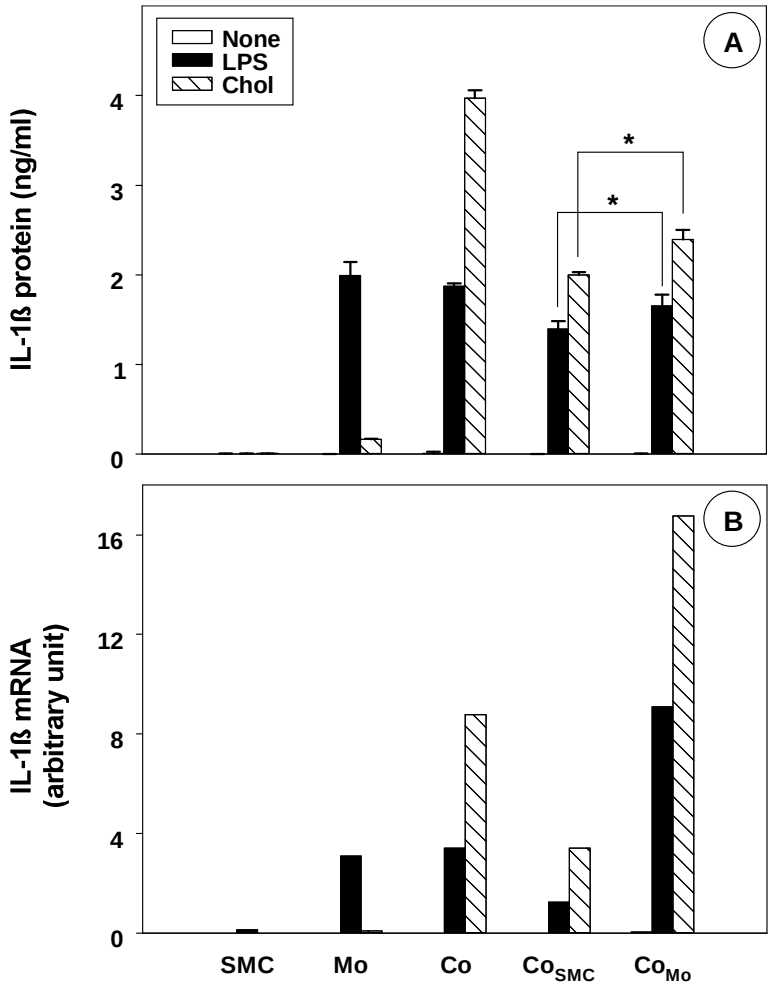

Figure 8. The monocytes in the monocyte-compartment of the cocultures express more IL-1ß mRNA than the SMC in the SMC-compartment. The experimental design is described in Figure 7, except that IL-1ß was analyzed.

Since both, the SMC-compartment and the monocyte-compartment contained large amounts of IL-1ß-protein, this result could not unequivocally demonstrate the source of IL-1ß in the cocultures. However, the high IL-1ß-levels in both compartments may be attributed to diffusion of cytokines from one compartment to the other one. In order to understand the impact of the diffusion, we performed a time course experiment, where the supernatants of both compartments were harvested after 0, 3, 6, 9, 12, 15, 24 and 72 hours stimulation. Under LPS-stimulated conditions (Figure 9, LPS), the IL-1ß-expression in the monocyte-compartment (closed circle) was detected earlier and was significantly higher than in the SMC-compartment (open circle) starting after 6 hours. In the Chol-stimulated condition (Figure 9, Chol), the IL-1ß-expression in the monocyte-compartment was also somewhat earlier and significantly higher than in the SMC-compartment, starting at 12 hours. In both cases, the IL-1ß was detectable around 3 to 6 hours earlier in the monocyte-compartment than in the SMC-compartment.

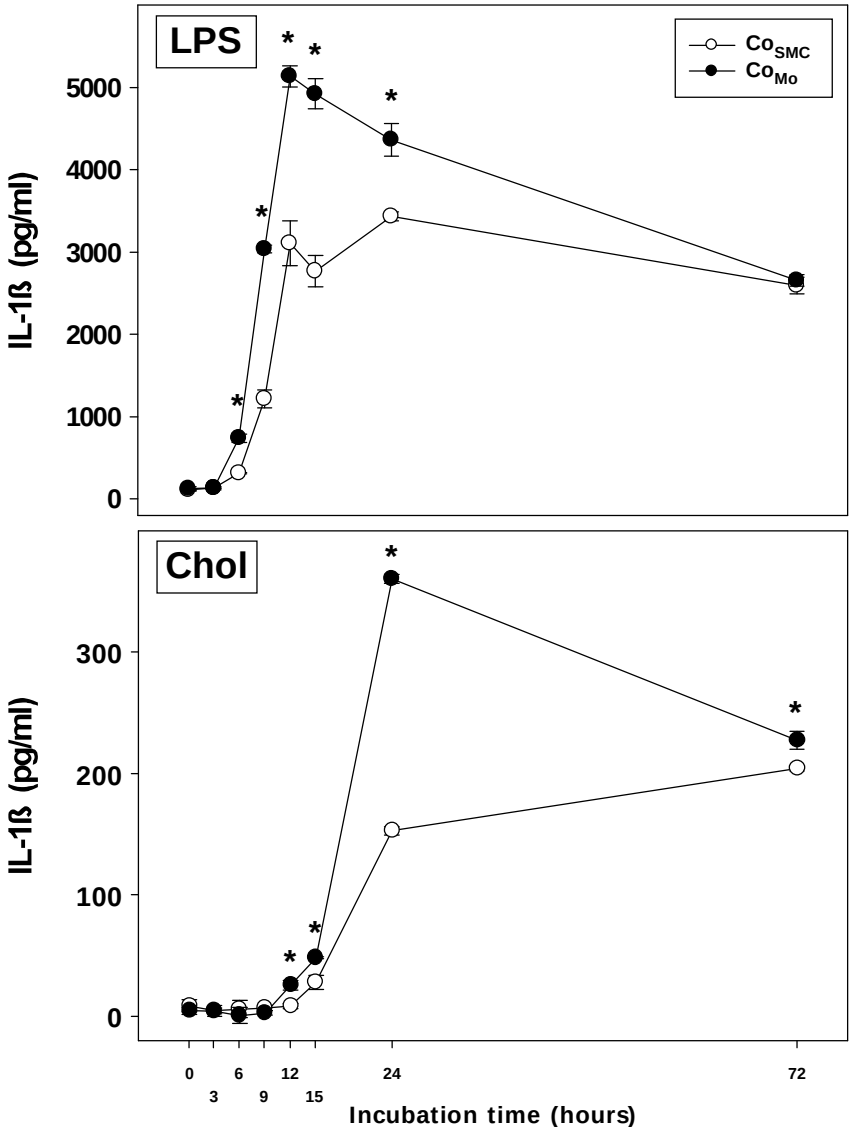

Figure 9. The IL-1ß-protein appears earlier in the monocyte-compartment than in the SMC-compartment. SMC (10,000/cm^2) were incubated overnight in 6-well plates. SMC-culture-medium was replaced by Mo-culture-medium containing the stimuli (100 ng/ml LPS; 20 µg/ml Chol). Freshly isolated monocytes (Mo; 5 Mo per 1 SMC) were placed into the trans-well inserts. The supernatants of the SMC- and monocyte-compartments were separately harvested after 0, 3, 6, 9, 12, 15, 24 and 72 hours. The IL-1-concentration was determined in ELISA and presented as mean ± SD. Significances were calculated by One Way ANOVA with subsequent post hoc analysis (LSD) (Co_{SMC} vs. Co_{Mo}; *, $p < 0.05$). The figure shows one of three experiments with similar results.

In Figure 8B it was shown that both, the monocyte-compartment and the SMC-compartment contained IL-1ß mRNA. However, it has been shown previously, that, although SMC can express IL-1ß mRNA, they are not capable of processing IL-1ß (Schönbeck et al., 1997). Monocytes can produce both IL-1ß mRNA and mature IL-1ß-

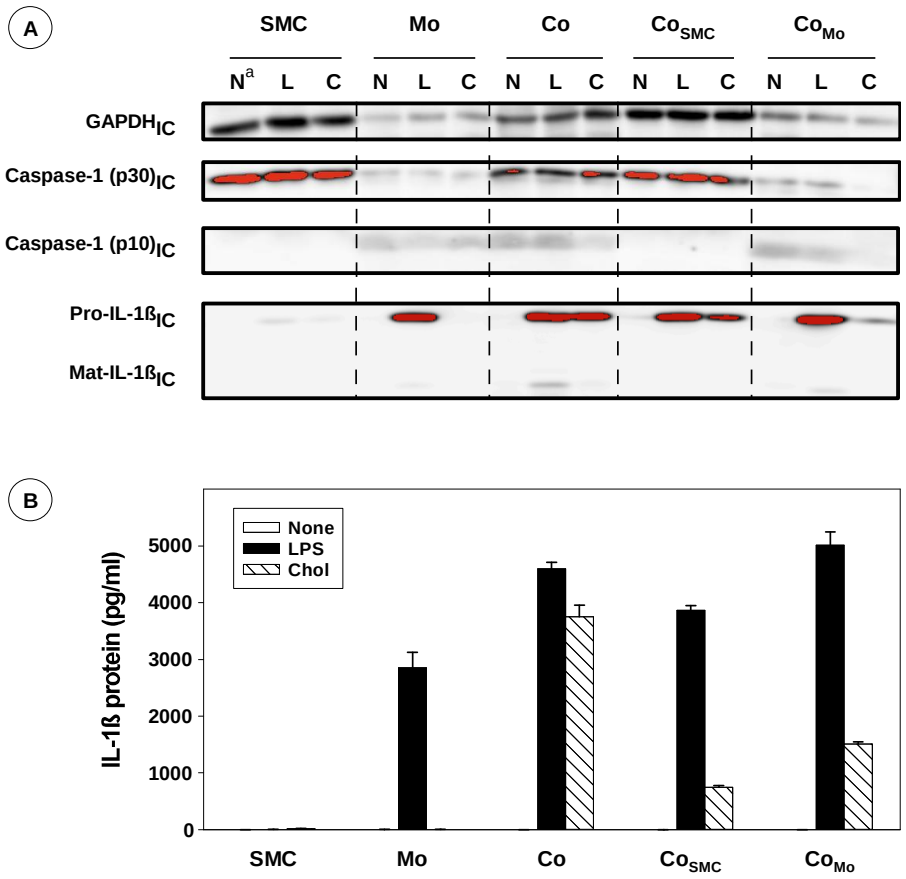

Figure 10. Cell lysates of monocytes derived from the monocyte-compartment contain active capase-1 (p10) and mature IL-1ß. The experimental design is described in Figure 7, except that after removal of the supernatants, the cell layers were harvested and used for Western blot. A) For Western blot analysis, the cell layers were lysed with 2% SDS and used for GAPDH ($GAPDH_{IC}$), caspase-1 (inactive caspase $(p30)_{IC}$ and active caspase $(p10)_{IC}$) and IL-1ß (Pro-IL-1ß$_{IC}$ and Mat-IL-1$_{IC}$) blots. B) The supernatants of the cultures used for western blotting were analyzed for IL-1-expression by ELISA. C: Chol-stimulated cells; IC: intracellular; L: LPS-stimulated cells; N: unstimulated cells. The figure shows one of three experiments with similar results.

protein. The evaluation of the IL-1ß-precursor-level and caspase-1-activation may add information about the source of IL-1ß in the cocultures. Thus, we analyzed expression of intracellular capase-1 and IL-1ß-protein in a separation experiment by Western blot. Both, the SMC and the monocytes expressed inactive caspase-1 (p30) (Figure 10A). However, no active caspase-1 (p10) was detected in the SMC of the monocultures, whereas monocytes produced active caspase-1. Active caspase-1 was detected also in the unseparated cocultures. Importantly, the SMC-compartment of the separated cocultures did not express active caspase-1, whereas the monocyte-compartment did so. In line with the previous publication, SMC produced only little IL-1ß-precursor after stimulation and no mature IL-1ß (Schönbeck et al., 1997). Furthermore, no IL-1ß was detected in supernatants from SMC by ELISA (Figure 10B), this result indicates that SMC did not perform IL-1ß maturation, which was in line with the previous data. The

ELISA-data shown in Figure 10B are in line with the data of the separation experiments shown in Figure 8. The present Western Blot analysis extends these findings: LPS-stimulated monocytes strongly produced IL-1ß-precursor and even mature IL-1ß could be detected intracellularly. In the cocultures, large amounts of IL-1ß-precursor were detected after LPS-stimulation and even after Chol-stimulation. Also, mature IL-1ß was detected in the LPS-stimulated cocultures and very little also in the Chol-stimulated cells. Notably, both the SMC- and the monocyte-compartments contained large amounts of IL-1ß-precursor, which is in line with the mRNA-data shown above (compare Figure 8B). However, no mature IL-1ß was detected in the SMC-compartment, whereas the LPS-stimulated monocyte-compartment contained mature IL-1ß.

Taken together, in the cocultures, SMC produced more IL-6 mRNA than monocytes, which indicates that SMC might be the major source of IL-6 in the cocultures. Furthermore, in the cocultures, monocytes produced more IL-1ß mRNA, active caspase-1 and mature IL-1ß than the SMC, which indicates that the monocytes might be the major source of IL-1ß in the cocultures.

4.2 Statin retains the capacity of monocyte-derived macrophages to respond to endotoxin via the geranylgeranylation-pathway

4.2.1 Medium-pretreated macrophages lose the capacity to respond to endotoxin, whereas statin-pretreated macrophages retain the capacity to respond to endotoxin

During the atherogenic process, the invasion of monocytes from the blood into the vessel wall is a very important step. The invading, as well as resident monocytes in the vessel wall may differentiate into macrophages. Thus, the interaction of macrophages and SMC could be interesting to investigate. However, before comparing cytokine production in the SMC-macrophage cocultures to that in the SMC-monocyte cocultures, the function of separately cultured monocytes and macrophages in monocultures was investigated. The macrophages in our experiments were obtained by overnight incubation of freshly isolated monocytes, since this approach has been frequently used in literature (Kiener et al., 2001; Kuijk et al., 2008b). Since monocytes and macrophages only produced little IL-6 or IL-1ß upon Chol-stimulation in our system, the capacity of these cells to respond to endotoxin was investigated. Isoprenylation has been shown to be importantly involved in cell inflammation, differentiation or proliferation. However, discrepant data exist and the involvement of isoprenylation in the inflammatory response of monocytes and macrophages is not fully understood. Literature shows that isoprenylation can be inhibited by using statin, which has been shown to enhance the IL-1ß-expression in the macrophages. Thus, we treated

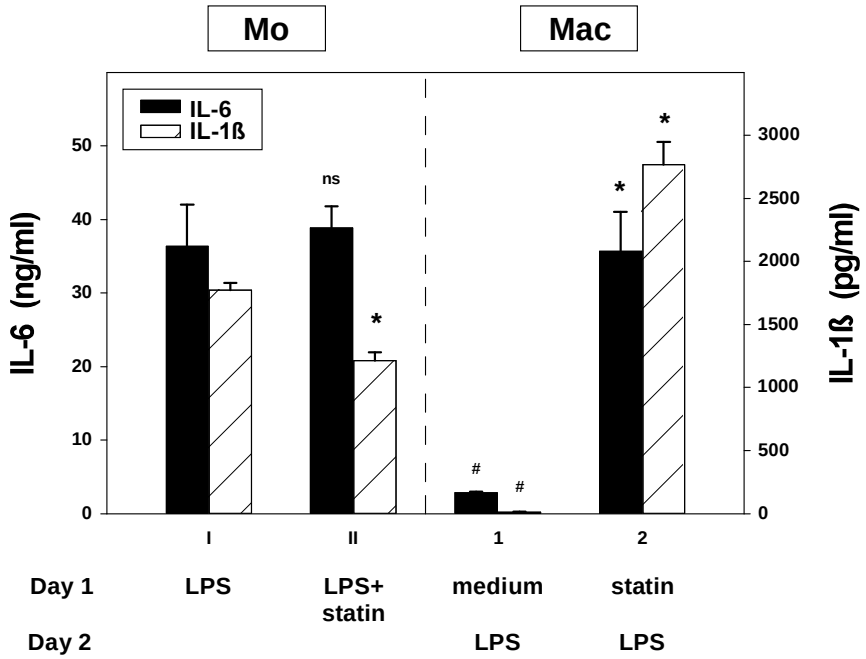

Figure 11. Medium-preincubated macrophages lose the capacity to respond to endotoxin, whereas statin-preincubated macrophages do not. Freshly isolated monocytes (100,000/cm^2) were incubated in 24-well plates (Mo, monocytes; left panel) and LPS (100 ng/ml) and fluvastatin (10 µg/ml) were added simultaneously to the cultures. Supernatants were harvested after 24 hours. In order to produce macrophage-cultures (Mac, macrophages; right panel), freshly isolated monocytes were incubated in the absence or presence of fluvastatin (10 µg/ml) for 24 hours, only then LPS (100 ng/ml) was added. Supernatants were harvested after further 24 hours. The IL-6 (black columns) and IL-1ß (hatched columns) concentration was determined in ELISA and presented as mean ± SD. The conditions in monocytes are labeled by Roman numerals (I, II) and the conditions in macrophages are labeled by Arabic numerals (1, 2). Significances were calculated by One Way ANOVA with subsequent post hoc analysis (LSD) ($^{\#}$, Mo vs. Mac, *, with statin vs. without statin, $p < 0.05$; ns, not significant). The data show one of five experiments with similar results.

monocytes and macrophages with statin to investigate the involvement of isoprenylation following the overnight differentiation. In Figure 11, the cytokine production of freshly isolated monocytes and overnight-preincubated macrophages is compared. Freshly isolated monocytes (Mo) expressed high levels of IL-6 (black columns) and IL-1ß (hatched columns) in response to LPS (condition I). In the present experiment, statin did not alter the IL-6-level in the monocyte cultures (condition II), but reduced the IL-1ß-expression to some degree. However, summarizing data of many experiments, these effects were not consistent. On the other hand, macrophages (Mac) incubated overnight without statin (condition 1) lost most of the capacity to produce IL-6 and IL-1ß, as compared to the monocytes. However, the statin-preincubated macrophages (condition 2) potently produced IL-6 and IL-1ß. These cytokine levels were 100- to 1,000-fold higher than the levels observed in macrophages prepared without statin. The cytokine production reached the cytokine levels of the freshly isolated monocytes or even surpassed it in some experiments. Taken together, these results indicated that macrophages incubated overnight without statin lose the capacity

to respond to endotoxin, whereas macrophages incubated overnight with statin do not lose this capacity.

4.2.2 Macrophages contain much less active caspase-1 (p10) than monocytes

We have shown above that medium-preincubated macrophages expressed little cytokines, whereas statin-preincubated macrophages expressed high levels of cytokines. It has been reported that THP-1-macrophages did not express mature IL-1ß due to a lack of active caspase-1 and that statin pretreatment induced the formation of active caspase-1 in these cells (Kuijk et al., 2008a). Thus, we investigated the intracellular caspase-1- and IL-1ß-expression in the monocytes and macrophages treated with or without statin by Western blot. Basically, all samples contained similar amount of protein, indicated by the comparable levels of GAPDH (Figure 12A). Figure 12 shows that both, monocytes and macrophages contained inactive caspase-1 (p30). The macrophages contained half the amount of p30, as compared to the monocytes. This suggestion was confirmed by summarizing the density analysis of this and 3 additional Western blot-data (Figure 12B). Statin-treatment did not potently enhance the expression of p30 in both, monocytes and macrophages. On the other hand, expression of caspase-1 (p10), which may reflect active caspase-1, was investigated.

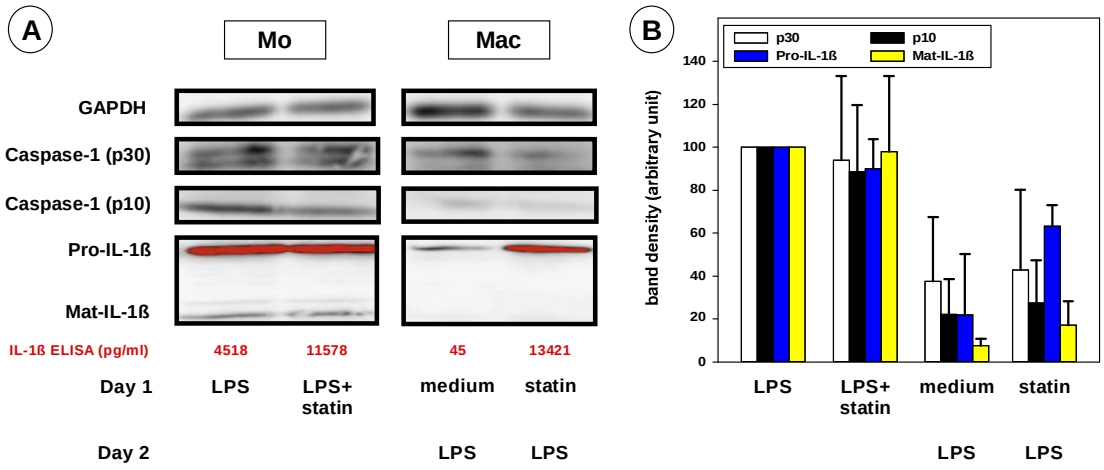

Figure 12. Cell lysates of macrophages contain less active caspase-1 (p10) than monocytes. Monocytes and macrophages were prepared as described in Figure 11, except that 6-well-plates were used. After removal of the supernatants, the cell layers were harvested and used for Western blot. A) For Western blot analysis, the cell layers were lysed with 2% SDS and used for GAPDH (GAPDH), caspase-1 (both, inactive caspase (p30) and active caspase (p10)) and IL-1ß (Pro-IL-1ß and Mat-IL-1) blots. B) The bands in the Western blot shown in 11A and three other experiments were analyzed in “TotalLab”, the p30, p10, Pro-IL-1ß and Mat-IL-1ß densities were normalized to GAPDH. The values after normalization in the LPS-stimulated monocytes were determined 100%. The mean and standard deviation of the values after normalization were calculated and presented. The data in A show one of four experiments with similar results.

The monocytes contained p10, whereas the macrophages contained much less. Normalized to GAPDH expression, the macrophages contained 22±16% of p10, as compared to the monocytes (100%). However, statin-treatment did not significantly enhance the expression of p10 (with statin vs. without statin; 28±20% vs. 22±16%, respectively). Since the Western blot measurement may be not sensitive enough to detect differences of activity of caspase-1, the amount of IL-1ß-precursor produced in the cells may also be important. The monocytes contained large amounts of intracellular IL-1ß-precursor, whereas the macrophages contained less. Statin-treatment did not alter the expression of IL-1ß-precursor in the monocytes. However, in the macrophages statin significantly increased the amount of IL-1ß-precursor. Furthermore, the monocytes even contained cell-associated mature IL-1ß, whereas the macrophages contained much less cell-associated mature IL-1ß. The IL-1ß ELISA measurement of the supernatants presented below the blots in Figure 12A showed high levels of IL-1ß suggesting that the IL-1ß-precursor of the statin-pretreated macrophages was processed and released. Taken together, the above results suggest that the macrophages produce and release less mature IL-1ß, but that statin-pretreatment enhances the IL-1ß-expression, probably by enhancing the maturation and release of IL-1ß.

4.2.3 Statin functionally alters the macrophages during the differentiation period

We have shown in Chapter 4.2.1 that LPS-treated macrophages pretreated without statin failed to express IL-1ß and IL-6, whereas the macrophages preincubated with statin were capable of producing high levels of these cytokines after the same endotoxin stimulation. For this statin effect, two explanations appear to be possible: 1) Statin may influence the differentiation from monocytes to macrophages during the first 24 hours of the macrophage-experiment (i.e., the differentiation period; compare Figure 4), thereby retaining the capacity of the cells to respond to endotoxin. 2) Statin and LPS may costimulate the macrophages to produce cytokines by some unknown interaction during the second 24 hours (i.e., the stimulation period). In order to investigate these two possibilities, we limited the presence of the statin to either the differentiation period or the stimulation period, respectively.

In the monocytes, similar to the data described above, statin did not potently change the IL-6- and IL-1ß-levels (Figure 13, conditions I and II). The macrophages pretreated without statin (condition 1) expressed only little cytokines, whereas macrophages pretreated with statin (condition 2) expressed high levels of cytokines. Limiting the presence of the statin to the differentiation period was achieved by washing the cells at day 2, in order to remove the statin before the addition of LPS. Washing of the macrophages preincubated without statin (condition 3) did not change the IL-6- and IL-1ß-production, as compared to the macrophages without washing (condition 1).

Macrophages pretreated with statin also underwent the washing procedure (condition 4). Importantly, although they lacked the statin during the stimulation period (i.e., the second 24 hours), these cells did not lose the capacity to produce IL-6 and IL-1ß, indicating that the statin-effect is obtained throughout the differentiation period (i.e., the first 24 hours).

In order to obtain further evidence for this suggestion, in parallel cultures we limited the presence of the statin to the stimulation period of the macrophages, by adding it together with LPS to cells preincubated overnight without statin, i.e. after the differentiation period (condition 5). Like the macrophages pretreated without statin (condition 1), these cells expressed only neglectable IL-6 and IL-1. This finding clearly indicates that statin does not costimulate the macrophages with LPS, however, suggests that statin functionally alters the cells during the differentiation period.

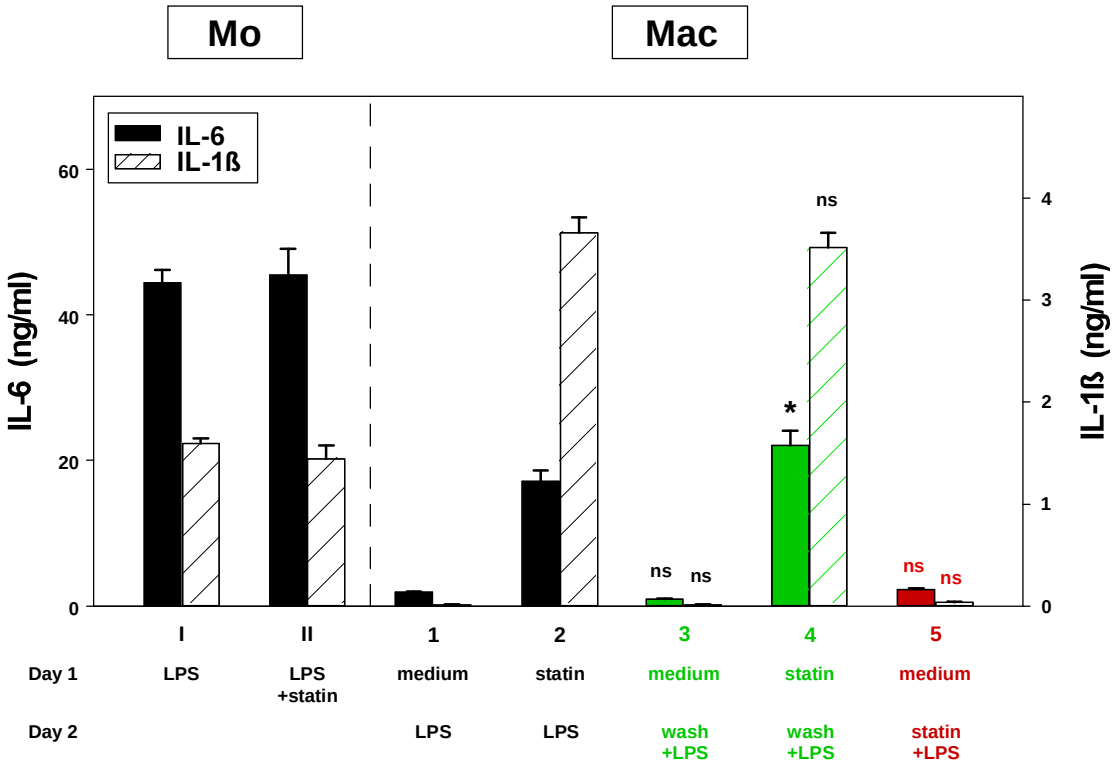

Figure 13. In order to retain the responsiveness of the cells, the presence of statin is essential during the macrophage differentiation period (day 1). The experimental design of condition I, II, 1 and 2 is described in Figure 11. In addition, based on the condition 1 and 2, parallel cultures, also incubated in the absence or presence of statin, were washed after the first 24 hours. Subsequently, LPS (100 ng/ml) was added (green filled and hatched columns, condition 3 and 4). To further cultures without statin-pretreatment, LPS together with statin was added without washing (red filled and hatched columns; condition 5). Supernatants were harvested after further 24 hours. The cytokine concentration was determined in ELISA and presented as mean ± SD. Significances were calculated by One Way ANOVA with subsequent post hoc analysis (LSD) (with wash vs. without wash, *, $p < 0.05$; ns, not significant (i.e., 1 vs.3 and 2 vs. 4, respectively); 1 vs. 5, red-colored ns). The data show one of five experiments with similar results.

4.2.4 Statin functionally alters the cells via the inhibition of the isoprenylation pathway, but not the cholesterol-synthesis pathway

We have shown above that macrophages differentiated in the presence of statin retained the capacity to respond to endotoxin. Literature has suggested the involvement of the isoprenoid pathway in cytokine production. In the isoprenoid pathway, statin inhibits the synthesis of mevalonic acid (Mev,compare Figure 3). In order to investigate the involvement of this pathway in the retainment-effect caused by statin during the differentiation of the macrophages, we added Mev in addition to statin. The standard conditions (Figure 14; conditions I, II, 1 and 2) worked as shown above. Addition of Mev to the freshly isolated monocytes (Figure 14; condition VI) slightly reduced the cytokine expression in the present experiment, however, this reduction was not observed in a second experiment. In contrast, addition of Mev to the statin-pretreated macrophages (condition 6) completely reversed the retainment-effect caused by the statin (compare condition 2). This reversal of the statin-effect was very potent, since the statin/Mev-pretreated cultures (condition 6) did not produce more IL-6 and IL-1 than the medium-pretreated macrophages (condition 1). These results

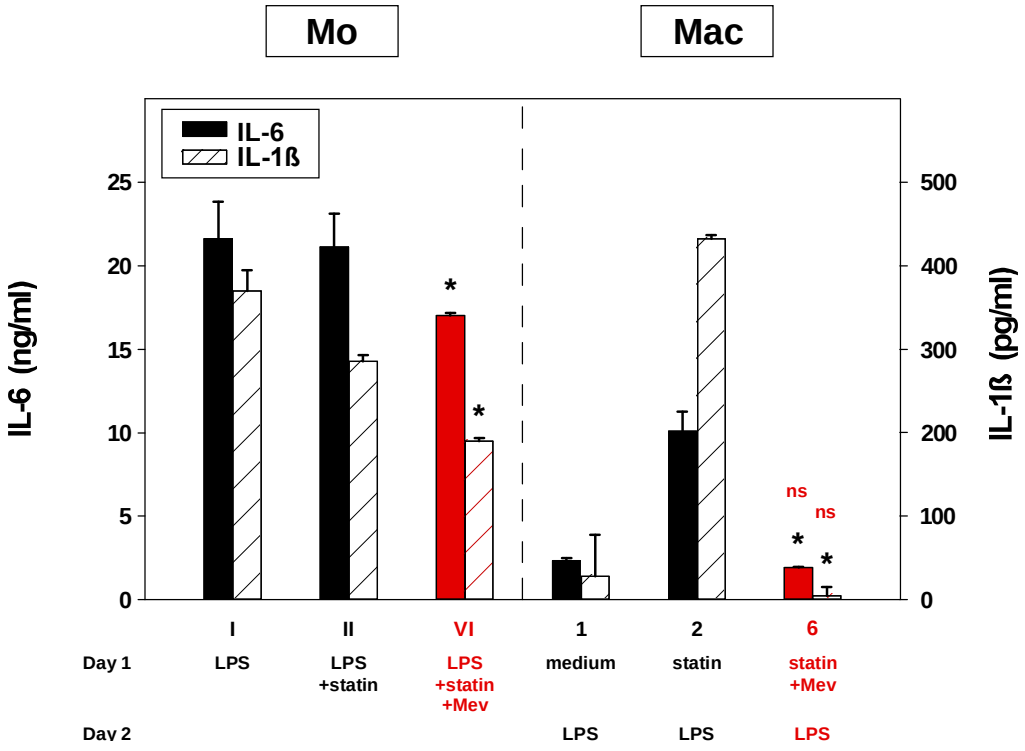

Figure 14. Mevalonic acid reverses the retainment-effect caused by the statin. The experimental design of condition I, II, 1 and 2 is described in Figure 11. In the parallel cultures to II and 2, mevalonic acid (Mev, 50 µM) was added in addition to statin (condition VI and 6). Supernatants were harvested, the cytokine concentration was determined in ELISA and presented as mean ± SD. Significances were calculated by One Way ANOVA with subsequent post hoc analysis (LSD) (with Mev vs. without Mev, *, $p < 0.05$) (condition 1 vs. 6, red-colored ns, not significant). The data show one of two experiments with similar results.

suggested that inhibition of the isoprenoid pathway may be involved in the retainment-effect caused by statin on the macrophages.

Since the addition of Mev completely reversed the retainment-effect in the macrophages, downstream products of the isoprenoid pathway, farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP) were also applied. Both, FPP and GGPP are important in isoprenylation of small GTPases, such as Ras and Rho family proteins, which may be importantly involved in the statin-effect. If farnesylation or geranylgeranylation are involved in the retainment-effect mediated by statin, FPP and GGPP, respectively, should block the retainment-effect. In the freshly isolated monocytes, addition of FPP (Figure 15; condition VII) did not alter the cytokine expression (compare condition II). In contrast, addition of FPP (condition 7) potently reversed the retainment-effect caused by statin in the macrophages (compare condition 2). Similar to the data obtained by addition of Mev, the statin/FPP-pretreated macrophages (condition 7) did not produce much more cytokine than the medium pretreated macrophages (condition 1).

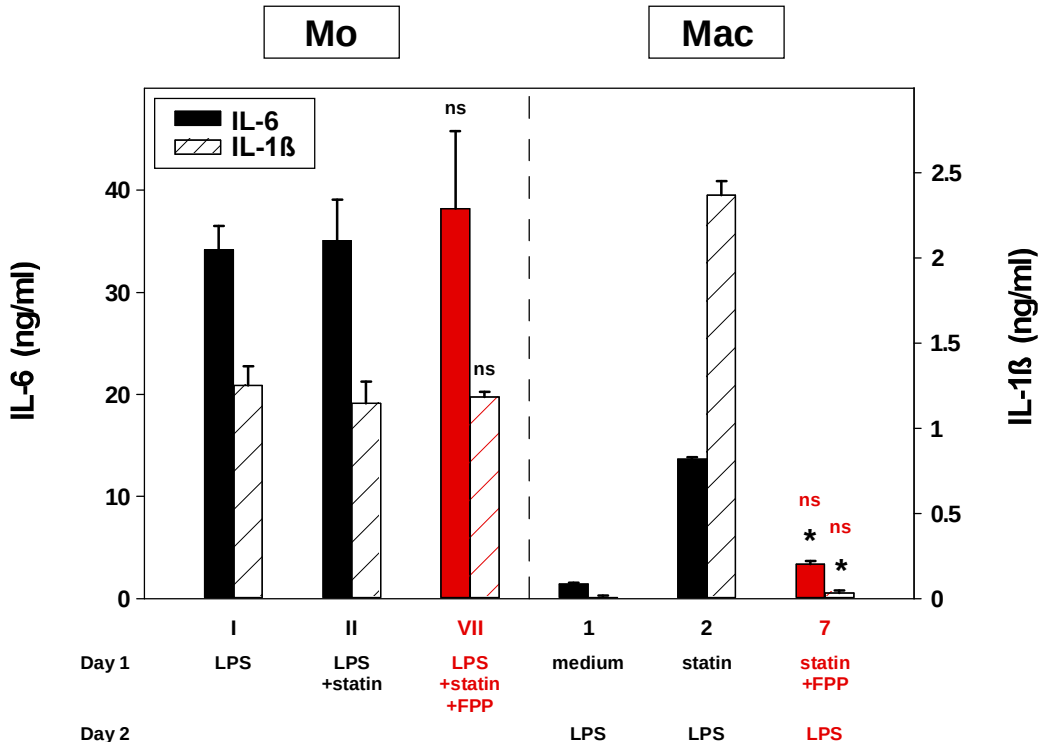

Figure 15. FPP reverses the retainment-effect. The experimental design is similar to the description in Figure 14, except that FPP (20 µM) instead of Mev was added in addition to statin (condition VII and 7). Supernatants were harvested, the cytokine concentration was determined in ELISA and presented as mean ± SD. Significances were calculated by One Way ANOVA with subsequent post hoc analysis (LSD) (with FPP vs. without FPP, *, $p < 0.05$) (condition 1 vs. 7, red-colored ns, not significant). The data show one of two experiments with similar results.

Like FPP, addition of GGPP (Figure 16, condition VIII) did not potently alter the cytokine expression in the freshly isolated monocytes (compare condition II). In the macrophages, addition of GGPP (condition 8) potently reversed the retainment-effect (compare condition 2). Similar to the FPP-data, the statin/GGPP-pretreated macrophages (condition 8) did not produce more cytokine than the medium-pretreated macrophages (condition 1).These data indicate that inhibition of farnesylation and/or geranylgeranylation may be involved in the retainment-effect caused by statin.

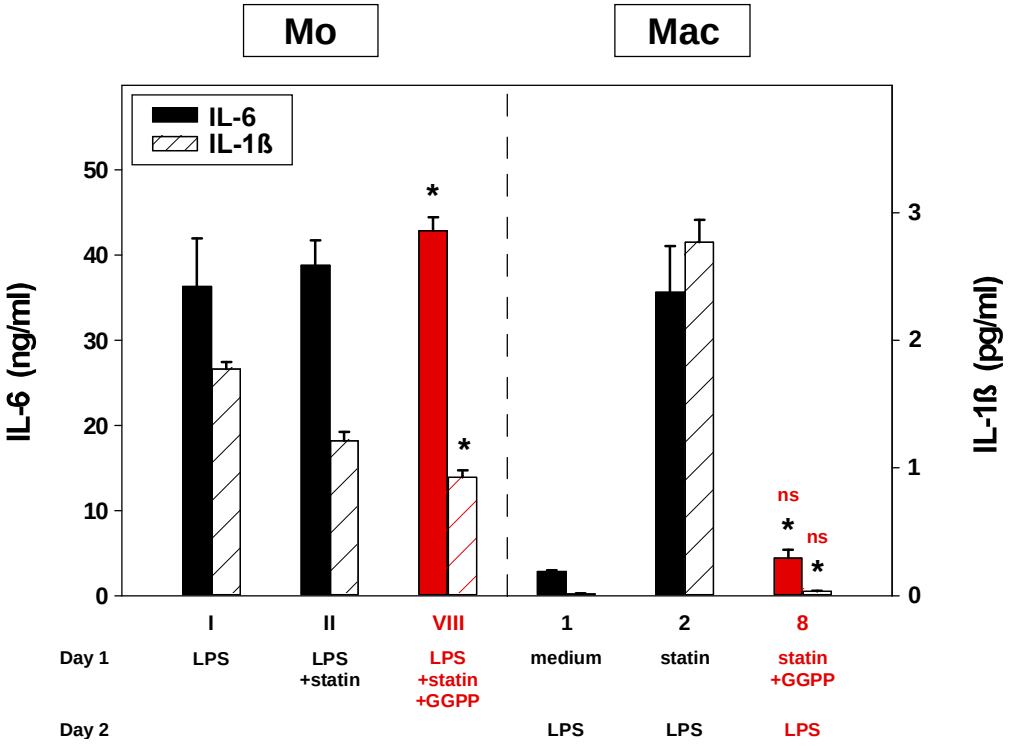

Figure 16. GGPP reverses the retainment-effect. The experimental design is similar to the description in Figure 14, except that GGPP (20 µM) instead of Mev was added in addition to statin (condition VIII and 8). Supernatants were harvested, the cytokine concentration was determined in ELISA and presented as mean ± SD. Significances were calculated by One Way ANOVA with subsequent post hoc analysis (LSD) (with GGPP vs. without GGPP, *, $p < 0.05$) (condition 1 vs. 8, red-colored ns, not significant). The data show one of six experiments with similar results.

We have shown above that addition of mevalonic acid, FPP or GGPP abolished the retainment-effect caused by statin in the macrophages. Since FPP is also an intermediate of squalene, which leads to cholesterol synthesis, the involvement of the cholesterol-synthesis pathway in the statin-mediated macrophage-retainment was analyzed. For this purpose, we incubated macrophages differentiated in the presence of statin with squalene. In the freshly isolated monocytes, addition of squalene (Figure 17, condition IX) did not alter the IL-6- and IL-1ß-expression, as compared to the condition without squalene (condition II). However, different from Mev, FPP and GGPP, in the overnight differentiated macrophages, squalene (condition 9) did not reduce the

retainment-effect mediated by statin (compare condition 2). This result indicates that the cholesterol-synthesis pathway is not involved in the retainment-effect.

Taken together, these results indicated that the statin retains the capacity of macrophages to respond to endotoxin mostly via the inhibition of the isoprenylation pathway, and that the cholesterol-synthesis pathway is not potently involved.

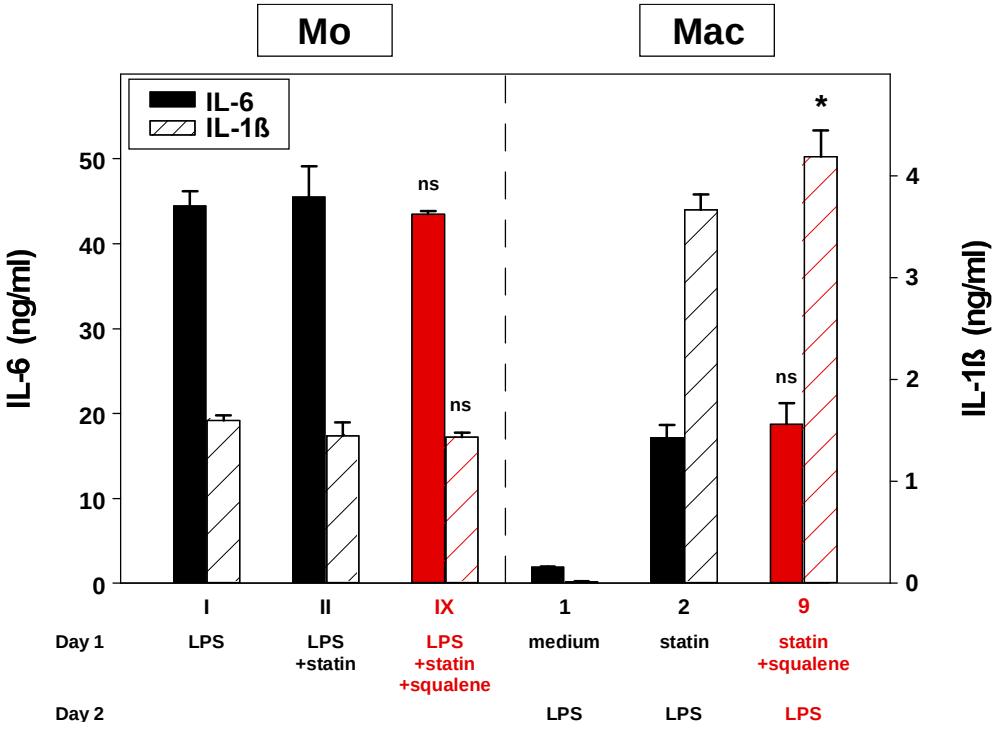

Figure 17. Squalene does not reverse the retainment-effect. The experimental design is similar to the description in Figure 14, except that squalene (20 µM) instead of Mev was added in addition to statin (condition IX and 9). Supernatants were harvested, the cytokine concentration was determined in ELISA and presented as mean ± SD. Significances were calculated by One Way ANOVA with subsequent post hoc analysis (LSD) (with squalene vs. without squalene, *, $p < 0.05$; ns, not significant). The data show one of two experiments with similar results.

4.2.5 Geranylgeranylation contributes to both IL-6- and IL-1ß-expression

We have shown above that statin alters the capacity of the macrophages to produce cytokines, possibly via the inhibition of the isoprenylation pathway, rather than via the cholesterol-synthesis pathway. The use of specific inhibitors, such as geranylgeranyl transferase-inhibitor (GGTI), farnesyl transferase-inhibitor (FTI) for the isoprenylation pathway, as well as zaragozic acid-A (ZAA) for the cholesterol-synthesis pathway, may provide further evidence for this suggestion. Thus, we replaced statin by GGTI, FTI or ZAA (Figure 18, green columns), as well as by combinations of two (blue columns) or

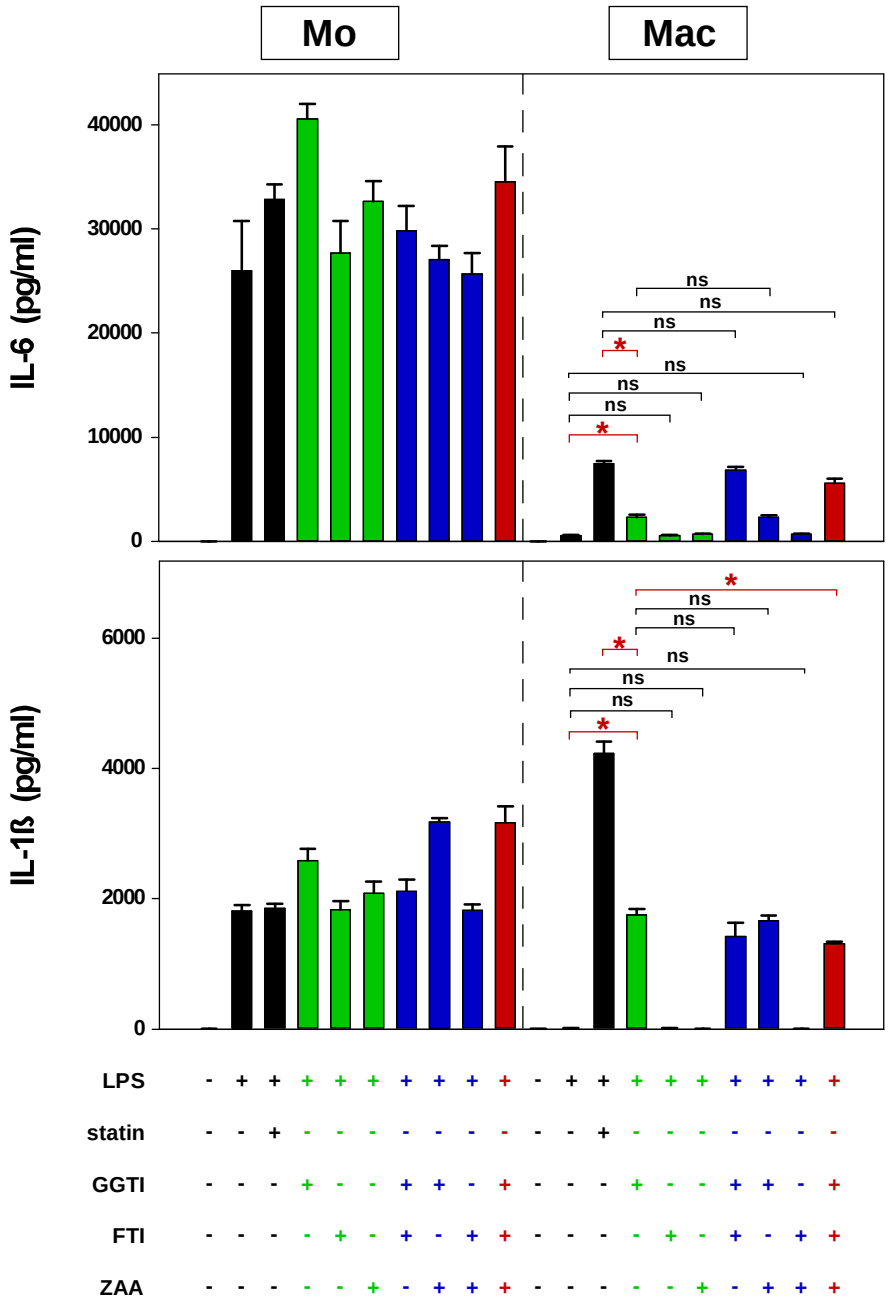

Figure 18. Like statin, GGTI provides some retainment of the cytokine production in the macrophages. The experimental design was similar to the description in Figure 11, except that in parallel cultures, statin was replaced by inhibitors (GGTI (8 µM), FTI (8 µM), ZAA (20 µM)) (green columns) or combinations of two (blue columns) or three (red columns) of these inhibitors. Supernatants were harvested after further 24 hours. The IL-6- or IL-1ß-concentration was determined in ELISA and presented as mean ± SD. Significances were calculated by One Way ANOVA with subsequent post hoc analysis (LSD) (*, $p < 0.05$; ns, not significant). The data show one of two experiments with similar results.

three (red columns) of these inhibitors. Similar to statin, in the freshly isolated monocytes, GGTI, FTI, ZAA and the combinations of these inhibitors had no clear effect on the IL-6- and IL-1ß-expression (Figure 18, Mo). Also, similar to statin, the

macrophages preincubated with GGTI expressed more IL-6 and IL-1ß, as compared to the cells preincubated with medium only. However, the IL-6- and the IL-1ß-levels in these macrophages were lower than in the statin-preincubated macrophages. Different from GGTI, the macrophages preincubated with FTI alone did not express more IL-6 or IL-1ß than the macrophages peincubated with medium, which may imply that inhibition of farnesylation is not involved. No retainment was also observed if the macrophages were preincubated with ZAA, which was in accordance with the squalene-data shown above.

In the inhibitor-combination experiments, the macrophages preincubated with both GGTI and FTI expressed a higher IL-6-level than the separate compounds, and obtained a similar level of IL-6 as the macrophages treated with statin (black column). On the other hand, the IL-1ß-level in the GGTI/FTI-coincubated macrophages was similar to the macrophages preincubated with GGTI alone, and was lower than the IL-1ß-level in the statin-preincubated macrophages. The combination with ZAA did not enhance the levels of IL-6 and IL-1ß in any tested combinations. Taken together, these results support the idea that statin functionally alters the cells via the inhibition of the isoprenylation pathway and that the cholesterol-synthesis pathway is not involved.

4.2.6 Blockade of the retainment-effect by addition of geranylgeranyl pyrophosphates is reversed by geranylgeranyl transferase-inhibitor

We have shown above that addition of GGPP completely blocked the retainment-effect caused by the statin. In line with this result, and similar to statin, GGTI potently enhanced the IL-6- and IL-1ß-expression in the macrophages, which indicated that inhibition of geranylgeranylation may be importantly involved in the retainment-effect caused by statin. In order to demonstrate the involvement of GGPP more directly, we added the GGPP together with statin to the macrophages in the absence or presence of GGTI. In statin/GGPP-preincubated macrophages, GGPP dose-dependently inhibited the IL-6- and IL-1ß-levels (Figure 19; red columns). The IL-6- and IL-1ß-levels were inhibited by high amounts of GGPP to the level present in the macrophages without statin, emphasising the importance of geranylgeranylation. In the GGPP-treated macrophages, addition of GGTI (yellow columns) recovered the IL-6-expression to the level similar to that of the statin-preincubated macrophages. The IL-1ß-level in the statin-GGPP-pretreated macrophages was also recovered by the treatment of GGTI. Taken together, these results show that GGPP blocks the retainment-effect caused by statin, supporting the idea that inhibition of geranylgeranylation potently contributes to the retainment-effect.

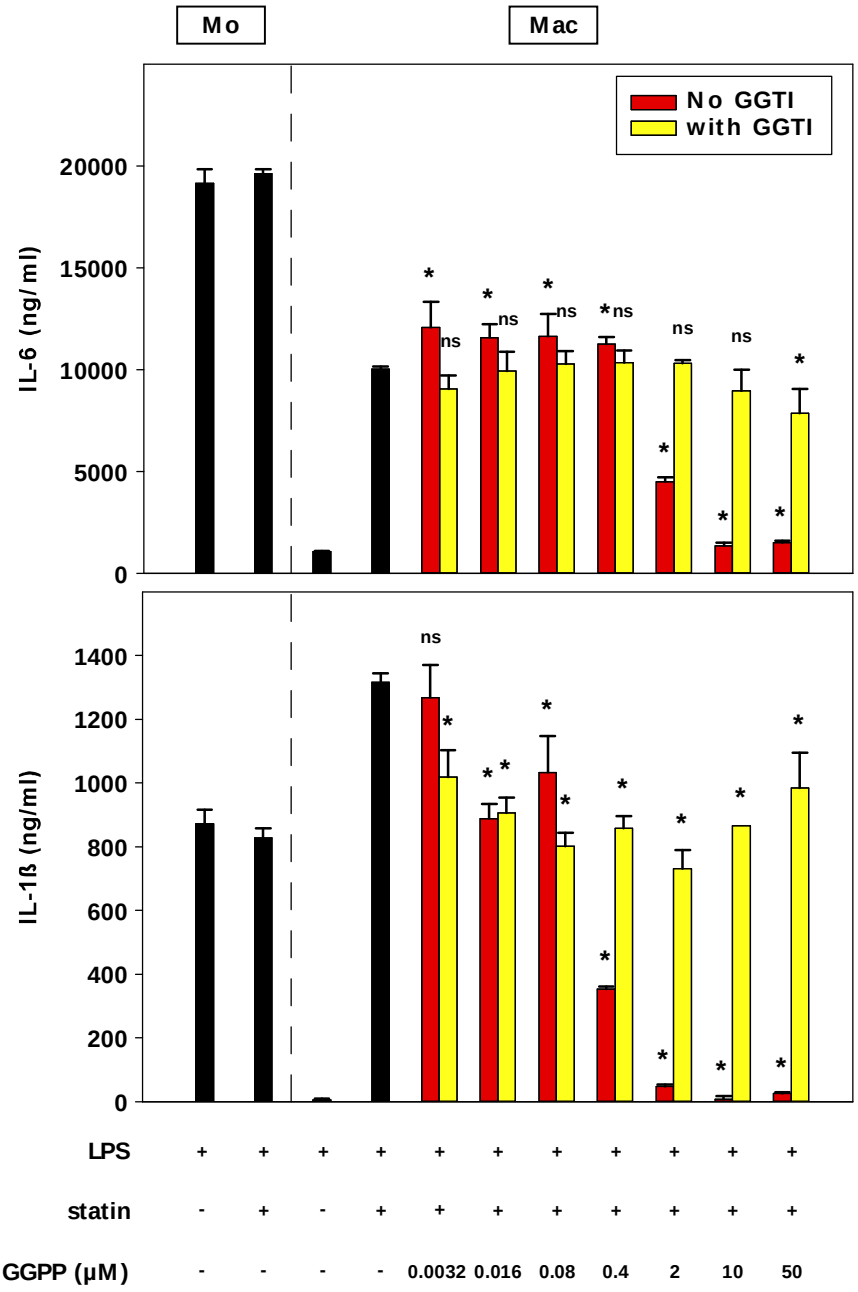

Figure 19. Inhibition of the retainment-effect by GGPP is reversed by addition of GGTI. The experimental design is similar to the description in Figure 14, except that instead of Mev, GGPP (3.2, 16, 80, 400 nM, 2, 10 and 50 µM) was added in the absence or presence of GGTI (8 µM) to the statin. This was performed only in the macrophage cultures, since only these cells showed the retainment by statin. Supernatants were harvested after further 24 hours. The IL-6- and IL-1ß-concentrations were determined in ELISA and presented as mean ± SD. Significances were calculated by One Way ANOVA with subsequent post hoc analysis (LSD) (statin vs. statin+GGPP, and statin vs. statin+GGPP+GGTI, *, $p < 0.05$; ns, not significant). The data show one of two experiments with similar results.

4.2.7 Inhibition of Rac1-, PI3K- and p38-activity blocks the cytokine expression in statin-pretreated macrophages

We have shown above that inhibition of geranylgeranylation may be involved in the retainment-effect observed in statin-pretreated macrophages. Activation of small GTPases may be involved in this process, since they are prenylation targets. It has been reported that inhibition of Rac1-activation by NSC23766 abolished the IL-1ß-expression in statin-treated THP-1 cells (Kuijk et al., 2008a), thus, we used this inhibitor in the present model to investigate the involvement of Rac1-activation in the

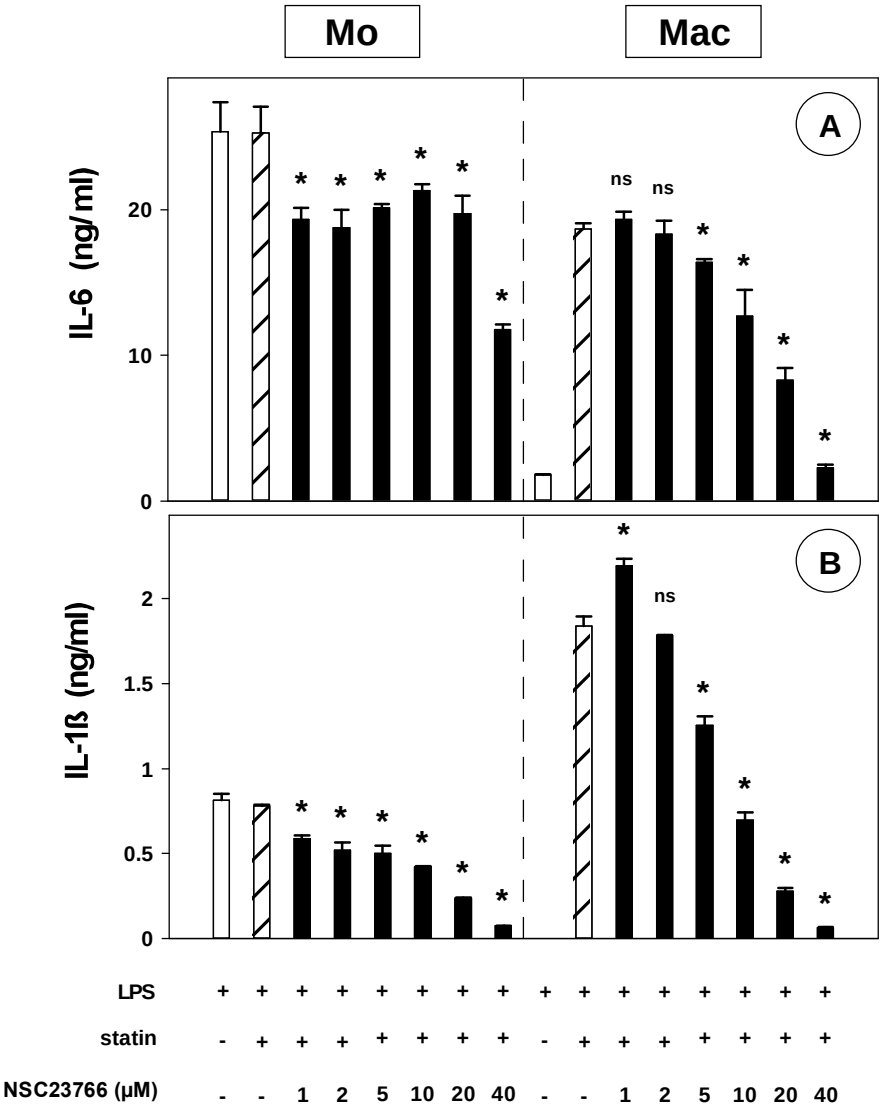

Figure 20. The retained cytokine production in statin-pretreated macrophages is reversed by the Rac1-inhibitor NSC23766. The experimental design is similar to the description in Figure 14, except that the Rac1-inhibitor NSC23766 (1, 2, 5, 10, 20 and 40 µM) instead of Mev was added in addition to statin. Supernatants were harvested after further 24 hours. The IL-6- and IL-1ß- concentration was determined in ELISA and presented as mean ± SD. Significances were calculated by One Way ANOVA with subsequent post hoc analysis (LSD) (with inhibitor vs. without inhibitor; *, $p < 0.05$; ns, not significant). The data show one of four experiments with similar results.

retainment-effect. The LPS-, as well as the LPS+statin-treated monocytes expressed high levels of IL-6. Treatment of NSC23766 reduced the IL-6-level to some degree (Figure 20). In addition, the LPS- and LPS+statin-treated monocytes expressed high levels of IL-1ß. Treatment of the monocytes with NSC23766 reduced the IL-1ß-level dose-dependently (Figure 20, IL-1ß). Since statin did not potently alter the IL-6- and IL-1ß-expression in the monocytes, the reduction by the Rac1-inhibitor may be independent from the statin effect. In contrast to the monocyte-data, only the statin-preincubated macrophages showed the capacity to produce IL-6 and IL-1ß in response to LPS. In the statin-preincubated macrophages, the Rac1-inhibitor blocked this retainment-effect dose dependently. The reduction of both, the IL-6- and IL-1ß-production, was present starting from 5 µM. Taken together, the data indicate that inhibition of Rac1-activation reverses the retainment-effect in the macrophages.

The above data indicate that Rac1 may be involved in the retainment-effect caused by statin in the macrophages. Although the Rac1-signaling pathway has not been finally established, interference of Rac1 with downstream mediators, such as PI3K, p38, ERK or JNK, has been suggested in some reports. Regulation of such pathways by Rac1 may contribute to regulation of inflammation and / or differentiation in monocytes and macrophages. Thus, we also investigated the effect of the PI3K-inhibitor LY294002, the p38-MAPK-inhibitor SB203580, the ERK1/2-inhibitor PD98059 and the JNK-inhibitor SP600125 on the retainment-effect observed in macrophages.

In statin-treated monocytes 20 and 40 µM of the PI3K-inhibitor LY294002 altered the IL-6-level (Figure 21). The IL-1ß-level in the statin-treated monocytes was enhanced by 1 to 20 µM of this inhibitor, whereas the IL-1ß-level was only reduced by 40 µM (Figure 21). In contrast, in the statin-pretreated macrophages, the PI3K-inhibitor dose-dependently blocked the high levels of IL-6- and IL-1ß-expression caused by the statin-treatment. Summarizing the data of 3 experiments, the IL-6 was significantly reduced by the inhibitor starting from 2 µM, whereas the IL-1ß was significantly reduced starting from 5 µM. The results indicate that activation of PI3K, which may function downstream of Rac1-activation, may be involved in the retainment-effect caused by statin on macrophages.

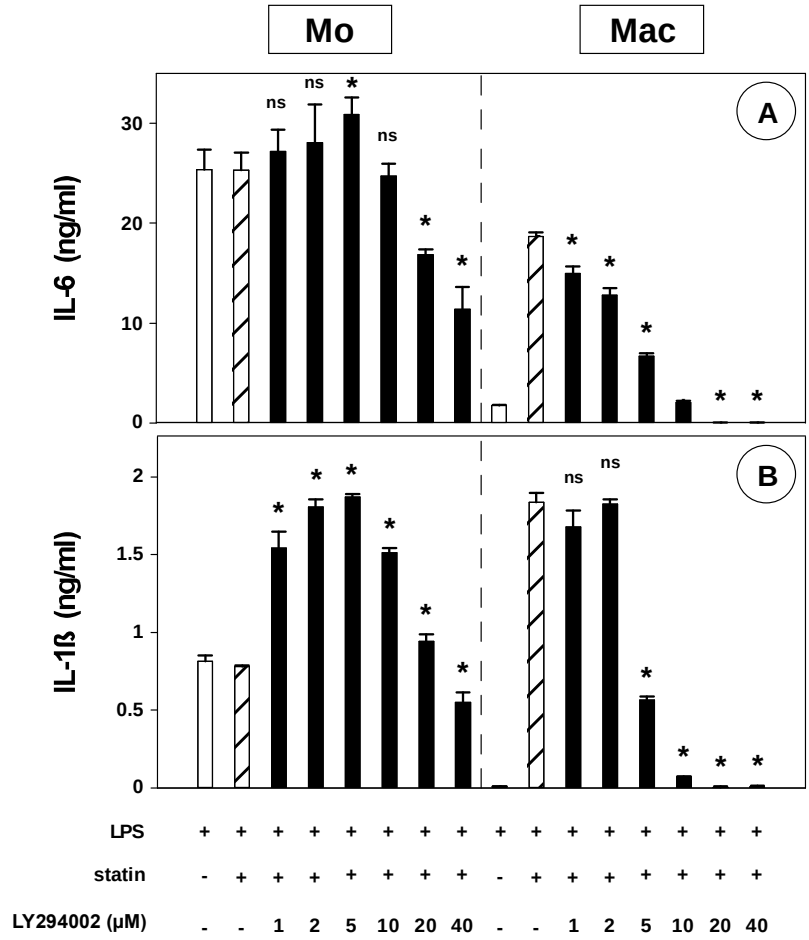

Figure 21. The retained cytokine production in statin-pretreated macrophages is reversed by the PI3K-inhibitor LY294002. The experimental design is similar to the description in Figure 14, except that the PI3K-inhibitor LY294002 (1, 2, 5, 10, 20 and 40 µM) instead of Mev was added in addition to statin. Supernatants were harvested after further 24 hours. The IL-6- and IL-1ß- concentration was determined in ELISA and presented as mean ± SD. Significances were calculated by One Way ANOVA with subsequent post hoc analysis (LSD) (with inhibitor vs. without inhibitor; *, $p < 0.05$; ns, not significant). The data show one of three experiments with similar results.

In monocytes, the p38-MAPK-inhibitor SB203580 potently reduced the IL-6-expression from 1 to 20 µM and the IL-6-expression was further reduced by 40 µM of the inhibitor (Figure 22). The IL-1ß-expression was also potently reduced by the low inhibitor concentrations (1 – 10 µM), however, in the monocytes treated with 20 or 40 µM of the inhibitor, no inhibition was present (Figure 22). On the other hand, in the statin-pretreated macrophages, the p38-MAPK-inhibitor dose-dependently reduced the IL-6- and IL-1ß-level. These results indicate that activation of p38-MAPK, which may function downstream of Rac1-activation, may be involved in the retainment-effect caused by statin on the macrophages.

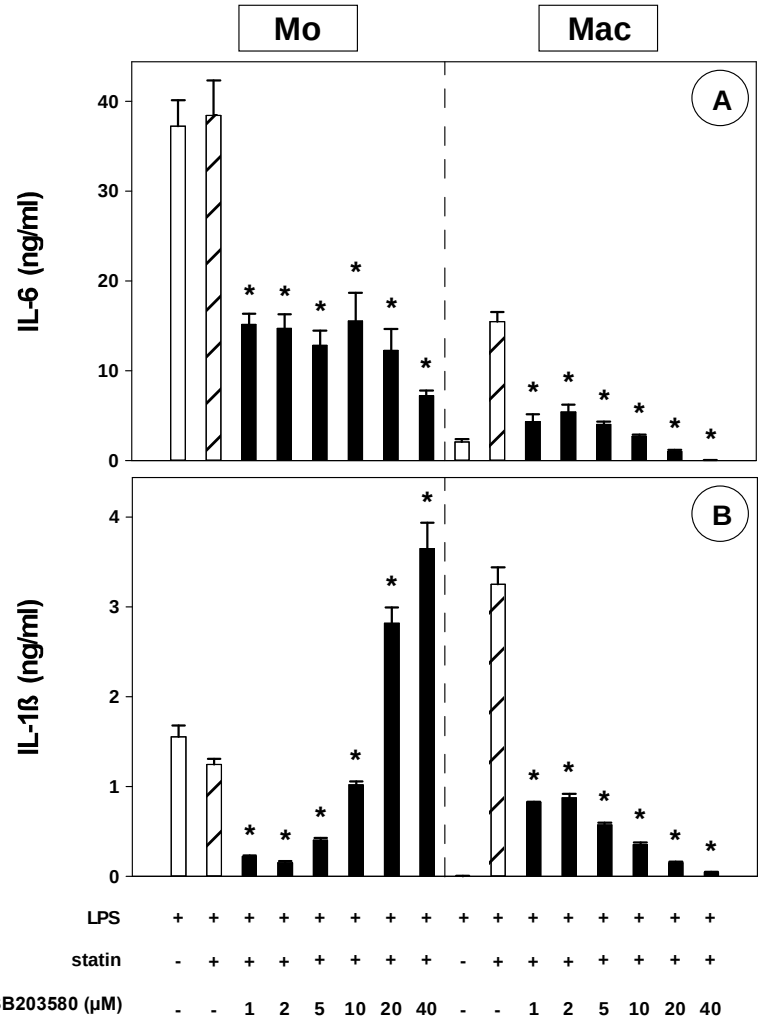

Figure 22. The retained cytokine production in statin-pretreated macrophages is reversed by the p38-MAPK-inhibitor SB203580. The experimental design is similar to the description in Figure 14, except that the p38-MAPK-inhibitor SB203580 (1, 2, 5, 10, 20 and 40 µM) instead of Mev was added in addition to statin. Supernatants were harvested after further 24 hours. The IL-6- and IL-1ß-concentration was determined in ELISA and presented as mean ± SD. Significances were calculated by One Way ANOVA with subsequent post hoc analysis (LSD) (with inhibitor vs. without inhibitor; *, $p < 0.05$; ns, not significant).The data show one of three experiments with similar results.

In the statin-treated monocytes the ERK1/2-inhibitor PD95059 did not potently alter the IL-6-expression from 1 to 20 µM (Figure 23). The IL-6-level was only reduced by 40 µM of the inhibitor. The IL-1ß-level was enhanced to some degree by 1 to 20 µM of the inhibitor, whereas addition of 40 µM the inhibitor did not potently alter the IL-1ß-expression (Figure 23). In the statin-pretreated macrophages, different from Rac1-, PI3K- and p38-inhibition-data mentioned above, the IL-6-level was not reduced by this inhibitor. The IL-1ß-levels in the statin-pretreated macrophages were not reduced by 1

to 20 µM of this inhibitor. Only 40 µM inhibitor reduced the IL-1ß-expression. These results indicate that activation of ERK1/2 may be not involved in the retainment-effect.

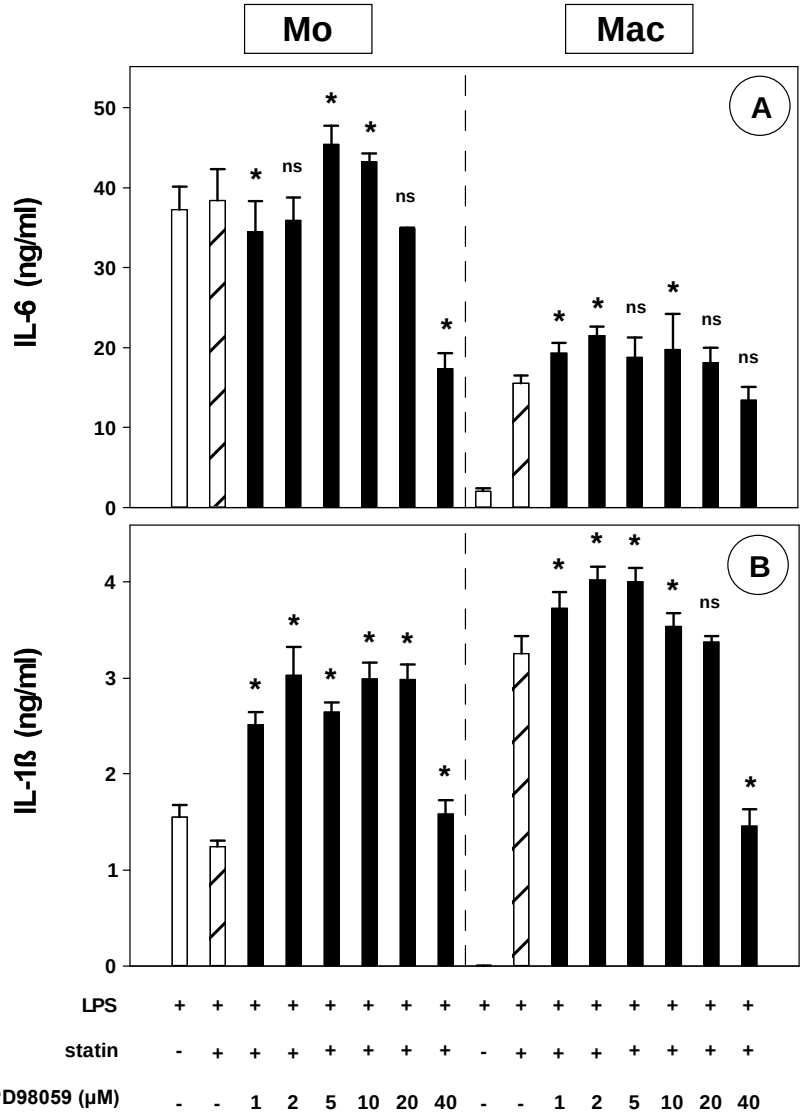

Figure 23. The retained cytokine production in statin-pretreated macrophages is not reversed by the ERK1/2-inhibitor PD98059. The experimental design is similar to the description in Figure 14, except that instead of Mev, the ERK1/2-inhibitor PD98059 (1, 2, 5, 10, 20 and 40 µM) was added. Supernatants were harvested after further 24 hours. The IL-6- and IL-1ß-concentration was determined in ELISA and presented as mean ± SD. Significances were calculated by One Way ANOVA with subsequent post hoc analysis (LSD) (with inhibitor vs. without inhibitor; *, $p < 0.05$; ns, not significant). The data show one of three experiments with similar results.

In the statin-treated monocytes 1 to 20 µM the JNK-inhibitor SP600125 did not reduce the IL-6-expression (Figure 24). The IL-6 was only reduced by 40 µM of the inhibitor. Furthermore, the IL-1ß-levels were enhanced by all concentrations of this inhibitor (Figure 24). In the statin-pretreated macrophages, only 40 µM of the inhibitor reduced

the IL-6-expression and the IL-1ß-level was only reduced by 20 or 40 µM of the inhibitor. Since only the high concentration of the inhibitor reduced the IL-6- and IL-1ß-expression in the statin-preincubated macrophages, these results indicate that activation of JNK may not be potently involved in the retainment-effect.

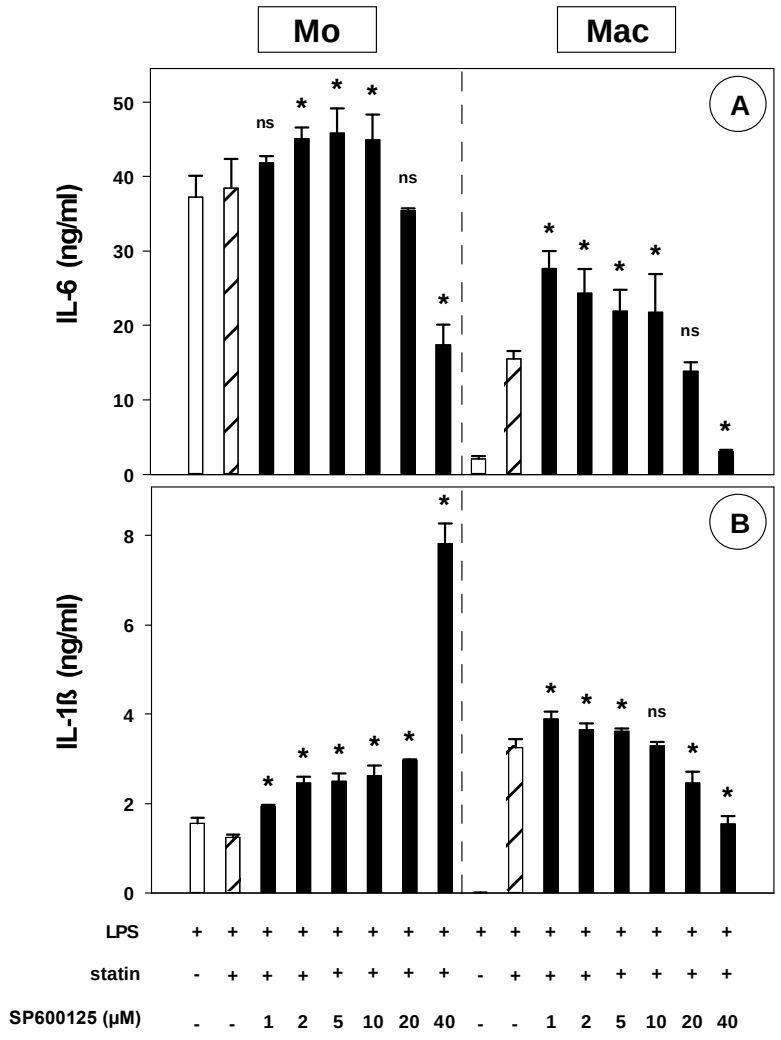

Figure 24. The retained cytokine production in statin-pretreated macrophages is not reversed by the JNK-inhibitor SP600125. The experimental design is similar to the description in Figure 14, except that instead of Mev, the JNK-inhibitor SP600125 (1, 2, 5, 10, 20 and 40 µM) was added. Supernatants were harvested after further 24 hours. The IL-6- and IL-1ß-concentration was determined in ELISA and presented as mean ± SD. Significances were calculated by One Way ANOVA with subsequent post hoc analysis (LSD) (with inhibitor vs. without inhibitor; *, $p < 0.05$; ns, not significant). The data show one of three experiments with similar results.

Taken together, the inhibition-data indicate that the retainment-effect caused by statin on macrophages may depend on Rac1-, PI3K- and p38-MAPK-regulation. The ERK1/2- and JNK-regulation pathways do not appear to be involved in the retainment-effect.

4.2.8 Inhibition of Rac1-, PI3K- and p38-MAPK-activation blocks the retainment-effect on the macrophages during the differentiation period

In chapter 4.2.7, we have shown that inhibition of Rac1-, PI3K- and p38-MAPK-activation blocks the cytokine expression retained by statin-pretreatment in the macrophages. However, it was not clear whether or not these inhibitors functioned by influencing the retainment-effect caused by statin during the differentiation period, or by influencing the inflammatory signaling pathways during the stimulation period. Therefore, we restricted the presence of the inhibitors to the differentiation period or the stimulation period.

In line with the data shown above, in the statin-treated monocytes, the Rac1-inhibitor NSC23766 did not potently reduce the IL-6-level, but reduced the IL-1ß-level to some degree (Figure 25, red columns). The PI3K-inhibitor LY294002 did not alter the IL-6-level and enhanced the IL-1ß-level (blue columns). The p38-MAPK-inhibitor SB203580 reduced both the IL-6- and IL-1ß-levels (green columns). On the other hand, in the statin-pretreated macrophages, the standard application (i.e., presence throughout the differentiation and the stimulation period) of all three inhibitors potently reversed the retainment-effect of the statin on the IL-6- and IL-1ß-expression (Figure 25; condition a). When the presence of the inhibitors was limited to the stimulation period of the macrophages, the IL-6-expression was not reduced by any of these inhibitors (condition b) and only the p38-MAPK-inhibitor blocked IL-1ß-production to some degree (condition b; green column). However, this inhibition was not as potent as observed in the standard treatment (compare condition a). In contrast, when we limited the presence of the inhibitors to the differentiation period by washing after the first 24 hours (i.e., before the addition of LPS) (condition c), the retainment of IL-6 and IL-1ß was abolished by all three inhibitors, to a similar degree observed in the standard procedure.

Taken together, the above data indicate that the inhibition of Rac1, PI3K and p38-MAPK may block the retainment-effect, observed during the differentiation period, rather than influencing the inflammatory signaling pathways during the stimulation period.

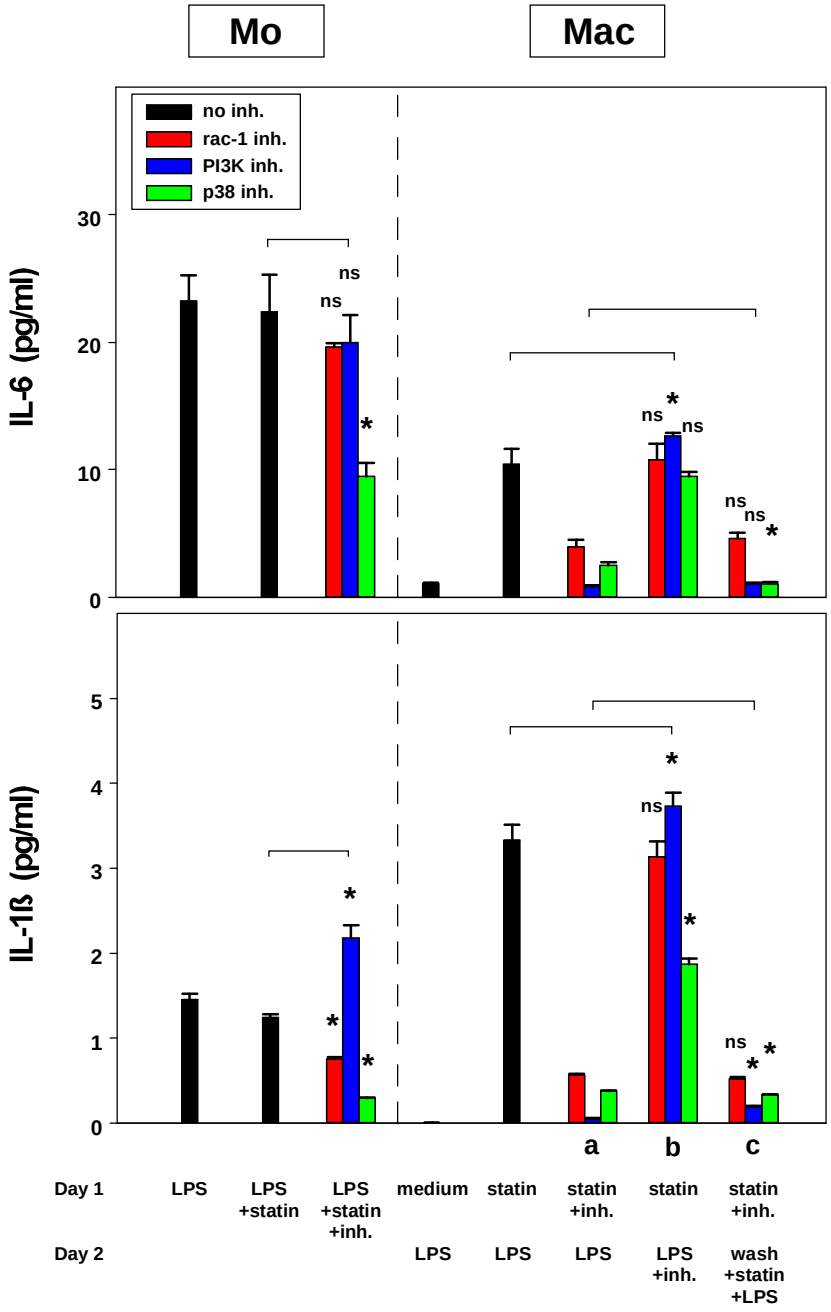

Figure 25. The blockade of the retainment by the Rac1-, PI3K- and p38-MAPK-inhibitors is obtained during the differentiation period (day 1), but not during the stimulation period (day 2). The experimental design for the standard conditions (black columns) was described in Figure 11. In the monocyte-cultures, the inhibitors (NSC23766, red columns; LY294002, blue columns; SB203580, green columns; 10 µM) were separately added simultaneously to the freshly isolated monocytes. Supernatants were harvested after 24 hours. In the macrophage-cultures, the inhibitors were added simultaneously with the statin. LPS (100 ng/ml) was added 24 hours later (condition a). In parallel macrophage-cultures, the inhibitors were added at day 2, together with LPS (condition b). In addition, further parallel macrophage-cultures were incubated as described in condition a, however, the inhibitors were removed prior to the addition of LPS at day 2 (condition c). Supernatants were harvested after further 24 hours. The IL-6- and IL-1ß-concentrations were determined in ELISA and presented as mean ± SD. Significances were calculated by One Way ANOVA with subsequent post hoc analysis (LSD) (*, $p < 0.05$; ns, not significant). The data show one of two experiments with similar results.

4.2.9 Statin retains the capacity of overnight-incubated macrophages to respond to endotoxin via activation of Rac1

We have shown above that inhibition of Rac1-activation potently inhibited the cytokine expression in statin-preincubated macrophages and that the presence of the Rac1-inhibitor is necessary during the first 24 hours (differentiation period). These results implicated that upregulation of Rac1-activation in the differentiation period may contribute to the retainment-effect caused by statin on the macrophage cytokine production. Thus, we investigated the activation status of Rac1 at this time point in Rac1 pulldown assay and Western blot analysis, rather than after the second 24 hours (stimulation period). The supernatants of these cultures did not contain IL-1ß (data not show), since the cells will be stimulated only after the differentiation period, however, the regulation pathways altered by statin may have taken place already. This suggestion is supported by the above chapter (4.2.3) showing that addition of statin during the stimulation period does not provide the retainment-effect (compare Figure 13). The pulldown assay shows that the statin-treated cells contained more active Rac1-GTP than the cells treated without statin (Figure 26A). Quantification of the Rac1-GTP bands showed that the expression of Rac1-GTP in the statin-treated cells was about 3-fold higher than in the cells treated without statin (Figure 26B). Parallel cultures to the pulldown analysis were stimulated, further incubated for 24 hours and IL-1ß-production was measured. As shown above, the statin-treatment caused the retainment-effect (none, 20 pg/ml IL-1ß; statin, 28085 pg/ml IL-1ß). Taken together, these data confirm the suggestion that Rac1-activation may be involved in the retainment-effect caused by the statin.

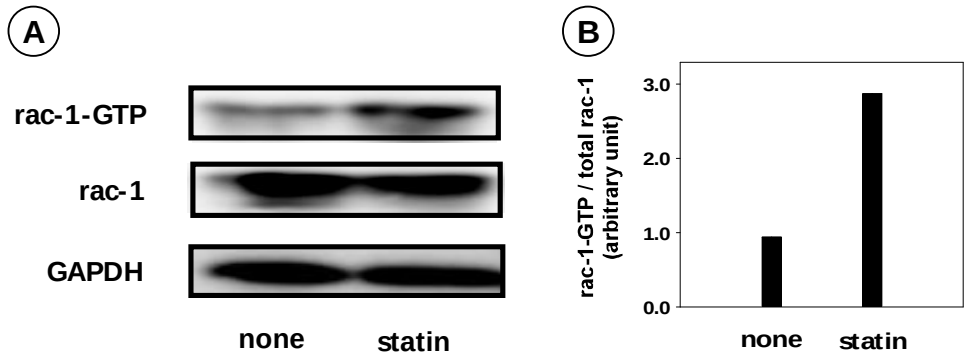

Figure 26. Preincubation with statin enhances the activation of Rac1. Monocytes were incubated in the absence or presence of statin for 24 hours in 75 cm^2 flasks. A) After removal of the supernatants, the cell layers were harvested in pulldown-lysis-buffer. Pulldown assay was performed and active Rac1-GTP, total Rac1 and GAPDH were analyzed in SDS-Page and subsequent in Western blot. B) The bands shown in 25A were quantified using "TotalLab" analysis software. The Rac1-GTP densities were normalized to total Rac1 densities. The data show one of four experiments with similar results.

5 Discussion

Atherosclerosis is the most common cause of cardiovascular diseases. Until now, the pathophysiology of atherosclerosis is not fully understood. Nevertheless, it is well accepted that inflammation may play a central role in atherogenesis. In early steps of atherosclerosis, endothelial dysfunction may result in leukocyte invasion into the vessel wall. The interaction between invading cells, such as monocytes, and cardiovascular cells, such as smooth muscle cells (SMC), may be important for the progression of atherogenesis. Our previous work has pointed out that interaction of monocytes and SMC with LPS or 25-hydroxycholesterol (Chol) stimulation synergistically enhances IL-6-expression (Chen et al., 2009; Fu et al., 2014).

In the LPS-stimulated cocultures, IL-1 has been suggested to be a central mediator for the synergistic IL-6-expression (Chen et al., 2009). However, in the Chol-stimulated cocultrues, the mechanism for this synergistic cytokine expression was not defined. Thus, we investigated factors which may be relevant in this interaction, such as the role of IL-1, the monocyte-SMC ratio and the cell source of cytokines.

In this study, we report that more than 80% of the synergistic IL-6- and IL-1ß-expression in the Chol-stimulated cocultures was inhibited by the treatment of IL-1Ra. These results indicate that IL-1 importantly contributes to the synergistic IL-6-production in the Chol-stimulated cocultures. Interestingly, the IL-1 appears to be an important activator for the synergistic IL-1ß-production itself. However, this was not unexpected, since literature showed IL-1 induces IL-1 itself (Dinarello et al., 1987; Warner et al., 1987). This induction may partially explain the source of the synergistic cytokine production in the Chol-stimulated cocultures. In the cocultures, the IL-1ß may be induced by the stimuli, then this IL-1 may activate cells to produce more IL-1ß, and in turn also activates cells to produce more IL-6. The mechanism to trigger the enhancement of IL-1ß in the Chol-stimulated cocultures is not clear. One possibility may be due to enhanced CD36 expression of the monocytes in these cocultures. Literature revealed that interaction of monocytes with SMC enhances the CD36 expression of the monocytes (Cai et al., 2004). Other investigations demonstrated that CD36 cooperates with TLR-4 and TLR-6 to activate the NLRP3 inflammasome and induces inflammation under oxLDL stimulation (Sheedy et al., 2013; Stewart et al., 2010). Another possibility may be ATP-dependence. ATP is an important coactivator of the inflammasome in monocytes (Netea et al., 2009). SMC may release ATP into the culture (Lohman et al., 2012; Pearson and Gordon, 1979), which in turn may contribute to the activation of inflammasome in monocytes.

We also investigated the influence of monocyte-SMC-ratio on the cytokine expression and synergisms in the cocultures. We report that in the Chol-stimulated cocultures, the IL-6- and IL-1ß-expression was monocyte-SMC ratio dependent. This result indicates

that in the Chol-stimulated cocultures, if more monocytes were added, more IL-6 and IL-1ß was expressed. Since the IL-6 in the Chol-stimulated cocultures may be induced by IL-1ß, the IL-1ß-level and the number of monocytes may correlate. Synergistic IL-6- and IL-1ß-production was obtained in the Chol-stimulated cocultures of the monocyte-SMC ratios 1 to 20 and 2 to 20, respectively. This result shows that both, low and high numbers of monocytes in the cocultures can induce synergistic cytokine expression. A positive-feedback loop may be derived from this result: during early steps of atherogenesis, small numbers of monocytes interacting with SMC already may be sufficient to induce synergistic IL-6 and IL-1ß under oxysterol activation. This IL-6- and IL-1ß-production may further induce chemokine production and adhesion molecules (Biswas et al., 1998; Struyf et al., 1998; Wang et al., 1995), which may induce more monocytes invading into vessel wall and interact with SMC.

In order to clarify the cell source of IL-6 and IL-1ß in the coculture system, we used inserts in cocultures to separate the cocultures into SMC-compartment and monocyte-compartment. We report that the IL-6 protein expression in the supernatants of the SMC-compartment and the mRNA expression in the SMC-compartment was higher than in the monocyte-compartment. In contrast, the IL-1ß-protein and -mRNA expression in the SMC-compartment was lower than in the monocyte-compartment. Although the protein expression in the supernatants already showed different cytokine levels in the two compartments, the different cytokine expression in the two compartments were not so potent, since cytokine molecules may diffuse through the membrane of the insert. Thus, the mRNA expression of IL-6 and IL-1ß was analyzed. The mRNA results suggest that in the cocultures SMC may be the major source of IL-6 and monocytes may be the major source of IL-1ß. Since previous investigations revealed that, monocytes can produce and release IL-1ß (Dinarello, 1996), whereas SMC produce IL-1ß mRNA and the IL-1ß-precursor (Libby et al., 1986; Schönbeck et al., 1997), but do not process and release the mature form of IL-1ß (Loppnow and Libby, 1992; Schönbeck et al., 1997). Thus, we postulate that in the Chol-stimulated cocultures, monocytes produce IL-1ß, which may in turn activate the SMC to produce IL-6. Furthermore, this postulation for the cell source of IL-1ß was further supported by time-course experiments and intracellular protein investigation. In the time course analysis, the IL-1ß was detected around 3 hours earlier in the monocyte-compartment than in the SMC-compartment. This result suggests that the IL-1ß detected in the SMC-compartment could be attributed to a diffusion process between these two compartments. We did not analyze the IL-6-expression in this time-course experiment, since both, SMC and monocytes, produce IL-6 in response to LPS, and the IL-6 has been detected in both compartments at the same time point. Further evidence is shown in the Western blot analysis, since only the monocyte-compartment contained the active caspase-1 and mature IL-1ß. The SMC-compartment contained only inactive caspase-1 and IL-1ß-precursor. These results are in line with the previous finding that

SMC do not process IL-1ß (Schönbeck et al., 1997) and support the suggestion that monocytes are the major source of IL-1ß in the cocultures.

In the early step of atherogenesis, after the invasion of monocytes into the vessel wall, the monocytes may be differentiated into macrophages. Thus, the interaction of SMC and macrophages may contribute to the progression of atherogenesis. However, it was unclear whether or not macrophages have the same capacity to produce cytokines important for the interaction, thus we compared the inflammatory response of monocytes and macrophages in monocultures. We report that, in contrast to the monocytes, macrophages prepared by overnight-differentiation lose the capacity to respond to endotoxin, whereas macrophages prepared in the presence of statin did not. The determination of monocytes after 24 hours preincubation to be macrophages, is derived from the literature (Netea et al., 2009). Although these authors did not characterize the cells by FACS analysis, the study reported a lack of IL-1ß-production in macrophages, which may be due to the less caspase-1 activity. On the other hand, the statin-effect in our study was in line with the previous finding that in THP-1 cells statin enhanced IL-1ß-production by increasing caspase-1 activity (Kuijk et al., 2008b). Thus, we proposed that, expression of caspase-1 may be importantly involved in the cytokine expression of overnight-differentiated macrophages. To address this question, we investigated intracellular caspase-1, as well as IL-1ß-expression by Western blot. We report that the overnight-differentiated macrophages contained much less active caspase-1 (p10) than the monocytes. In addition, the macrophages contained no mature IL-1ß. Together with the IL-1ß-data form supernatants, these data indicate that the hypo-expression of IL-1ß in the macrophages may be due to the lack of IL-1ß processing by caspase-1. However, not only the processing step was influenced after the overnight differentiation, the macrophages also contained much less IL-1ß-precursor than the monocytes. This indicates that, in contrast to monocytes, the LPS-induced signaling pathways in the macrophages may be different or the signaling pathways may be partially blocked during the overnight differentiation. In addition, we report that the statin-pretreated macrophages contained similar amounts of active caspase-1 (p10), as compared to the macrophages pretreated without statin. However, this finding is contrary to the literature (Kuijk et al., 2008b). This difference of active caspase-1-expression may due to the different cells used in our experiments. In the analysis of IL-1ß, statin-pretreatment enhanced the level of IL-1ß-precursor in the macrophages. This result indicates that the isoprenoid pathway may be involved in the alteration of the LPS-induced signaling pathway in the overnight differentiation of macrophages. The statin-pretreated macrophages contained no intracellular mature IL-1ß. Since high levels of IL-1ß were detected in the supernatants, this indicates that unlike the macrophages pretreated without statin, the statin-pretreated macrophages have the capacity to process IL-1ß and release it. Since the levels of active caspase-1 (p10) was not enhanced by the statin-pretreatment, these results imply that, in the

statin-pretreated macrophages, the IL-1ß-processing may be caspase-1-independent, and some other proteases, such as trypsin, plasmin (Matsushima et al., 1986) or caspase-8 (Maelfait et al., 2008), may participate in this IL-1ß-processing step. On the other hand, the expression of p10 does not directly reflect the activation of caspase-1, since caspase-1 is able to cleave the IL-1ß-precursor only by forming an inflammasome complex with ASC and NLRP3 proteins. Thus, statin-pretreatment may influence the expression of ASC or NLRP3, subsequently influencing the processing of IL-1ß-precursor.

Since statin is present in both, the differentiation period (first 24 hours) and the stimulation period (second 24 hours) of the macrophages, one question is raised: dose statin enhance the cytokine expression in the macrophages by influencing the differentiation of macrophages, or does it co-stimulate the macrophages with LPS. We report that, in order to produce high level of cytokines, the presence of statin is necessary in the differentiation period, not in the stimulation period. This result indicates that statin retains the capacity of macrophages in response to endotoxin, rather than co-stimulating the macrophages with endotoxin. This result supports the idea that the isoprenoid pathway may be involved in the monocyte vs. macrophage differentiation. In addition, this idea has been further supported by my colleague's data, which showed that statin-pretreatment decreased the expression of CD163 on the surface of the macrophages (Julia Großmann, Reem Abdosh; personal communication).

Since statin inhibits the isoprenoid pathway by inhibiting synthesis of mavalonic acid, this molecule and its downstream molecules may reverse the retainment-effect caused by statin in the macrophages. Our experiments showed that addition of mevalonic acid, FPP or GGPP, but not squalene, blocked the retainment-effect on the macrophages. This result suggests that statin retains the capacity of macrophages to respond to endotoxin via the inhibition of the isoprenoid pathway. Since squalene leads to synthesis of cholesterol, but GGPP contributes to isoprenylation of proteins, this result further suggests that the isoprenylation pathway, but not cholesterol-synthesis pathway is involved in the retainment-effect. To support this suggestion, we used geranylgeranyl transferase-inhibitor (GGTI), farnesyl transferase-inhibitor (FTI), zaragozic acid-A (ZAA), as well as the combinations of these inhibitors to replace statin in the cell cultures. We report that, by use of the single inhibitor, only GGTI can enhance the IL-6- and IL-1ß-levels like statin does. This result indicates that the retainment-effect may partially be attributed to the inhibition of geranylgeranylation of small GTPases. Moreover, the inhibition of farnesylation is not involved in the retainment. In addition, combination of GGTI and FTI further enhanced the IL-6-level, which was similar to the IL-6-level in the statin-pretreated macrophages. However, the IL-1ß-level was not further enhanced by this combination. This is interesting, since FTI alone was not able

to enhance IL-6-expression. This finding indicates that the small GTPases, which can be both, geranylgeranylated or farnesylated, such as RhoB, may contribute to the IL-6-production, but not to the IL-1ß-production.

Since the role of GGPP in the retainment-effect caused by statin was not completely established, we added GGPP in the absence or presence of GGTI to the statin-preincubated macrophages. GGPP dose-dependently blocked the retainment-effect. On the other hand, addition of GGTI reversed the blockade by GGPP. These results clearly indicate the role of geranylgeranylation in the retainment-effect.

Since the inhibition of geranylgeranylation of small GTPases may be involved in the retainment-effect caused by statin on the macrophages, we focused on small GTPases, which may be involved in this effect. Basically, Ras family proteins can be farnesylated, whereas Rho, Rab and Rap family proteins can be geranylgeranylated (Van Aelst and D'Souza-Schorey, 1997). The Rho family members Rac1, Rhoa and Cdc42 have been investigated in many studies. Literature has shown that in the statin-treated THP-1 cells, Rac1-activation was involved in the hypersecretion of IL-1ß (Kuijk et al., 2008a). Thus, we proposed that in our statin-pretreated macrophages, activation of Rac1 may be also importantly involved in the retainment-effect caused by statin. To address this question, we used the Rac1-inhibitor NSC23766 in statin-treated cells. In these cells, we reported that NSC23766 dose-dependently inhibited the cytokine expression in the statin-pretreated macrophages. This result indicated that statin retains the capacity of macrophages to respond to endotoxin possibly via the activation of Rac1. Since the activation of Rac1 may be involved in the retainment-effect in the macrophages, activation of downstream molecules of Rac1, such as PI3K, p38-MAPK, ERK1/2 and JNK may be involved. Our experiments demonstrated that the PI3K-inhibitor LY294002 and the p38-MAPK-inhibitor SB203580 dose-dependently inhibited the IL-6- and IL-1ß-expression in the statin-pretreated macrophages, whereas the ERK1/2-inhibitor PD98059 and the JNK-inhibitor SP600125 did not. These results indicate that the activation of PI3K and p38-MAPK may be involved in the retainment-effect. However, although the inhibitors we used in our study are commonly used in literature for inhibition of PI3K, p38, ERK1/2 and JNK, it is important to be aware that these inhibitors may exert inhibition effects on other targets depending on the concentration. For instance, the PI3K-inhibitor LY294002 also inhibits mTOR, CK2 (Gharbi et al., 2007), as well as NFκB-activation (Kim et al., 2005) and the p38-MAPK-inhibitor SB203580 also shows inhibition on phosphorylation of PKB (Lali et al., 2000). This may explain the reduced IL-1ß-expression in the monocytes upon addition of the p38-MAPK-inhibitor.

Although the inhibitors of Rac1-, PI3K- and p38-activation blocked the cytokine expression, it was still not clear whether these inhibitors reversed the retainment-effect

during the differentiation period, or inhibited the inflammatory signaling pathway during the stimulation period. Thus, we limited the presence of these inhibitors to either differentiation period or stimulation period. We report that the presence of these inhibitors was necessary in the differentiation period. These results suggested that inhibition of Rac1-, PI3K- and p38-activation during the differentiation period may block the retainment-effect on the macrophages. The p38-MAPK-inhibitor also reduced the IL-1ß-level during the stimulation period, however, not as potent as the treatment during the differentiation period. This result implies that, activation of p38 may not only be involved in the retainment-effect caused by statin, but it may also be involved in the LPS-induced inflammatory pathway during the stimulation period.

Since the inhibition-data suggest that Rac1-activation may be involved in the retainment-effect caused by statin, in order to support this suggestion, we investigated the Rac1-activation in the monocytes treated with or without statin for 24 hours. Here we report that statin-treatment enhanced the expression of Rac1-GTP. How exactly statin-treatment results in activation of Rac1 is not completely understood. Literature suggests that statin impairs binding activity of Rac protein to RhoGDI, this impairment may account for activation of Rac1 (Cordle et al., 2005). Another possibility is that activation of Rac1 may be regulated by Rho protein crosstalk, for instance RhoA may regulate activation of Rac1 (Guilluy et al., 2011). In a research of mevalonate kinase deficiency, people suggest that unprenylated RhoA contributes to hypersecretion of IL-1ß through activating of Rac1 (van der Burgh et al., 2014). However, in this study they did not show how exactly the unprenylated RhoA activates Rac1.

Taken together, our data demonstrate the role of IL-1ß in the interaction of SMC and monocytes, and the cell source of IL-6 and IL-1ß. These findings strengthen the importance of interaction of SMC and monocytes in the progression of atherosclerosis. On the other hand, we find that statin retains the capacity of macrophages to respond to endotoxin possibly via the isoprenylation-pathway. Since statins are widely used in the treatment of atherosclerosis, this finding may provide a different perspective of pleiotropic effects of statin in the treatment.

6 Summary

Atherosclerosis is the most common cause of cardiovascular diseases, which cause the highest mortality in the Western world. In recent years, atherosclerosis has been identified to be a chronic inflammatory disease. Thus, cytokines are thought to be central regulators of atherogenesis. In the early steps of atherogenesis, functions of endothelial cells are dysregulated and different adhesion molecules are released. Then the circulating leukocytes, such as monocytes, are attracted by these adhesion molecules and invade into the vessel wall. There, invading monocytes may differentiate into macrophages and interaction of monocytes or macrophages with local vessel wall cells, such as smooth muscle cells (SMC), may importantly contribute to the progression of atherosclerosis.

In order to investigate the interaction, a coculture model containing both SMC and monocytes was used and the IL-6 and IL-1ß were analyzed. On the other hand, the capacity of monocytes and macrophages to produce cytokines was also investigated. Cytokines were analyzed in ELISA and Western blot. The mechanisms which may be involved in differential regulation of cytokine products of Mo and Mac were investigated by supplementation or inhibition of the isoprenoid pathway, as well as pulldown assay.

We found that IL-1 importantly contributed to the synergistic IL-6-expression in Chol-stimulated cocultures. The synergistic effect was present at both, low and high monocyte-SMC ratios. In the cocultures, SMC preferentially expressed IL-6, whereas monocytes preferentially expressed IL-1ß. Macrophages differentiated from monocytes by overnight incubation lost the capacity to respond to endotoxin. However, overnight differentiation in the present of statin retained the capacity to respond to endotoxin of the macrophages. The geranylgeranylation was involved in the retainment-effect caused by statin on macrophages, as shown by inhibition with GGTI, as well as by supplementation with mevalonic acid, farnesyl pyrophosphates or geranylgeranyl pyrophosphates. The activation of Rac1, PI3K and p38 MAPK signaling pathways may be involved in the retainment-effect.

In conclusion, our data indictate a possible role of IL-6 and IL1ß in the chlolesterol-induced inflammation. In addition, our data identify a differential effect of statin on monocytes and macrophages, which may be of potential relevance for the comprehension of the role of statins in cardiovascular diseases.

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8 Theses

1. Atherosclerosis is a chronic inflammatory disease, cytokines such as IL-6 and IL-1 may be involved in this process. Interaction of smooth muscle cells (SMC) and monocytes or macrophages may be important for atherogenesis

2. In LPS- and 25-hydroxycholesterol-(Chol)-stimulated cocultures, IL-6 was synergistically enhanced

3. In LPS- and Chol-stimulated cocultures, IL-1 mediated the synergistic IL-6-production to a large degree

4. The synergistic effect is obtained in low and high Mo-SMC ratios

5. In the cocultures, SMC preferentially express IL-6, whereas monocytes preferentially express IL-1ß

6. In contrast to freshly isolated monocytes, overnight-differentiated macrophages, stimulated by LPS expressed only little cytokine. Macrophages prepared in the presence of statin still produced the cytokines

7. Statin retains the capacity of macrophages to respond to endotoxin, rather than it costimulates the cells in the presence of endotoxin

8. Statin retains the capacity of macrophages to respond to endotoxin via the geranylgeranylation pathway

9. Statin retains the capacity of macrophages to respond to endotoxin possibly via the activation of Rac1

10. The retainment of inflammatory function in macrophages may contribute to the beneficial effect of statins

Disclosure Statement

Herewith I declare that I have written this thesis independently and that I have only used the cited aids.

Previous Promotion

Herewith, I declare that I have not attended to any other promotion procedure previously.

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