Lipids, inflammation and atherosclerosis

Stitzinger, M.

Chapter 8

SUMMARY AND PERSPECTIVES

Despite significant progress in the management of atherosclerosis and its complications, cardiovascular disease resulting from atherosclerosis remains the major cause of death in the Western world. Atherosclerosis is a progressive disease involving the development of vascular atherosclerotic lesions characterized by both lipid accumulation and by inflammation[1-4]. The main aim of this thesis was to investigate interactions between lipids, inflammation and atherosclerosis.

The expression of pro-inflammatory cytokines is decisive for the progression of atherosclerosis[5]. Many pro-inflammatory cytokines, such as IL-12 and IFN-γ, and various chemokines exert their pro-atherosclerotic effects during various stages of lesion development[6,7]. A reduction in the expression of pro-inflammatory cytokines may, in addition to regulation of plasma lipid levels, be considered as one of the main targets for the treatment of atherosclerosis. A limited number of members of the cytokine family, such as IL-10 and IL-9, displays anti-inflammatory features via inhibition of the production of pro-inflammatory cytokines[8-12]. However, although atherosclerosis is considered as a chronic inflammatory disease and IL-9 affects many inflammatory processes, the role of IL-9 in atherosclerosis has not been elucidated yet. Therefore, in **Chapter 2**, we investigated the effect of IL-9 treatment and of vaccination against endogenous IL-9 on atherosclerotic lesion development in LDLr deficient mice, a well established mouse model for atherosclerosis. Daily IL-9 treatment resulted in a significant reduction of atherosclerotic lesion size, whereas blocking endogenous IL-9 caused a marked increase in atherosclerosis. Furthermore, IL-9 not only affected the production of pro-inflammatory cytokines, but also affected two other hallmarks of atherosclerosis: β-VLDL induced foam cell formation in peritoneal macrophages and the adhesion of monocytes to endothelial cells. Based on these data, IL-9 and its receptor are interesting new targets for the development of anti-inflammatory drugs for the treatment of atherosclerosis.

In **Chapter 3**, we focused on the inhibitory effect of IL-9 on β-VLDL induced foam cell formation. Foam cells, which play a prominent role in the initiation and progression of the atherosclerotic lesion, result from excessive accumulation of cholesteryl esters. *In vitro* studies have demonstrated that foam cells can be formed by receptor mediated uptake of β-VLDL or experimentally modified lipoproteins such as acLDL and oxLDL[13-15]. In addition to the uptake of modified lipoproteins, cholesterol efflux is an important process in the development of foam cells, because foam cell formation is the result of an imbalance in cholesterol homeostasis[16]. Various factors including inflammatory mediators can affect foam cell formation.

Most studies suggest a foam cell inducing effect of pro-inflammatory mediators such as LPS and TNF-α[17,18] and a suppressive effect of anti-inflammatory mediators such as TGFβ1[19,20]. We confirmed our earlier results that IL-9 was able to inhibit foam cell formation using β-VLDL induced lipid accumulation in RAW 264.7 murine macrophage cells as a model. IL-9 did not affect cholesterol efflux or gene expression of cholesterol efflux pumps, but IL-9 exerted its inhibitory effect on foam cell formation by reducing the gene expression of the LDLr, LRP-1 and SR-BI, all tentative receptors for β-VLDL. Furthermore, IL-9 induced the gene expression of nuclear receptor PPARα and reduced the gene expression of transcription factors SREBP-1 and SREBP-2, which might explain the observed effects on the gene expression of the lipoprotein receptors. These findings provide the first evidence for a direct effect of IL-9 on the gene expression of key players in foam cell formation providing a possible mechanism for the anti-atherogenic effect of IL-9.

Several studies have shown that IL-9 promotes many processes involved in asthma[21,22]. Moreover, high IL-9 levels are related to malignancies of lymphocytes and to enhanced survival of lymphomas[23-25]. Therefore, one should be cautious to treat atherosclerosis patients with a high dosage of IL-9. More research is needed to fully understand the mechanism of action of IL-9 and to translate the observed effects of IL-9 in animal models to the human situation.

Inflammatory mediators regulate the expression of many genes involved in foam cell formation. LPS, a very potent inducer of inflammation, is able to affect macrophage expression of receptors for lipoproteins, such as LRP-1, and of cholesterol efflux mediators, including ABCA1 and SR-BI[17,26-28]. Lipid loading of macrophages, in turn, alters the response of these macrophages to inflammatory mediators[29]. We investigated the effect of foam cell formation on the response of macrophages to LPS with a specific emphasis on the expression of lipid related genes in **Chapter 4**. We used β-VLDL induced lipid accumulation by RAW 264.7 murine macrophage cells as a model for foam cell formation. In both macrophages and foam cells, LPS affected gene expression of LRP-1, ABCG1, PPARγ, LXRβ, SREBP-1 and SREBP-2 in a similar manner. However, the effect of LPS on the expression of LDLr, ABCA1, apoA-I, apoE and PPARα gene expression and SR-BI protein level was potentiated, attenuated or even reversed by pre-incubation of macrophages with β-VLDL. In macrophages, LPS inhibited gene expression of apoE and ABCA1, whereas in foam cells gene expression of apoA-I and ABCA1 were upregulated by LPS, suggesting that apolipoprotein mediated cholesterol efflux is attenuated in macrophages and stimulated in foam cells by LPS. These data provide a better understanding of the complex interaction between lipid metabolism and inflammation in macrophages, which is important to unravel the mechanism underlying atherogenesis.

As the bio-active component of the outer membrane of Gram-negative bacteria, LPS stimulates monocytes and macrophages to produce high amounts of pro-inflammatory cytokines, which, in turn, can evoke cytotoxic effects, organ failure and eventually death[30-33]. It has been demonstrated that the LPS induced cytokine response primarily involves TLR4 and plasma membrane CD14, which initiate downstream signaling to NF-κB

followed by activation of LPS responsive genes[34,35]. In addition, LPS uptake, clearance, and catabolism are mediated by the family of scavenger receptors, which results in neutralization of LPS[36]. In **Chapter 5**, we set out to examine the role of one of these scavenger receptors, SR-BI, in the response to LPS using SR-BI wild-type and SR-BI deficient mice. Challenging these mice with a sublethal dose of LPS showed that SR-BI protects against LPS induced TNF-α production. Although LPS uptake by the liver, the main organ for LPS clearance, was not affected by SR-BI expression, serum decay of LPS in the first 10 minutes was impaired by the absence of SR-BI expression. The coinciding higher serum cholesterol levels, HDL in particular, can explain the prolonged presence of LPS in SR-BI deficient mice. SR-BI deficient mice displayed higher oxidative stress levels than wild-type mice. After treatment with the HDL-lowering anti-oxidant probucol, oxidative stress levels were reduced to a similar level in both types of mice, but SR-BI deficient mice still responded to LPS with a higher TNF-α production than wild-type mice. Although macrophage content in the liver was the same, SR-BI deficient macrophages appeared to be more activated, as determined by gene expression of MARCO, an innate activation marker. In addition, a higher activation status of circulating monocytes in SR-BI deficient was observed using a whole blood assay for LPS induced TNF-α production. These results indicate that SR-BI modulates LPS induced TNF-α production and forms a host defense mechanism against inflammation.

The results obtained in **Chapter 4** and in **Chapter 5** shed new light on the close interactions between lipids and inflammation. Interestingly, normal macrophages do not produce apoA-I, but we showed that LPS induced apoA-I gene expression in foam cells. Furthermore, other genes involved in apolipoprotein mediated cholesterol efflux were upregulated by LPS in foam cells. Although inflammation stimulates the initiation of foam cells, further progression of foam cells seems to be inhibited by inflammation. The mechanism of action of SR-BI is highly complex, which is illustrated by an earlier study that showed that macrophage SR-BI is either pro-atherogenic (small fatty streak) or anti-atherogenic (advanced lesion), depending on the stage of lesion development[37]. We demonstrated that in addition to being a key player in cholesterol homeostasis, SR-BI protects against LPS induced inflammatory effects, probably via preventing activation of monocytes and macrophages.

In addition to inflammation, the accumulation of lipids, especially cholesterol, in the vessel wall is one of the hallmarks of atherosclerosis. Cholesterol in the atherosclerotic lesion is mainly derived from (modified) lipoproteins and lipoprotein remnants, which are taken up from the circulation into the vessel wall[1]. Therefore, high serum cholesterol levels due to the consumption of a Western-type high fat diet are a major risk factor for atherosclerosis. Serum cholesterol levels are largely influenced by synthesis and secretion of VLDL and HDL and by removal of cholesterol from the body via bile[38,39]. These processes occur in the liver and are performed by parenchymal cells, which thus are essential in maintaining serum lipid homeostasis[40]. In **Chapter 6** and **Chapter 7**, microarray analysis was used to determine the effect of Western-type diet feeding on liver parenchymal cells and the effect of IL-10 treatment of liver

parenchymal cells during Western-type diet feeding, both in LDLr deficient mice. To gain insight in the specific pathways and genes involved in the response to increased dietary lipid levels, in **Chapter 6**, changes in parenchymal cell gene expression upon feeding a Western-type diet for 0, 2, 4, and 6 weeks were determined. In agreement with previous studies, Western-type diet feeding induced an atherogenic lipoprotein profile, since the circulating serum cholesterol levels of both LDL and VLDL were markedly induced. Time dependent gene expression profiling identified FABP5 and four novel FABP5-like transcripts as important proteins in the primary response of liver parenchymal cells to Western-type diet induced high cholesterol levels, since their expression was 16- to 22-fold increased within the first 2 weeks of Western-type diet consumption. In the secondary response of liver parenchymal cells to the Western-type diet, the expression of key genes involved in lipogenesis and glycolytic pathways, such as pyruvate kinase, was markedly stimulated in liver parenchymal cells.

In previous studies, overexpression of IL-10 resulted in attenuation of atherogenesis, indicating a protective role for endogenous IL-10, which was underlined by studies showing an enhanced atherosclerotic lesion development in IL-10 deficient mice[41-45]. The protective role of IL-10 can partly be explained by the inhibitory effect of IL-10 on inflammation and on the expression of adhesion molecules[43,45]. In addition, several animal studies demonstrated that IL-10 influences lipid metabolism resulting in lower serum total cholesterol levels[41,44], but the mechanism of action remained elusive. In **Chapter 7**, LDLr deficient mice were put on a Western-type diet for two weeks to induce atherogenic cholesterol levels, subsequently received adenoviral IL-10 treatment and Western-type diet was continued for another two weeks, after which liver parenchymal cells were isolated for microarray analysis. The adenoviral treatment resulted in high serum IL-10 levels and a coinciding reduction of serum cholesterol and especially LDL levels. Microarray analysis revealed that in parenchymal cells, adenoviral IL-10 treatment led to a more than 2-fold change in the expression of over 900 genes. In addition to the classical target genes including TNF-α and IL-6, more genes involved in lipid metabolism were affected by IL-10 than anticipated from statistical expectations. Interestingly, amongst these genes, ABCG5 and ABCG8 were both upregulated upon adenoviral IL-10 treatment. ABCG5 and ABCG8 are involved in the excretion of cholesterol from the body and their upregulation by IL-10 might explain the cholesterol lowering effect observed in LDLr deficient mice on a Western-type diet.

Microarray analysis of liver parenchymal cells in **Chapter 6** and **Chapter 7** revealed that the expression of many genes in these cells is affected by Western-type diet feeding and by IL-10 treatment, respectively. These effects coincided with changes in serum cholesterol levels, indicating that liver parenchymal cells may be interesting target cells for the treatment of atherosclerosis. The effects of IL-10 on liver parenchymal cells suggest that IL-10 exerts its anti-atherosclerotic effect in both inflammation and lipid mediated manners. Microarray analysis identified several novel genes in liver parenchymal cells, which were affected by IL-10. Further research is necessary to unravel the role of these genes in lipoprotein homeostasis and in atherosclerosis.

In stead of regarding atherosclerosis as a purely lipid mediated or inflammatory disease, research has, recently, focused more on the relation between these two hallmarks of atherosclerosis. The research described in this thesis provides a better understanding of the interaction between lipids, inflammation and atherosclerosis leading to novel insights in the pathogenesis of cardiovascular disease. This knowledge is crucial for the development of new strategies to overcome this disease.

Both the interleukins IL-9 and IL-10 display effects on lipid metabolism that, together with their anti-inflammatory properties, may explain their protective effect in atherosclerosis. Because of their pleiotropic effects, potential adverse effects can occur after systemic treatment of cardiovascular patients with these cytokines. However, the local delivery of viral vectors encoding IL-9 or IL-10 may form a promising new treatment.

By using microarray technology, the expression of many genes can be analysed simultaneously making it a powerful tool in the search for new target genes in the treatment of cardiovascular disease. In this thesis, microarray analysis of liver parenchymal cells in response to IL-10 and/or a high cholesterol diet revealed several genes, such as FABP5, as potential new targets genes. Further studies of the metabolic role of these genes in lipid and lipoprotein metabolism are needed in order to evaluate their precise function before utilizing their potential beneficial effect on cardiovascular disease.

REFERENCES

1. Lusis AJ (2000) Atherosclerosis. *Nature*. **407**: 233-241.
2. Glass CK and Witztum JL (2001) Atherosclerosis. the road ahead. *Cell*. **104**: 503-516.
3. Hansson GK (2005) Inflammation, atherosclerosis, and coronary artery disease. *N Engl J Med*. **352**: 1685-1695.
4. Libby P and Theroux P (2005) Pathophysiology of coronary artery disease. *Circulation*. **111**: 3481-3488.
5. Tedgui A and Mallat Z (2006) Cytokines in atherosclerosis: pathogenic and regulatory pathways. *Physiol Rev*. **86**: 515-581.
6. Hauer AD, Uyttenhove C, de Vos P, Stroobant V, Renauld JC, van Berkel TJ, van Snick J and Kuiper J (2005) Blockade of interleukin-12 function by protein vaccination attenuates atherosclerosis. *Circulation*. **112**: 1054-1062.
7. Gupta S, Pablo AM, Jiang X, Wang N, Tall AR and Schindler C (1997) IFN-gamma potentiates atherosclerosis in ApoE knock-out mice. *J Clin Invest*. **99**: 2752-2761.
8. Fiorentino DF, Bond MW and Mosmann TR (1989) Two types of mouse T helper cell. IV. Th2 clones secrete a factor that inhibits cytokine production by Th1 clones. *J Exp Med*. **170**: 2081-2095.
9. Lacraz S, Nicod LP, Chicheportiche R, Welgus HG and Dayer JM (1995) IL-10 inhibits metalloproteinase and stimulates TIMP-1 production in human mononuclear phagocytes. *J Clin Invest*. **96**: 2304-2310.
10. de Waal Malefyt R, Abrams J, Bennett B, Figdor CG and de Vries JE (1991) Interleukin 10(IL-10) inhibits cytokine synthesis by human monocytes: an autoregulatory role of IL-10 produced by monocytes. *J Exp Med*. **174**: 1209-1220.
11. Pilette C, Ouadrhiri Y, Van Snick J, Renauld JC, Staquet P, Vaerman JP and Sibille Y (2002) IL-9 inhibits oxidative burst and TNF-alpha release in lipopolysaccharide-stimulated human monocytes through TGF-beta. *J Immunol*. **168**: 4103-4111.
12. Grohmann U, Van Snick J, Campanile F, Silla S, Giampietri A, Vacca C, Renauld JC, Fioretti MC and Puccetti P (2000) IL-9 protects mice from Gram-negative bacterial shock: suppression of TNF-alpha, IL-12, and IFN-gamma, and induction of IL-10. *J Immunol*. **164**: 4197-4203.

13. Goldstein JL, Ho YK, Brown MS, Innerarity TL and Mahley RW (1980) Cholesteryl ester accumulation in macrophages resulting from receptor-mediated uptake and degradation of hypercholesterolemic canine beta-very low density lipoproteins. *J Biol Chem*. **255**: 1839-1848.
14. Brown MS, Goldstein JL, Krieger M, Ho YK and Anderson RG (1979) Reversible accumulation of cholesteryl esters in macrophages incubated with acetylated lipoproteins. *J Cell Biol*. **82**: 597-613.
15. Arai H, Kita T, Yokode M, Narumiya S and Kawai C (1989) Multiple receptors for modified low density lipoproteins in mouse peritoneal macrophages: different uptake mechanisms for acetylated and oxidized low density lipoproteins. *Biochem Biophys Res Commun*. **159**: 1375-1382.
16. Linton MF and Fazio S (2003) Macrophages, inflammation, and atherosclerosis. *Int J Obes Relat Metab Disord*. **27 Suppl 3**: S35-40.
17. Funk JL, Feingold KR, Moser AH and Grunfeld C (1993) Lipopolysaccharide stimulation of RAW 264.7 macrophages induces lipid accumulation and foam cell formation. *Atherosclerosis*. **98**: 67-82.
18. Hsu HY and Twu YC (2000) Tumor necrosis factor-alpha -mediated protein kinases in regulation of scavenger receptor and foam cell formation on macrophage. *J Biol Chem*. **275**: 41035-41048.
19. Panousis CG, Evans G and Zuckerman SH (2001) TGF-beta increases cholesterol efflux and ABC-1 expression in macrophage-derived foam cells: opposing the effects of IFN-gamma. *J Lipid Res*. **42**: 856-863.
20. Argmann CA, Van Den Diepstraten CH, Sawyez CG, Edwards JY, Hegele RA, Wolfe BM and Huff MW (2001) Transforming growth factor-beta1 inhibits macrophage cholesteryl ester accumulation induced by native and oxidized VLDL remnants. *Arterioscler Thromb Vasc Biol*. **21**: 2011-2018.
21. Nicolaides NC, Holroyd KJ, Ewart SL, Eleff SM, Kiser MB, Dragwa CR, Sullivan CD, Grasso L, Zhang LY, Messler CJ, Zhou T, Kleeberger SR, Buetow KH and Levitt RC (1997) Interleukin 9: a candidate gene for asthma. *Proc Natl Acad Sci U S A*. **94**: 13175-13180.
22. Zhou Y, McLane M and Levitt RC (2001) Th2 cytokines and asthma. Interleukin-9 as a therapeutic target for asthma. *Respir Res*. **2**: 80-84.
23. Vink A, Renauld JC, Warnier G and Van Snick J (1993) Interleukin-9 stimulates in vitro growth of mouse thymic lymphomas. *Eur J Immunol*. **23**: 1134-1138.
24. Merz H, Houssiau FA, Orscheschek K, Renauld JC, Fliedner A, Herin M, Noel H, Kadin M, Mueller-Hermelink HK, Van Snick J and et al. (1991) Interleukin-9 expression in human malignant lymphomas: unique association with Hodgkin's disease and large cell anaplastic lymphoma. *Blood*. **78**: 1311-1317.
25. Nagato T, Kobayashi H, Kishibe K, Takahara M, Ogino T, Ishii H, Oikawa K, Aoki N, Sato K, Kimura S, Shimizu N, Tateno M and Harabuchi Y (2005) Expression of interleukin-9 in nasal natural killer/T-cell lymphoma cell lines and patients. *Clin Cancer Res*. **11**: 8250-8257.
26. LaMarre J, Wolf BB, Kittler EL, Quesenberry PJ and Gonias SL (1993) Regulation of macrophage alpha 2-macroglobulin receptor/low density lipoprotein receptor-related protein by lipopolysaccharide and interferon-gamma. *J Clin Invest*. **91**: 1219-1224.
27. Baranova I, Vishnyakova T, Bocharov A, Chen Z, Remaley AT, Stonik J, Eggerman TL and Patterson AP (2002) Lipopolysaccharide down regulates both scavenger receptor B1 and ATP binding cassette transporter A1 in RAW cells. *Infect Immun*. **70**: 2995-3003.
28. Liao W, Rudling M and Angelin B (1999) Endotoxin suppresses mouse hepatic low-density lipoprotein-receptor expression via a pathway independent of the toll-like receptor 4. *Hepatology*. **30**: 1252-1256.
29. Groeneweg M, Kanters E, Vergouwe MN, Duerink H, Kraal G, Hofker MH and de Winther MP (2006) Lipopolysaccharide-induced gene expression in murine macrophages is enhanced by prior exposure to oxLDL. *J Lipid Res*. **47**: 2259-2267.
30. Van Amersfoort ES, Van Berkel TJ and Kuiper J (2003) Receptors, mediators, and mechanisms involved in bacterial sepsis and septic shock. *Clin Microbiol Rev*. **16**: 379-414.
31. Raetz CR (1990) Biochemistry of endotoxins. *Annu Rev Biochem*. **59**: 129-170.
32. Cohen J (2002) The immunopathogenesis of sepsis. *Nature*. **420**: 885-891.
33. Dobrovolskaia MA and Vogel SN (2002) Toll receptors, CD14, and macrophage activation and deactivation by LPS. *Microbes Infect*. **4**: 903-914.

34. Lien E, Means TK, Heine H, Yoshimura A, Kusumoto S, Fukase K, Fenton MJ, Oikawa M, Qureshi N, Monks B, Finberg RW, Ingalls RR and Golenbock DT (2000) Toll-like receptor 4 imparts ligand-specific recognition of bacterial lipopolysaccharide. *J Clin Invest*. **105**: 497-504.
35. Chow JC, Young DW, Golenbock DT, Christ WJ and Gusovsky F (1999) Toll-like receptor-4 mediates lipopolysaccharide-induced signal transduction. *J Biol Chem*. **274**: 10689-10692.
36. van Oosten M, van Amersfoort ES, van Berkel TJ and Kuiper J (2001) Scavenger receptor-like receptors for the binding of lipopolysaccharide and lipoteichoic acid to liver endothelial and Kupffer cells. *J Endotoxin Res*. **7**: 381-384.
37. Van Eck M, Bos IS, Hildebrand RB, Van Rij BT and Van Berkel TJ (2004) Dual role for scavenger receptor class B, type I on bone marrow-derived cells in atherosclerotic lesion development. *Am J Pathol*. **165**: 785-794.
38. Gibbons GF (1990) Assembly and secretion of hepatic very-low-density lipoprotein. *Biochem J*. **268**: 1-13.
39. Miettinen TA and Kesaniemi YA (1989) Cholesterol absorption: regulation of cholesterol synthesis and elimination and within-population variations of serum cholesterol levels. *Am J Clin Nutr*. **49**: 629-635.
40. Kmiec Z (2001) Cooperation of liver cells in health and disease. *Adv Anat Embryol Cell Biol*. **161**: III-XIII, 1-151.
41. Von Der Thusen JH, Kuiper J, Fekkes ML, De Vos P, Van Berkel TJ and Biessen EA (2001) Attenuation of atherogenesis by systemic and local adenovirus-mediated gene transfer of interleukin-10 in LDLr-/- mice. *Faseb J*. **15**: 2730-2732.
42. Mallat Z, Besnard S, Duriez M, Deleuze V, Emmanuel F, Bureau MF, Soubrier F, Esposito B, Duez H, Fievet C, Staels B, Duverger N, Scherman D and Tedgui A (1999) Protective role of interleukin-10 in atherosclerosis. *Circ Res*. **85**: e17-24.
43. Liu Y, Li D, Chen J, Xie J, Bandyopadhyay S, Zhang D, Nemarkommula AR, Liu H, Mehta JL and Hermonat PL (2006) Inhibition of atherogenesis in LDLR knockout mice by systemic delivery of adeno-associated virus type 2-hIL-10. *Atherosclerosis*. **188**: 19-27.
44. Yoshioka T, Okada T, Maeda Y, Ikeda U, Shimpo M, Nomoto T, Takeuchi K, Nonaka-Sarukawa M, Ito T, Takahashi M, Matsushita T, Mizukami H, Hanazono Y, Kume A, Ookawara S, Kawano M, Ishibashi S, Shimada K and Ozawa K (2004) Adeno-associated virus vector-mediated interleukin-10 gene transfer inhibits atherosclerosis in apolipoprotein E-deficient mice. *Gene Ther*. **11**: 1772-1779.
45. Caligiuri G, Rudling M, Ollivier V, Jacob MP, Michel JB, Hansson GK and Nicoletti A (2003) Interleukin-10 deficiency increases atherosclerosis, thrombosis, and low-density lipoproteins in apolipoprotein E knockout mice. *Mol Med*. **9**: 10-17.