Vaccination against atherosclerosis

Es, T. van

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A novel therapeutic approach

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Welke fakkel wij ook ontsteken
en hoever zij haar licht ook verspreiden moge:
Onze horizon blijft steeds de diepere duisternis
-Schoppenhauer-

Aan hen die er niet meer zijn,
maar altijd bij mij zullen blijven

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Chapter 1

General introduction and outline of the thesis

Thomas van Es

1 ATHEROSCLEROSIS

1.1 GENERAL

Cardiovascular disease (CVD) is still the number one cause of death in the Western world. There are many different clinical manifestations of cardiovascular disease such as angina pectoris, cardiomyopathy, endo-myocarditis and aneurysm.

Atherosclerosis affects the medium and large sized arteries of the heart and blood vessels and is the underlying cause of many clinical symptoms of CVD. Major risk factors for CVD, such as smoking, obesity and a high fat diet are well recognized. In 2004, 45445 people died of the consequences of CVD, accounting for 33%[1] of all reported deaths in the Netherlands, whereas 28% (38824 people) of all reported deaths death were related to cancer.[2] This states the importance of cardiovascular disease in the Dutch society.

The current treatment of atherosclerosis is mostly aimed at the reduction of risk factors by life style advice (stop smoking, more exercise, and lower cholesterol intake) and by subscribing drugs, such as statins, that lower plasma cholesterol levels. Additionaly drugs that lower blood coagulation and blood pressure are prescribed. However, these interventions cannot prevent that CVD is still the leading cause of death in the Western world. Therefore, there is an urge to develop new therapies targeting the different molecular pathways and stages of atherosclerosis.

1.2 PATHOGENESIS OF ATHEROSCLEROSIS

1.2.1 Initiation of the lesion

The first stage of atherosclerosis is fatty-streak formation. Fatty streak formation is asymptomatic and is already found during the first decades of life in medium and large sized arteries, at predisposed sites. The typical atherosclerotic prone sites are characterized by low shear stress and high oscillatory shear stress, which increase adhesion of leukocytes and the expression of inflammatory genes.[3]

Under normal conditions, healthy endothelium is able to respond to physical and chemical signals by the production of a wide range of factors that regulate vascular tone, cellular adhesion, thrombus resistance, smooth muscle cell proliferation, and vessel wall inflammation.[4] It is generally accepted that the activation of the endothelial cell layer, as a response to modulated gene expression, caused by hyperlipidemia, hypertension, diabetes mellitus and smoking, forms the first step in atherosclerosis, the fatty-streak formation.[5, 6]

Circulating lipoproteins, in particularly very low-density lipoproteins (VLDL) and low-density lipoproteins (LDL) infiltrate into the arterial intima and become modified through processes, such as oxidation, glycation, aggregation, association with proteoglycans or incorporation into immune complexes.[7-10] The modified LDL particles are highly immunogenic and activate the endothelial cells.

Stimulated endothelial cells undergo a switch from a quiescent phenotype towards a phenotype that initiates a defense response. Most cardiovascular risk factors, such as smoking and high blood pressure, activate the molecular machinery in the endothelium, resulting in the expression of chemokines, cytokines and adhesion molecules designed to interact with leukocytes and platelets and designed to target inflammatory cells to specific tissues to clear invading microorganisms or to respond to vascular injury.[11] Activated endothelial cells express adhesion molecules, like vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), P-selectin and E-selectin.[3, 12] Leukocytes (i.e. monocytes and lymphocytes) express counter receptors for these adhesion molecules and decelerate via interaction with P- and E-selectin. Once slowed down, a more firm adhesion is facilitated via interaction with VCAM-1 and ICAM-1 with very late antigen-4 (VLA-4) and lymphocyte function associated antigen-1 (LFA-1), respectively, which are expressed on leukocytes.[13-15]

Once a firm adhesion is established, leukocytes migrate through the interendothelial junction into the subendothelial space (diapedesis) (Figure 1.2A). This process is facilitated by additional adhesion molecules, such as platelet/endothelial cell adhesion molecule-1 (PECAM-1) and junctional adhesion molecule-1 (JAM-1).[16, 17] Furthermore, activated endothelial cells produce several chemokines and interleukins (IL), which enhance diapedesis [18] and the recruitment of leukocytes into the lesion. CCL2 (MCP-1), produced by vascular endothelial cells, is an important chemoattractant for monocytes and T cells. These cells play an important role in lesion development.[19-22] Activated vascular cells but also macrophages within the lesion continue to produce chemokines such as, CCL5 (RANTES), CXCL10 (IP-10) and CCL11 (eotaxin) to further enhance the immune response (Figure 1.2B).[23-25] Within the lesion, monocytes differentiate into macrophages by stimulation of macrophage-colony-stimulating factor (M-CSF), which is produced by endothelial and smooth muscle cells.[26] The macrophages become activated by the uptake of modified LDL, thereby transforming into lipid loaded "foam cells". This process will be discussed in more detail later on.

The above-described process will lead to focal fatty streak formation and remain asymptomatic for a long time and may even be reversible at this stage. However, during the progression of life these fatty streaks may develop into more advanced lesions, depending on the exposure to several risk factors. A schematic overview of the above-described processes are depicted in figure 1.1A.

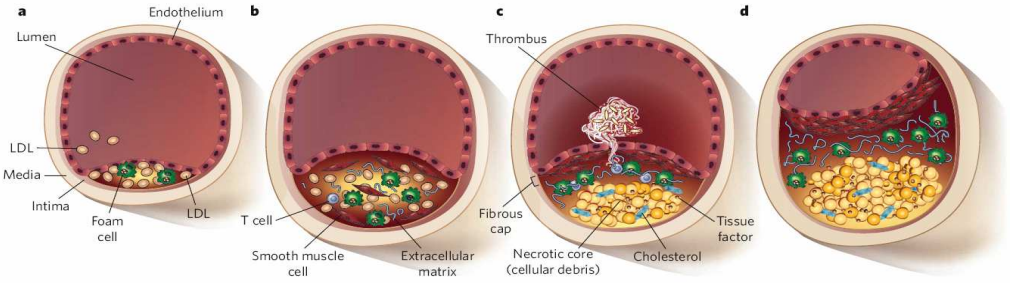

FIGURE 1.1: DEVELOPMENT OF ATHEROSCLEROSIS.
A, fatty streak formation: Endothelial cells in lesion prone areas become activated leading to permeabilety for lipoproteins, such as LDL. Within the intima, LDL is modified by processes, like oxidation and becomes immunogenic. Monocyte derived macrophages migrate into the intima and start to phagocytose the modified LDL particles and become activated. Macrophages, trapped in the intima and loaded with cholesterol are now called "foam cells". B, progression of the plaque: vascular smooth muscle cells become activated. They migrate and produce extracellular matrix proteins, in particular collagen, to form a cap structure to protect the lesion from the blood flow. More leukocytes are recruited to the intima, such as T cells and monocytes and these cells enhance the inflammation. To compensate for the narrowed lumen, outward remodeling takes place. C, necrotic core and thrombus formation: "foam cells" become apoptotic and eventually form a necrotic core, consisting of cellular debris and free cholesterol. The necrotic core is highly immunogenic, which results in the recruitment of more inflammatory cells to the intima. The fibrous cap formed by the smooth muscle cells protects the lesion from the bloodflow, however this may not prevent rupture. When the plaque ruptures, a thrombus will form and can cause clinical symptoms, such as acute coronary syndrome. D, obstructive lesion: when the plaque does not rupture, the lesion can grow by the ongoing inflammation. When outward remodeling is not sufficient, the lesion becomes obstructive and causes clinical symptoms, such as angina pectoris. More details are described in the text. (Adapted from Rader and Daugherty).[236]

1.2.2 Progression of the lesion

Atherosclerotic lesion progression is associated with the continuous influx of inflammatory cells due to the local production of chemokines in the plaque. T cells interact with activated macrophages ("foam cells"), which express class II and class I histocompatiblity complexes (MHC II and MHC I) and present antigens to T cells. $CD4^+$ and $CD8^+$ T cells are associated with all stages of atherosclerotic lesion development and activation of T cells results in a broad range of immune responses and the acquisition of many features of a chronic inflammatory state.[27] Activated T cells produce several cytokines such as IFN-γ and TNF-α.[28] These cytokines activate other cells within the lesion, such as endothelial cells, macrophages and smooth muscle cells.

The next step in atherosclerotic lesion development is the formation of a necrotic core. This necrotic core consists of extracellular lipids and cellular debris derived from apoptotic cells. This process involves pro- and anti-apoptotic proteins, including death receptors, proto-oncogens and tumor suppressor genes. Oxidized sterols, present in oxLDL, promote apoptosis and necrosis of foam cells in the plaque, thereby releasing oxidized and insoluble lipids within the lesion.[29]

The necrotic core becomes covered by a fibrous cap, which consists of smooth muscle cells and extracellular matrix proteins like collagen. The formation of the cap structure is facilitated by cytokines and growth factors, which are produced by activated macrophages and T cells. The cytokines and growth factors stimulate smooth muscle cells to proliferate and migrate to the cap.[5, 6]Activated smooth muscle cells can migrate from the media to the intima, where they are able to internalize lipids and transform into smooth muscle cell derived foam cells and can produce matrix proteins.[30]

In this stage of lesion development, the outward remodeling of the vessel takes place to compensate for the increase in lesion size. The process of outward remodeling is necessary to prevent severe narrowing of the vessel and to preserve blood flow.[31] The above-described process is schematically depicted in figure 1.1B and C.

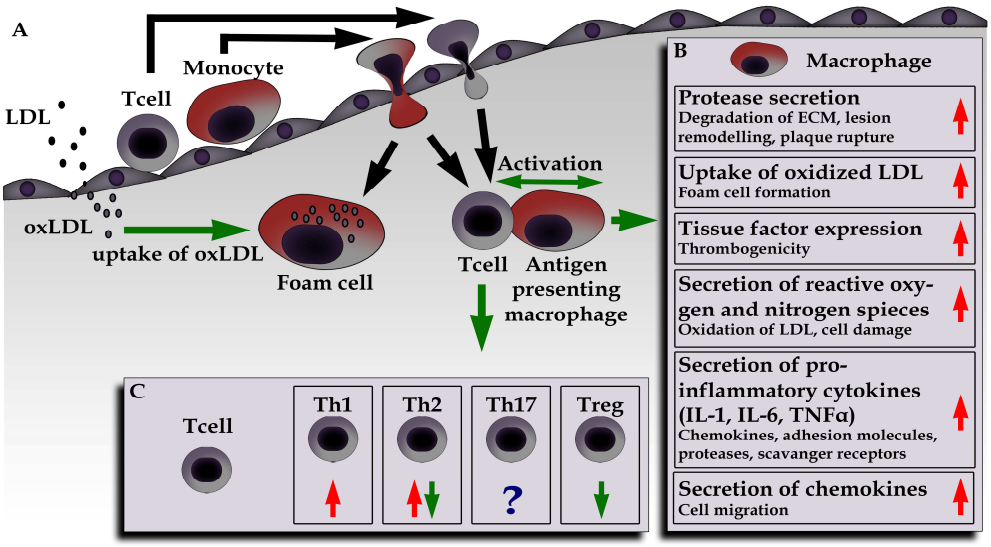

FIGURE 1.2: THE ROLE OF MACROPHAGES AND T CELLS IN ATHEROSCLEROSIS.
A, Monocyte and T cells are recruited to the intima of the vessel wall were they become activated via different stimuli. Depending on the microenvironment within the intima, the inflammation can be enhanced or dampened. B, Within the intima, macrophages take up modified LDL and become activated and exert several pro-atherogenic properties. C, Macrophages are able to take up modified LDL and present it to T cells, which recognize the antigen and become activated. Depending on the microenvironment, T cells can develop into different subtypes of T cells population. B and C, During atherosclerosis, T cells and macrophages tightly regulate each others activation and function, which make them important immune cells in bridging the innate (macrophages) and adaptive (T cells) immune system.

1.2.3 Lesion stability and rupture

When outward remodeling can not compensate for the reduced volume, the advanced atherosclerotic lesion leads to insufficient blood flow to distal tissues, causing clinical symptoms, such as angina pectoris (Figure 1.1D). However, acute cardiovascular events are associated with myocardial infarction and stroke as a result of a ruptured plaque and a subsequent thrombotic event (Figure 1.1C).[32, 33]

Plaque rupture is the final outcome of plaque destabilization. During lesion development activated macrophages, T cells and other immune cells accumulate around the necrotic core and in the shoulder regions of the plaque.[6, 34] Furthermore, activated macrophages, T cells, and mast cells have been observed at sites of plaque rupture, indicating their potential relation to the rupture process.[35-37] T cells, predominantly of the T helper 1 phenotype, produce high amounts of IFN-γ, which inhibit the production of collagen by vascular smooth muscle cells and their cell proliferation, thereby negatively influencing plaque stability.[28, 38]

Activated macrophages produce several proteases, which destabilize the plaque, such as matrix metalloproteinases (MMPs), cysteine proteases, and chymases.[39-41] Members of these families of enzymes are found in the atherosclerotic plaque and can degrade the matrix. Especially MMP-1, MMP-8, MMP-9 and MMP-13 may be important.[42] In addition, macrophages induce apoptosis of vascular smooth muscle cells, thereby negatively influencing the collagen production and subsequent plaque stability.[43, 44]

Since the weakened cap structure cannot withstand the hemodynamic forces, the unstable atherosclerotic lesion may rupture and consequently expose thrombogenic plaque material (lipids/necrotic core) to the blood. The subsequent aggregation of platelets and coagulation forms a thrombus, which obstructs blood flow and results in clinical symptoms such as myocardial infarction and stroke.[34]

Plaques that are prone to rupture contain high numbers of activated immune cells. Furthermore, patients with acute coronary syndromes (ACS) demonstrate signs of inflammation, with elevated levels of circulating cytokines, acute phase reactants, and not only activated T cells.[45, 46] Therefore the immune system and inflammation play an important role throughout atherosclerotic plaque development, but are also crucial in the final stage of atherosclerosis.

2 ATHEROSCLEROSIS: AN INFLAMMATORY DISEASE

During the last years, it has become more clear that atherosclerosis resembles a chronic autoimmune-like disease. Inflammation plays a pivotal role in the process of atherosclerotic lesion development. As atherosclerosis already starts in early life and gradually develops during life, it can be considered a chronic disease. Inflammation is tightly regulated by cells involved in the innate and adaptive

immune response, both with their specific role in host defense.[47,48] This is illustrated in different mouse models for atherosclerosis with a specific depletion of components of the innate as well as the adaptive immune system.[49, 50] For example, when LDL receptor deficient mice were cross-bred with lymphocyte-deficient (RAG1 deficient) mice, a reduction in lesion size was observed.[51]

Further evidence supporting the relation between atherosclerosis and inflammation is found in gene polymorphisms involved in inflammation, such as TLR4 polymorphisms.[52-54] There are associations found between an increased risk of cardiovascular events and autoimmune diseases, such as rheumatoid arthritis and systemic lupus erythematosus.[55] However, besides inflammation, atherosclerosis is also associated with metabolic and hemodynamic factors, thereby placing atherosclerosis as an unique disease.

Metabolic and hemodynamic factors are likely to play a role in the initiation of autoimmunity by activation of endothelial cells and subsequent recruitment of immune cells. This process leads to initial atherosclerosis via the innate immune system and gradually evolves into a chronic, autoimmune like, inflammatory disease via the adaptive immune system. Therefore, the regulation and crosstalk between innate and adaptive immune cells is very important in the initiation and development of atherosclerosis.

Although several exogenous stimuli, such as cytomegalovirus (CMV) and *Chlamydia pneumoniae* have been identified in atherosclerosis,[56] there is also evidence that endogenous stimuli are involved in the process of atherosclerosis.[57, 58]

2.1 INNATE IMMUNE SYSTEM

The innate immune response is the fist line of defense against pathogenic stimuli and is characterized by a natural selection of germline-encoded receptors, which focuses on highly conserved motifs in pathogens. It provides the first line of defense for the host and is characterized by fast (minutes to hours) and blunt (lacking exquisite structural specificity) responses. It is a very conserved system, which is already present in many lower organisms.

Important cells involved in the innate immune response are macrophages, neutrophils, mast cells and natural killer (NK) cells. The exact role of neutrophils in atherosclerotic lesion initiation is not known yet, but neutrophils are found in ruptured or eroded plaques, indicating that they are recruited in a later phase in response to injury.[59] However, upon endothelial activation, P-selectin and E-selectin are upregulated, which both bind neutrophils suggesting a non-confirmed role in the initial stages of atherosclerosis.[60-63]

Another kind of innate immune cells are mast cells. These cells are present in atherosclerotic lesions and are activated at sites of plaque rupture, indicating that mast cells are involved in the rupture process of advanced lesions.[64] Mast cells store granules, which contain growth factors, chymases and pro-inflammatory cytokines.[65,66] Once stimulated, the mast cells degranulate and exocytose the granule-associated effector substances into their microenvironment, thereby negatively influencing plaque stability.[67, 68]

NK cells are found to play a role in early atherosclerosis.[69, 70] Their role however, is not yet completely understood. Reduced atherosclerosis was observed in LDLr$^{-/-}$ mice after bone marrow transplantation from transgenic mice overexpressing the Ly49A receptor, which results in dysfunctional NK cells.[70] Noteworthy, in these transgenic mice, not only NK cells are affected, but also NK T cells, CD8^{+} cytotoxic cells and other lymphocytes expressing granzyme A. Therefore, it is difficult to determine the exact role of NK cells in the initiation of atherosclerosis.

The key inflammatory cell during atherosclerotic plaque formation is the macrophage. Macrophages are part of the innate immune system and have an important "bridge" function between the innate and adaptive immune system by presenting innate immune signals to the adaptive immune system. In atherosclerosis, macrophages play an important role in the various phases of lesion formation and progression.[12] Infiltrated monocytes differentiate into macrophages and start to express cytokines and receptors[71] by stimulation via M-CSF, which is produced by endothelial and smooth muscle cells.[26] Monocyte-derived macrophages express pattern recognition receptors (PRRs), which are involved in the innate immune response. PRRs recognize a restricted pattern of ligands called pathogen-associated molecular patterns (PAMPs). PAMPS consist of many different ligands such as lipopolysaccharides, aldehyde-derivatized proteins, bacterial DNAs and denatured DNAs, resulting in endocytoses and lysosomal degradation of the ligand[72,73] and activation of nuclear factor-κB, resulting in an inflammatory response.[74]

Two important groups of PRRs are the scavenger receptors (ScRs) and the toll-like receptors (TLRs), which are both expressed by macrophages.[47, 75] The ScR family, which includes CD36, CD68, SR-A and SR-B, mediates the internalization of modified lipoproteins (e.g. oxLDL) via endocytosis and contributes to foam cell formation, a hallmark of the atherosclerotic lesion.[9,76,77] The uptake of modified lipoproteins and their constituents via ScRs is important in triggering the production of the mediators of innate immunity such as, IL-1β and TNF-α.[78] Additionally, they are important in the activation of the adaptive immune system via presentation of internalized material on MHC class II molecules.[79]

The other important pattern recognition receptor family, the TLRs, are involved in the activation of macrophages.[80] Studies with TLR4 and ApoE double knock out mice, demonstrate a reduction in atherosclerotic plaque development, thereby further illustrating the importance of the innate immune system in atherogenesis.[81] Activation of ScRs and TLRs on macrophages within the plaque results in a proinflammatory environment capable of activation of other vascular and immune cells, thereby enhancing lesion progression.

Within the lesion, foam cells contribute to the production of reactive oxygen species, cytokines and other molecules, thereby amplifying and sustaining the inflammatory response in the plaque.[80] Additional cells in the early plaque, such as smooth muscle cells and endothelial cells become activated by these molecules and start producing interleukins and chemokines, i.e. IL-1β, IL-6, IL-18, TNF-α and CCL2.[82-84] Besides production of these cytokines, macrophages and other vascular cells start to produce T cells attractants, like CCL5, CXCR3, CXCL10 and CXCL11.[24] The pro-atherosclerotic effect of T cells in atherosclerosis is demonstrated by blocking for example CCL5 or CXCR3 and thereby attenuating atherosclerosis.[85,25]

Besides attracting T cells, macrophages also express MHC class II in association with a specific epitope. This is a pivotal step in bridging the innate immune response to the adaptive immune response. Within the lesion MHC class II expressing macrophages (and also dendritic cells, discussed in more detail later on) can be detected close to T cells, which suggests that there is an ongoing immune activation of the adaptive immune response in the plaque.[86-88]

2.2 ADAPTIVE IMMUNE SYSTEM

The adaptive immune system is characterized by a slower response than the innate immune system and exerts a high specificity for its target. The high variation of antigen specificity of, for example T cell receptors (TCRs) and immunoglobulins, is a result of somatic rearrangements in blast cells. When T cells recognize a specific antigen, which is presented by an antigen-presenting cell (APC), an adaptive immune response against that specific antigen is initiated. Many cells are involved in the adaptive immune response, in particular DCs, T cells, B cells and macrophages. Upon stimulation, these cells demonstrate versatile effects, for instance a helper T cell response, regulatory T cell response, cytotoxic T cell response, stimulation of B cells and subsequent antibody production, production of chemokines and cytokines, which enhance and regulate the innate and adaptive immune cells.

The role of the adaptive immune system, especially the T and B cells, is nicely demonstrated in studies in which these cells were not functional by for example using severe combined immunodeficient (SCID) mice, resulting in decreased lesion formation.[89]

2.3 AUTOANTIGENES

Many exogenous and endogenous antigens have been suggested to be presented in atherosclerosis. As mentioned before, T cells recognize specific antigens presented by APCs with their TCR. Upon activation, T cells that recognize a specific antigen will start proliferating, resulting in clonal T cell expansion. Clonal T cell expansion has been detected in atherosclerotic lesions of mice and humans, suggesting specific TCR activation by an (auto) antigen.[90-92]

Since inflammation has a prominent role in atherosclerosis, it has been suggested that exogenous stimuli may initiate atherosclerotic lesion development. Many virus and bacteria related antigens have been identified in atherosclerosis, but most extensively studied are the Cytomegalovirus (CMV)[93] and *Chlamydia pneumoniae*.[94, 95] Both pathogens are associated with aggravating atherosclerosis in mice and humans, since antibodies against these pathogens have been correlated with the severity of cardiovascular disease in patients.[93, 96-98] Furthermore, experimental data have shown that infection with *Chlamydia pneumoniae* enhances atherosclerosis.[97] However, large clinical trials on the treatment of cardiovascular patients with antibiotics directed against *Chlamydia pneumoniae* did not result in a reduction of cardiovascular events in antibiotic treated patients.[99, 100]

Another group of antigens is of endogenous origin, but is related to exogenous antigens via a process called molecular mimicry.[101] An example are the heat shock proteins (HSPs), a group of highly conserved proteins. These proteins are highly conserved and immune responses induced against bacterial HSP60 may cross-react with responses against endogenous hsp60. Endogenous HSP60 is induced when cells are exposed to different stress stimuli.[102] HSP60 is expressed on endothelial cells, vascular smooth muscle cells and mononuclear cells in human atherosclerotic plaques.[103] Furthermore, circulating antibodies against HSP60 were detected in patients with atherosclerosis and HSP60 specific T cells were detected within the atherosclerotic plaque.[104,105] Therefore an immune response against HSP60 may contribute to endothelial damage and subsequent enhancement of atherosclerosis.[106,107] Interestingly, antibodies against HSP60 and its prokaryote homologue HSP65 are also detected in other autoimmune diseases such as rheumatoid arthritis.[108] In relation to this, patients with rheumatoid arthritis have a 2- to 5- fold higher risk of cardiovascular morbidity and mortality.[109]

A third group of antigens is derived from endogenous sources. This group mainly contains altered self-proteins and the autoimmune response against these proteins is directed against the neo-epitopes of the altered proteins. T cells do not react with native LDL, as there is immunological tolerance against self-antigens. However, modifications of LDL lead to non-self epitopes (neo-epitopes) and increased autoreactivity of T cells in mice and human.[110, 111] LDL in the atherosclerotic lesion can be modified by various processes as described before, and are accountable for the development of neo-epitopes.[112] An example of a neo-epitope related to LDL is oxLDL. OxLDL specific T cells are identified in human plaques and circulating antibodies against epitopes of oxLDL are detected in serum samples of patients with cardiovascular disease.[110, 113] Furthermore, lymph nodes and spleens of ApoE-deficient mice gave rise to an oxLDL specific T and B cell line displaying a strong humoral and cellular immune response against these modified lipoproteins, indicating the role of oxLDL in immune activation.[114, 115]

2.4 DENDRITIC CELLS

Although activated macrophages effectively present antigens to T cells, dendritic cells (DCs) are the most potent APCs of the immune system and are the key players in the regulation of the adaptive immune response. [116]

Immature DCs efficiently take "samples" of their antigenic microenvironment through macropinocytoses and receptor mediated endocytosis. Depending on the triggered PRRs, DCs present the antigen in context of either MHC class I or MHC class II and produce cytokines to evoke an appropriate immune response.[117] Therefore, DCs are crucial for an adequate clearance of the infection, but DCs are also responsible for pathogenic immunological responses.

Dendritic cells are a component of the vasculature associated lymphoid tissue and low numbers are found in the intima of healthy, but susceptible arteries before atherosclerotic lesion development is initiated. [118] Furthermore, DCs increase in number in the intima during the progression of atherosclerosis.[119] This indicates a role for DCs in the initiation and regulation of arterial inflammation. Immature DCs capture antigens at the site of inflammation and migrate to secondary lymphoid organs, such as the spleen and lymph nodes. The migration is orchestrated by various chemokines, such as CCR-2, -5, -6, -7 and CXCR1 and CXCR2.[120, 121] Within the secondary organs the maturated DCs are able to stimulate antigen specific T cells, which is further enhanced by the fact that mature DCs starts to express co-stimulatory molecules, such as CD40, thereby enabling them to interact with CD40L expressing T cells.

Some macrophages take up antigens in tissues and differentiate into migratory cells resembling dendritic cells that emigrate to lymph nodes.[122]

However, in hypercholesterolemic conditions these monocyte derived DCs are possibly not able to migrate out of the atherosclerotic lesion to the secondary lymph organs, thereby directly activating residential cells in the lesion such as T cells, which leads to aggravated atherosclerosis.[122-124]

2.5 T CELLS

T cells are activated in the lymphoid organs by APCs by recognizing a specific antigen and by costimulatory signals such as CD40L-CD40 and CD80/CD86-CD28 interactions.[125] The microenvironment determines the type of T cell response. For example IL-12 production by APCs lead to the development of T helper 1 (Th1) cells, which have been shown to aggravate atherosclerosis.[126] Activated T cells migrate from the lymphoid organs to the site of inflammation e.g. the atherosclerotic lesion via chemokine signaling and are reactivated upon recognition of the antigen presented by APCs. Interference in T cell migration via the inhibition of the CXCR3 and CCL5 pathway, results in reduced Th1 cell influx into the lesion and subsequently in reduced atherosclerosis. [85, 127]

Most T cells within the atherosclerotic lesions are $CD3^+$ $CD4^+$ TCR $\alpha\beta^+$ cells.[128, 129] Although $CD8^+$ T cells are also found in atherosclerotic plaques, their role in atherosclerosis is not yet clear. Furthermore, $CD4^-$ $CD8^-$ TCR γδ T cells are found in plaques and may play a role in relation to IL-17 production.[129] IL-17 will be discussed later in more detail.

In relation to the topic of this thesis, the discussed T cell populations will be limited to Th1, T helper 2 (Th2), T helper 17 (Th17) and regulatory T (Treg) cells (Figure 1.3).

2.5.1 T helper 1 cells

Data obtained from patients with CVD illustrated a predominant Th1 pattern within atherosclerotic plaques. This is also observed in mouse models for atherosclerosis. T cells differentiate into Th1 cells through stimulation of naive T cells with Th1 polarizing cytokines, such as IL-12 and IL-18.[130, 131] The production of interferon (IFN)-γ hallmarks Th1 cells, which has pleiotropic pro-atherosclerotic effects. IFN-γ promotes activation of macrophages and endothelial cells to produce more adhesion molecules, proinflammtory cytokines and chemokines, which results in more T cell recruitment. Furthermore, IFN-γ promotes the production of proteases and inhibits collagen production, thereby interfering in plaque stability.[132] Another Th1 associated cytokine is TNF-α, which also exerts pleiotropic pro-atherogenic effects.[133]

The role of Th1 cells in the aggravation of atherosclerosis has already been demonstrated. For example, vaccination against IL-12 in LDLr$^{-/-}$ mice results in a reduction in IFN-γ expression within the lesion and reduced atherosclerotic lesion development.[131] Buono *et al.* demonstrated in LDLr and IFN-γ deficient mice a 75% reduction in lesion size, indicating the pro-atherosclerotic nature of Th1 cells.[134] Furthermore, IL-18 deficient ApoE$^{-/-}$ mice also showed reduced atherosclerosis.[130]

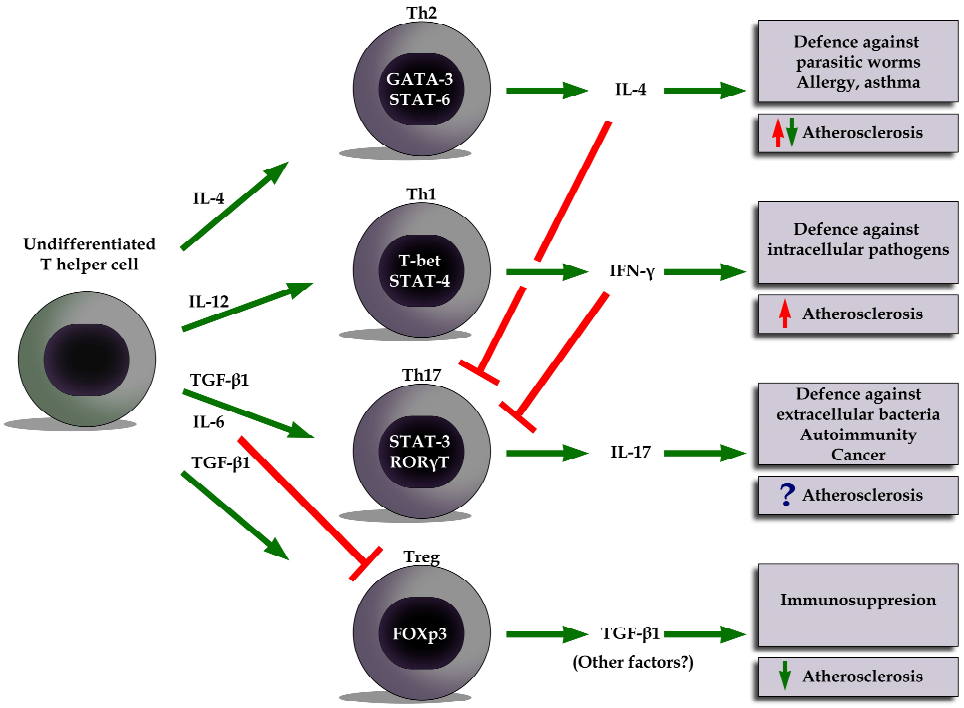

FIGURE 1.3: DIFFERENTIATION OF T HELPER CELLS.
Naive T cells differentiate into either Th1, Th2, Th17 or Treg cells upon stimulation with specific interleukins. Every Th cell subset has its own specific function in the immune response. However, a disproportional proliferation of a Th cell subset may lead to autoimmune diseases or chronic inflammation. The role of T helper cells in atherosclerosis is also depicted in this figure. See text for a more detailed description of the different Th subsets. (Adapted and modified from Tato and O´Shea)[237]

2.5.2 T helper 2 cells

Traditionally, Th2 cells are considered anti-atherosclerotic due to the production of IL-4, IL-5, IL-9, IL-13, IL-10 and IL-3.[135] An athero-protective role for these cells is suggested since the Th2 interleukins inhibit Th1 cells. Overexpressing IL-10 in LDLr$^{-/-}$ mice for example, exerts anti-atherosclerotic effects.[136, 137] Furthermore, IL-5 exerts an indirect anti-atherosclerotic effect by stimulation of B-1 cells. These B-1 cells produce natural occurring T15/EO6 IgM antibodies directed against oxLDL. These natural antibodies can block the uptake of oxLDL by macrophages and

thereby attenuate atherosclerosis.[138] This effect is nicely illustrated by Binder *et al.*, where IL-5 deficient bone marrow is transplanted into a LDLr$^{-/-}$ recipient mice leading to aggravated atherosclerosis and a decrease in natural autoantibodies.[139]

Conflicting data exist on the role of IL-4 in atherosclerotic plaque development. An athero-protective effect on initial plaque development was found after injection of IL-4 into mild hypercholesterolemic mice.[140] On the other hand IL-4 deficient ApoE$^{-/-}$ mice demonstrate reduced atherosclerosis, illustrating a pro-atherosclerotic effect of IL-4.[141] Moreover, IL-4 is associated with increasing MMP-1 production by macrophages and may therefore have negative effects on plaque stability.[142]

For long, it was thought that a disturbed balance between Th1 and Th2 cells was causative for many autoimmune diseases, including atherosclerosis. However, the aforementioned findings on Th2 cells show that the classical Th1/Th2 balance cannot adequately explain the inflammatory process in atherosclerosis.

Recently, two new T cell subsets were identified: the Th17 cell and the Treg cell. These cells provide more complexity and may provide an opportunity to explain the observed conflicting experimental effects.

2.5.3 Regulatory T cells

The regulatory T cells (Tregs) are important in maintaining immune homeostasis and preventing autoimmunity.[143] Tregs develop in the thymus and display a diverse TCR repertoire specific for autoantigens. Tregs migrate from the thymus into the peripheral tissues and exert their anti-inflammatory response by recognition of specific autoantigens.[144]

There are various mechanisms of immune suppression by Tregs.[182] Firstly, the suppression of immune cells by cytolysis, via granzymes and perforins. Secondly, suppression by metabolic disruption, via "consuming" IL-2. Thirdly, suppression by inhibitory cytokines, such as TGF-β and IL-10. Finally, suppression by targeting DCs, via the interference in maturation and function of DCs, for example by cell-cell inhibition via CTLA4 (on Tregs) and CD80/CD86 (on APCs).[146]

Tregs can generally be divided into two groups, the natural occurring Treg (nTreg) cells and the inducible T regulatory (iTreg) cells.[147, 148] nTreg cells express CD4, CD25 (IL-2Rα), cytotoxic T lymphocyte antigen (CTLA)-4 and forkhead box P3 (Foxp3).[145] nTreg cells exert their immunosupressive action predominantly by expressing membrane bound TGF-β, which suppress cells via cell-cell contact in paracrine fashion.[146, 149] Furthermore, nTreg cells are able to bind to CD80 and/or CD86 via CTLA-4, thereby suppressing the immune system.[150]

There are also Foxp3 negative Treg cells leaving the thymus, which can be induced in the periphery to become immunosuppressive Treg cells, called inducible Tregs (iTregs). Depending on the suppressive action, these cells can be divided in Tr1 cells, which predominantly produce IL-10[151] and Th3 cells, which exert their immunosuppressive function predominantly via TGF-β. Interestingly, Th3 cells do transiently express FoxP3.[152, 153]

Mallat *et al.* hypothesized that adaptive or natural regulatory T cells may play an important role in the regulation of pathogenic T cells in atherosclerosis. There are several studies underlining this hypothesis. Depletion of Treg cells by treatment with anti-CD25 antibodies results in aggravated atherosclerosis in ApoE$^{-/-}$ mice.[125] Furthermore, van Puijvelde *et al*. demonstrated that the induction of Treg cells via tolerance induction against oxLDL or HSP60 leads to attenuated atherosclerosis.[154] Moreover, Foxp3 expression has been detected in human atherosclerotic plaques, indicating that Treg cells are present.[155]

2.5.4 T helper 17 cells

Th17 cells are a novel T cell subset with a separate lineage. Langrish *et al.* showed that IL-23 selectively induces the proliferation of *in vivo*-primed IL-17-expressing Th cells and that these cells do not produce IL-4 or IFN-γ, indicating a separate Th subset.[156] Harrington *et al.* and Park *et al.* further established the idea of a separated lineage of Th cells distinct from the T helper type 1 and 2 lineages. A naive precursor T cell is potently inhibited by IFN-γ and IL-4 in differentiation towards Th17 cells, whereas committed Th17 cells are resistant to suppression by Th1 or Th2 cytokines.[157] Together these data provide evidence for a new Th subset, which is regulated by cytokines of Th1/Th2 cells and is involved in autoimmunity.

Hence the name, IL-17 is the most prominent cytokine produced by Th17 cells. The IL-17 family consists of six members, IL-17A, IL-17B, IL-17C, IL-17D, IL-17E and IL-17F. IL-17A and F are most related to each other and share a 50% homology in protein sequence.[158] As IL-17A was the first member of the IL-17 family which was identified, it is mostly designated as IL-17.

The IL-17R family consists of five members, designated as IL-17RA, IL-17RB, IL-17RC, IL-17RD and IL-17RE.[158] The best-studied IL-17R is IL-17RA, also designated as IL-17R, and is expressed ubiquitously through the body, which explains the pleiotropic effects of IL-17. The primary source of IL-17A and F are Th17 cells.[159] However, there are other cells of the innate and adaptive immune system which produce IL-17A and F. It has been shown that CD8$^+$ T cells produce IL17A and F as well as the γδ T cells.[160, 161] In many different cell types, binding of IL-17A and/or F to its receptor results in the upregulation of a number of pro-inflammatory interleukins and chemokines.[158, 162-165] IL-17A and F also exert a

chemotactic effect in recruiting and activating neutrophils, providing a mechanism by which Th17 cells can mediate the crosstalk between innate and adaptive immune responses.[158,165,166] IL-17 exhibits pleiotropic biological effects on various atherosclerotic lesion-associated cell types, such as endothelial cells, vascular smooth muscle cells and macrophages.[167-169] Upon activation by IL-17 these cells produce pro-inflammatory cytokines, chemokines and matrix metalloproteinases (MMPs), including IL-6, CXCL8, CCL2 and MMP-9.[169, 170] However, the role of IL-17 in atherosclerosis is not investigated yet. In this thesis, the role of IL-17 and its receptor in atherosclerosis will be discussed in chapter 3 and 4.

Besides IL-17, Th17 cells also produce IL-21, IL-22 and CCL20. IL-21 is expressed by Th17 cells[171], but also by other cell types, such as IL-6 stimulated T cells.[172,173] The receptor for IL-21 is expressed only on lymphoid cells and predominantly on B cells.[174] IL-21 has pleiotropic effects, such as stimulating proliferation and differentiation of $CD8^+$ T cells[175] and it promotes differentiation and isotype switching in B cells.[176] IL-21 induces CXCL8 expression in macrophages[177], which is involved in the recruitment of monocytes to the early lesion, thereby aggravating atherosclerosis.[178] Furthermore, IL-21 regulates the differentiation of $CD4^+$ T cells to Th17 cells in an autocrine manner.[171, 179]

IL-22 is a member of the IL-10 family [180] and the IL-22 receptor subunits are primarily expressed on epithelial and parenchymal tissues. [181] IL-22 protects against liver damage in an acute inflammation model.[182]

Finally, Th17 cells produce CCL20.[183, 184] Interestingly, its receptor CCR6, is also expressed by Th17 cells.[183, 185] This may imply an autocrine mechanism to regulate its own recruitment in inflamed tissues.The development pathway of the Th17 cell lineage is actively investigated. Initially is was thought that IL-23 was the driving force behind Th17 development.[156]

IL-23 is a heterodimeric interleukin consisting of a p40 and a p19 subunit. IL-23 is closely related to IL-12 as they both share the p40 subunit. [186] The receptor of IL-23 consists of a heterodimeric complex consisting of IL-12Rβ1 shared with the IL-12 receptor and a unique IL-23R. The IL-23R shares many features with IL-12Rβ2, which is the other part of the IL-12 receptor. The IL-23R is mainly expressed on effector T cells and not on naive T cells, suggesting an important role for IL-23 in ongoing inflammation.[186, 187]

IL-23 is mainly produced by macrophages and dendritic cells. Like the subunits of IL-27, EBI3 and p28, p19 must be expressed together with p40 in the same cell in order to be functionally excreted as IL-23.[186] Recently, it is demonstrated that the development of gut inflammation in T-cell-deficient mice depends on IL-23, in that the loss of IL-23 but not IL-12 was associated with a decrease in gut inflammation. Most striking is the role of IL-23 in autoimmune diseases, such as EAE and RA. The role of IL-23 is identified by the observation

that IL-23p19-deficient animals do not develop EAE and do not develop IL-17-producing T cells.[188] In patients with Crohn's disease, a single nucleotide polymorphism (SNP) in the coding sequence of IL-23R results in a strong protection against this disease, indicating a pathogenic role of IL-23 in chronic inflammation. Since IL-23 can expand a population of IL-17-producing pathogenic cells, an important role of IL-23 in the development of autoimmune diseases was suggested.[156] These data imply that IL-23 may be responsible for the differentiation of Th17 cells. However, *in vivo* experiments demonstrated that IL-23 functioned more like a maintenance interleukin for the Th17 cell population.[189] Therefore, it has been proposed that IL-23 may play a role in maintaining or stabilizing the Th17 cell phenotype, or in the survival of Th17 cells (Figure 1.4).[190]

Interestingly, as research continued, two "old" cytokines with opposing effects, IL-6 and TGF-β, were associated with Th17 cell differentiation.[171, 191] IL-6 is a pro-inflammatory cytokine and was shown to aggravate atherosclerosis.[192] On the other hand, TGF-β is an anti-inflammatory cytokine, which is associated with the differentiation of natural Treg cells and attenuation of atherosclerosis.[193 194]

Most research on Th17 cells is done in mice. However, there are differences observed in Th17 development between mice and humans. Recently, some studies showed that also TGF-β, in combination with IL-1β, IL-6 or IL-21 is able to induce the differentiation of human Th17 cells,[195, 196] indicating some overlap. More research has to be performed to further clarify the different aspects of Th17 cell development in different organisms.

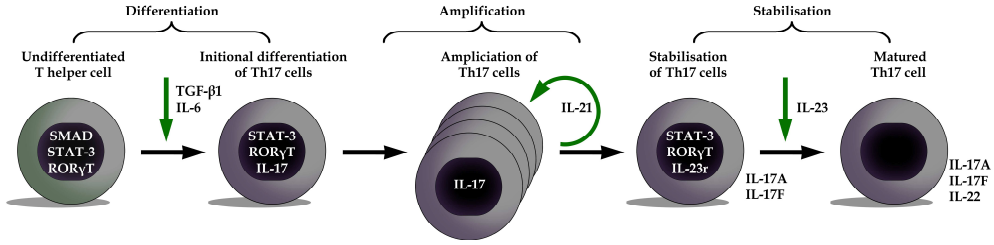

FIGURE 1.4: DETAILED OVERVIEW OF TH17 CELL DIFFERENTIATION.
Since the discovery of Th17 cells many research has been done to elaborate the developmental pathway of Th17 cells. The insight in the role of IL-23 in Th17 cell development changed over time and is now assigned as a stabilizing factor for Th17 cell differentiation. TFG-β and IL-6 are associated with the differentiation of naive T cells to Th17 cells. Furthermore, the recently identified IL-21 is assigned to amplify Th17 cell development via an autocrine positive feedback. (Adapted and modified from Bettelli and Kuchroo)[190]

The discovery of Th17 cells has changed the view on initiation and development of autoimmune diseases. There is, however, almost no literature available which associates Th17 cells with cardiovascular disease, although Cheng

et al. recently identified increased Th17 cells in patients with acute coronary syndrome.[197]

Nevertheless, there are indications that Th17 cells may play an important role in atherosclerosis. The prominent effector cytokine, IL-17 is discussed in chapter 3 and 4 of this thesis. Furthermore, the fact that IL-23 is involved in several autoimmune diseases, together with the finding that IL-23 is involved in the Th17 pathway, which is likely to be pro-atherosclerotic, indicates a role for IL-23 in atherosclerosis. Again, there are hardly any reports addressing IL-23 in atherosclerosis. In chapter 5 the role of the IL-23R in atherosclerosis will be discussed. Finally, we also studied the role of IL-27, which can suppress the development of Th17 cells,[198] as is described in chapter 6.

2.5.5 Th17 and Treg cells, two different subsets with a close relationship

Both, the Th17 lineage as well as the Treg lineage requires TGF-β for their development. Therefore, a reciprocal relationship between these two cell populations is likely.[191] TGF-β is needed for both cell types and IL-6 has a pivotal role in shifting the balance toward Th17 cells.[171, 191] Furthermore Laurence *et al.* demonstrated that IL-2, which is necessary for Treg cells, inhibits Th17 cells in a STAT5 dependent way.[189] Interestingly, IL-21 synergizes with TGF-β to promote the differentiation of Th17 cells in mice and, as mentioned before, IL-21 has an autocrine loop to enhance its own production by inducing proliferation and recruitment of Th17 cells. [171, 179]

There is also an interesting relation between Treg cells and Th17 cells on the level of transcription factors (Figure 1.5). The Th17 cell specific transcription factors, RORγ and RORα, are able to bind and antagonize Foxp3, a Treg transcription factor, and vice versa.[199, 200] To underline this fact, conditional deletion of Foxp3 protein in Treg cells *in vivo* results in an increase in RORγ and subsequent upregulated IL-17 and IL-21 expression.[201, 202] These data may be interesting for the treatment of chronic inflammation and autoimmunity.

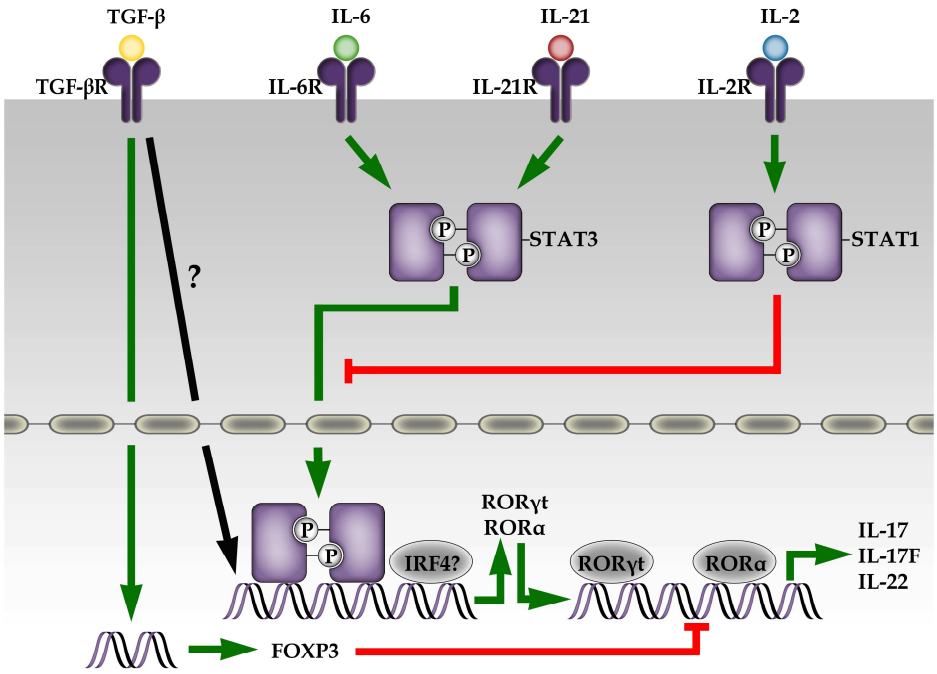

FIGURE 1.5: REGULATION OF TH17 AND TREG SPECIFIC TRANSCRIPTION FACTORS.
The transcription factor STAT3 is crucial for Th17 development. IL-6 and IL-21 activate this pathway, however the exact role for TGF-β on transcriptional level remains to be elucidated (black arrow). Recently, IL-27 has been shown to inhibit Th17 cells and this process is probably mediated by STAT1 dependent STAT3 inhibition. Furthermore, IL-2 is able to suppress Th17 development in a STAT1 dependent way. These data suggest a reciprocal relationship between Th17 and Treg cells. (Adapted and modified from Dong)[238]

2.6 B CELLS

B cells are also an important cell type of the adaptive immune system and are pivotal in the production of antibodies against specific antigens. Depletion of B cells results in aggravated atherosclerosis.[203, 204] A specific subpopulation of B cells, B-1 cells, produce IgM class antibodies (T15/EO6) directed against antigens that exert an atheroprotective function.[139, 205] Therefore, it is speculated that B cells are atheroprotective.[206]

However, B cells are also able to present antigens and produce cytokines. Positive correlations have been found between atherosclerosis burden in the carotid artery and activated B cells in the circulation.[207, 208] Furthermore, these B cells produce IgG antibodies against autoantigens and are associated with aggravating atherosclerosis.[209]

3 COMMUNICATION BETWEEN IMMUNE CELLS, A CRUCIAL STEP

The immune system is a complex system capable of defending its host against many different pathogenic organisms. However, although tightly regulated, the immune system can become pathogenic when targeting autoantigens or by overexerting the immune response against a certain pathogenic antigen. The adaptive immune system depends on signals of the innate immune response to adjust and adapt the response against the antigen. However, when this line of communication is disturbed or deregulated, it may result in chronic or autoimmune diseases.

Within atherosclerosis, the adaptive and innate immune response have an important role in all stages of the disease. The innate immune system initiates atherosclerosis and the adaptive immune system is involved in development of the lesion. An important way of communication between immune cells and non-immune cells is done by interleukins. A number of interleukins of special interest for this thesis, as they are involved in autoimmune diseases or in the polarization of autoimmune associated immune cell population, are discussed below.

3.1 INTERLEUKIN 15

Interleukin 15 (IL-15) is a pro-inflammatory cytokine which is first described in 1994 by Grabstein *et al.* as a T cell activating factor with structural resemblance to IL-2.[210] Although, little primary homology on protein level is found, high homology on secondary level is observed between IL-15 and IL-2. In addition, IL-15 is designated as a member of the α-helix bundle cytokine family, which also includes IL-2.[210]

The IL-15 receptor shares two subunits, the ß and γ_c subunit, with the IL-2 receptor. The third subunit is formed by a unique α-chain, IL-15Rα.[211] The third subunit of the IL-2R is the IL-2Rα (CD25).

IL-15 is expressed by several immune cells, in particular by monocytes and macrophages, but also by some non-lymphoid cells, such as fibroblasts.[212] IL-15 is also involved in expansion and survival of Natural Killer T (NKT) cells, which form an important link between the innate and adaptive immune response and enhance atherosclerosis.[213] IL-15 is also expressed in a biologically active form in association with IL-15Rα on the surface of monocytes and activated macrophages. This surface expressed IL-15 is approximately five times more effective than soluble IL-15 in the induction of T cell proliferation and is able to signal in a *cis* and *trans* fashion to neighboring cells.[214-216] Since soluble IL-15 is difficult to detect in biological fluids, it is suggested that the membrane bound IL-15 exerts a more important function in inflammation.[217]

IL-15 is expressed in human and murine atherosclerotic lesions[218, 219] and may therefore affect T cells within the plaque. Besides activating T cells, IL-15 is a strong chemo-attractant for T cells and Natural killer (NK) cells[220, 221] and it enhances CD44 mediated T cell adhesion to endothelial cells.[220, 222] Sanchi *et al*. demonstrated that in the presence of IL-15, activated endothelium at sites of chronic inflammation is able to recruit and activate peripheral blood T cells to the site of inflammation.[223] Furthermore, IL-15 can indirectly aggravate atherosclerosis by autocrine regulation of the production of pro-inflammatory cytokines by macrophages, such as TNF-α, IL-6 and IL-1β[224] and fibroblasts produce matrix metalloproteinases upon stimulation by IL-15.[225]

These data suggest a direct and indirect role for IL-15 in atherosclerosis. The role of IL-15 in atherosclerosis will be addressed in chapter 2 of this thesis.

3.2 INTERLEUKIN 27

Recently, a new interleukin was identified with structural resemblance with IL-12 and IL-23, called IL-27 and is composed of Epstein-Barr virus induced gene 3 (EBI3) and p28.[226] It is produced by activated antigen presenting cells and by resident macrophages.[227] The IL-27 p28 is poorly secreted unless it is co-expressed with its partner EBI3 and thus creating a situation where expression of IL-27 can be tightly controlled during an immune response.[187] IL-27 is therefore an important regulator of the adaptive immune response by interpreting signals of the innate immune system.

The receptor for IL-27 is a heterodimeric complex of gp130 (part of the IL-6 receptor) and the novel IL-27R (also designated as WSX-1 or TCCR).[228] IL-27 receptor is expressed on lymphocytes, such as B cells and Tregs, but also on natural killer (NK) cells, NK T cells, activated endothelial cells, activated epithelial cells, activated DCs, monocytes and mast cells.[187]

Interestingly, IL-27 is also associated with several autoimmune diseases. Initially IL-27 was assigned to have proinflammatory properties, based on early reports of the group of Goldberg *et al.* They illustrated that vaccination against p28 resulted in the suppression of EAE and adjuvant induced arthritis.[229, 230] Recent studies showed a more complex dualistic role for IL-27 with anti- and pro-inflammatory properties.

IL-27 is able to induce differentiation of naive $CD4^+$ T cells to Th1 cells, but is also able to suppress the development of Th17 cells in EAE models thereby attenuating the disease.[198, 227, 231] Furthermore, IL-27 inhibits the development of Tregs and Th2 cells.[232, 233] IL-27 has an unexpected activity in the immune system, as in some events it has a proinflammatory activity and in other events it shows anti-inflammatory activities by suppression of immune hyperactivity. Yoshimura

et al. proposed a mechanism where IL-27 stimulates STAT1 and STAT3 in naive Th cells, whereas only STAT3 is activated in activated Th cells.[234] Thus, IL-27 is capable to inhibit or stimulate T cells, depending on the IL-27R expression of the target cells and subsequent signal transduction. However, more research needs to be done to study the divergent effects of IL-27 on different cell types under different conditions.

The role of IL-27 in atherosclerosis is not yet investigated. The complex role of IL-27 in atherosclerosis is studied in this thesis and will be discussed in chapter 6.

4 VACCINATION AS RESEARCH AND THERAPEUTIC TOOL

The current treatment of atherosclerosis is focused on reducing risk factors, such as hypercholesterolemia by the administration of statins and a change in life style. Since the inflammatory aspects of atherosclerosis are getting more and more elucidated, novel strategies may arise as potential therapy, such as vaccination. Vaccination is an ideal tool to generate a desired immune response against an antigen. In atherosclerosis, vaccinations may be directed against one or several autoantigens involved in this disease. This approach is already successfully demonstrated by vaccinating mice against oxLDL[154] and HSP60[235] (chapter 7), which resulted in attenuated atherosclerosis. Another possibility is the targeting of certain cell types to restore or shift a balance towards a favorable outcome. For example, vaccination against IL-12 resulted in a decrease of Th1 cells and subsequently in the reduction of atherosclerosis.[131]

A relative new development is DNA vaccination and this may prove to be a promising strategy in the future. DNA vaccination is based on, hence the name, DNA and can be produced at relatively low costs. Furthermore, the manufacturing and storage conditions are less stringent compared to protein-based vaccines and thereby facilitating a broader distribution and availability of anti-atherosclerotic medicine. As atherosclerosis and its related symptoms are getting pandemic proportions, these issues may be considered.

Furthermore, DNA vaccination may provide a good and again relatively cheap research tool to investigate the function of certain cell types, by inducing a cytotoxic response against these cells. Signaling molecules, such as interleukins, can be neutralized by raising a humoral response against them. Additionally, the effect of depletion or neutralization of the targets can be studied in different phases of the disease. This contributes to a better understanding of the disease and ultimately to its cure.

Detailed information and perspectives about vaccination against atherosclerosis is described in chapter 9 of this thesis as a perspective review.

5 OUTLINE OF THE THESIS

In this thesis, the role of several key interleukins and inflammatory cells is studied in relation to atherosclerosis.

In chapter 2 a DNA vaccination strategy is used, which makes use of a living carrier, the *Salmonella typhimurium* to induce a cytotoxic T cell response. In this study, we investigated the effect of IL-15 neutralization in atherosclerosis. We observed a strong reduction in atherosclerosis, which suggest an important role for this cytokine in the initiation of atherosclerosis.

Another proinflammatory interleukin, IL-17, is studied in chapter 3. In this study we made use of a novel vaccination strategy, where we inject a DNA vaccine in the muscle. By neutralizing IL-17 in LDLr$^{-/-}$ mice with a HEL-IL-17 DNA vaccine, a dramatic decrease in atherosclerotic lesion development was observed.

In chapter 4 the role of IL-17 signaling is studied by performing a bone marrow transplantation of IL-17 receptor deficient bone marrow into LDLr$^{-/-}$ recipient mice. This study illustrates an important role for IL-17 signaling in atherosclerosis.

In chapter 5, another bone marrow transplantation is described with p19 (a subunit of IL-23) deficient bone marrow to study the contribution of IL-23 signaling in atherosclerosis. However, there is no change in plaque size observed in this experiment.

In chapter 6, again a DNA vaccination strategy was used, although with another immunodominant T helper cell epitope, PADRE, to break the tolerance against the p28 subunit of IL-27. In this study, the effect of IL-27 depletion in atherosclerosis results in aggravated atherosclerosis, indicating an atherosclerotic protective role for IL-27.

To study whether regulatory T cells can be induced against an atherosclerotic related autoantigen, we induced tolerance against HSP60 as described in chapter 7. The tolerance induction results in an increased number of Treg cells and attenuated atherosclerosis. These results further establish the protective role of regulatory T cells in atherosclerosis.

Dendritic cell based vaccination is described in chapter 8. Here we used a novel mRNA based vaccine against the specific regulatory T cell transcription factor, Foxp3. Via electroporation, the dendritic cells are “loaded” with the mRNA and subsequently injected in LDLr$^{-/-}$ mice. We demonstrate again that regulatory T cells have a protective role in atherosclerose, as we observed aggravated atherosclerosis in Foxp3 vaccinated mice.

In chapter 9, a prospective review describes the possibilities of vaccination in atherosclerosis. Based on our work and the work of others, we think that immunomodulation can provide a very useful approach against atherosclerosis.

Furthermore, we discuss, to our opinion, the best approach to vaccinate against atherosclerosis.

Finally, in chapter 10, all results described in this thesis will be discussed in relation to the mechanism of atherosclerosis and possible future treatment of this disease in patients.

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Chapter 2

IL-15 aggravates atherosclerotic lesion development in LDL receptor deficient mice

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ABSTRACT

Interleukin 15 (IL-15) is a pro-inflammatory cytokine involved in inflammatory diseases and IL-15 is expressed in atherosclerotic plaques.

To establish the role of IL-15 in atherosclerosis we studied the effect of IL-15 on atherosclerosis associated cells *in vitro* and *in vivo* by neutralizing IL-15 using a DNA vaccination strategy.

Upon feeding a Western type diet, LDLr$^{-/-}$ mice do express higher levels of IL-15 within the spleen and the number of IL-15 expressing cells among blood leukocytes and spleen cells is increased. Addition of IL-15 to macrophages induces the expression of TNF-α and CCL-2. After the mice were vaccinated against IL-15, we observe a reduction in plaque size of 75%. Unexpectedly, the relative number of macrophages within the plaque was 2-fold higher in IL-15 vaccinated mice than that in control mice. Vaccination against IL-15 leads to an increased cytotoxicity against IL-15 overexpressing target cells, resulting in a reduction in IL-15 overexpressing cells in the blood and within the spleen.

Hypercholesterolemia leads to upregulation of IL-15 within the spleen and peripheral blood mononuclear cells. DNA vaccination against IL-15 does markedly reduce atherosclerotic lesion size, indicating that blockade of IL-15 by vaccination may be considered to be a promising strategy in the treatment of atherosclerosis.

INTRODUCTION

Atherosclerosis is characterized as a dyslipidemic induced chronic inflammatory disease of the arterial wall.[1] During the various stages of lesion development, monocytes and T cells are recruited to the arterial wall.[2] Already in the early stages of atherogenesis, macrophages and T cells are present in the intima of the atherosclerotic plaque.[3]

Interleukin 15 (IL-15) is a pro-inflammatory cytokine which is expressed by different immune cells such as monocytes and macrophages and promotes T cell proliferation independently of antigen-specific T cell receptor activation.[4] IL-15 is also expressed in a biologically active form on the surface of monocytes and activated macrophages. This surface expressed IL-15 is approximately 5 times more effective than soluble IL-15 in the induction of T cell proliferation.[5] IL-15 expression is associated with chronic inflammatory diseases such as rheumatoid arthritis.[6] In addition, IL-15 is found to be expressed in human and murine atherosclerotic lesions[7,8] and may therefore affect T cells within the plaque.

The IL-15 receptor shares two subunits, the ß and γ_c subunit, with the IL-2 receptor, while the third subunit is formed by a unique α-chain, IL-15Rα.[9] Because the IL-15 and IL-2 receptor share two subunits, IL-15 shares biological activities with IL-2, such as the induction of proliferation of T cell subsets. There are however opposing effects of IL-2 and IL-15. IL-2 is primarily involved in the maintenance of regulatory T cells and IL-15 plays mainly a role in the survival of T cells and thus in memory cell formation.[10-12] IL-15 not only activates T cells, it is also a strong chemoattractant for T cells and Natural killer (NK) cells[13, 14] and enhances CD44 mediated T cell adhesion to endothelial cells.[15] IL-15 is also involved in expansion and survival of Natural killer T (NKT) cells, which form an important link between the innate and adaptive immune response and enhance atherosclerosis.[16] IL-15 finally exerts an autocrine regulation of the production of pro-inflammatory cytokines by macrophages, such as TNF-α, IL-6 and IL-1β.[17]

We studied the role of IL-15 in atherosclerotic lesion formation by applying an *in vivo* blockade of IL-15 using oral vaccination, which resulted in a 75% reduction in lesion size, thereby establishing an important role for IL-15 in atherogenesis.

METHODS

ANIMALS, MATERIALS, BACTERIAL STRAINS AND CELL LINES

All animal work was approved by Leiden University and was in compliance with the Dutch government guidelines. LDL receptor deficient ($LDLr^{-/-}$) mice were purchased from Jackson Laboratories. The mice were kept under standard laboratory conditions and food and water were provided *ad libitum*. Recombinant murine IL-15 was purchased from PeproTech and biotinylated polyclonal mouse anti-IL-15 was obtained from R&D systems. The attenuated *Salmonella typhimurium* (Dam-;AroA-,strain:SL7207) was provided by Dr. Kriszitana M. Zsebo (Remedyne Corporation,Santa-Barbara,CA). The macrophage cell line (RAW246.7), the endothelial cell line (H5V) and mouse fibroblasts were cultured in DMEM with 10% FCS, 2 mmol/L glutamin, 0.1 U/L penicillin, and 100 mg/L streptomycin. Vascular smooth muscle cells were isolated from a murine aorta and cultured as described previously.[18]

IN VITRO ASSESSMENT OF IL-15

Cells were added to a 24-well plate ($2.5x10^5$ RAW cells/ml, $1.0x10^5$ cells for H5V and vSMC). Where stated, 100 ng/ml recombinant IL-15 was added to the culturing medium and culturing medium alone served as a control. Cells were incubated for 24 hours, and thereafter the cells were used for qPCR and the supernatant was used for ELISA. All experiments were performed in triplicate.

RNA ISOLATION AND QPCR

Total RNA was isolated using Trizol (Boehringer Mannheim) and reverse transcribed (RevertAidP^{TMP} M-MuLV reverse transcriptase, Fermentas). qPCR was analyzed with SYBRgreen mastermix (Perkin&Elmer) and a final concentration of 300 nM primers (Table 2.1), using acidic ribosomal phosphoproteinP0(36B4) as an internal standard.

ELISA

A mouse TNF-α set (PharMingen) was used to detect TNF-α in culture supernatant according to manufacturer's protocol.

CONSTRUCTION OF THE DNA VACCINE

Murine IL-15 (AI503618) was cloned into the eukaryotic expression plasmid pcDNA3.1 (Invitrogen). The 605bp. fragment encoding the entire IL-15 gene was amplified using PCR primers: 5′-GAA GCC CAT CGC CAT AGC–3′ and 5′-GAG CAG CAG GTG GAG GTA-3′ and subsequent cloned into pcDNA3.1 with *Eco*RV, generating pcDNA3.1-IL-15. Subsequently, *S. typhimurium* was electroporated with pcDNA3.1-IL-15 or an empty pcDNA3.1 plasmid.[19]

TABLE 2.1: SEQUENCE OF PRIMERS. Primer sequences for the genes analyzed with qPCR.

GENE	FORWARD PRIMER	REVERSE PRIMER
IL-15	5′-TGAGGCTGGCATTCATGTCTT-3′	5′-ATCTATCCAGTTGGCCTCTGTTTT-3′
IL-1β	5′-TGGTGTGTGACGTTCCCATTA-3′	5′-AGGTGGAGAGCTTTCAGCTCATAT-3′
IL-10	5′-TCTTACTGACTGGCATGAGGATCA-3′	5′-GTCCGCAGCTCTAGGAGCAT-3′
CXCL1	5′-GGCGCCTATCGCCAATG-3′	5′-CCTGAGGGCAACACCTTCAA-3′
CCL2	5′-GCATCTGCCCTAAGGTCTTCA-3′	5′-TTCACTGTCACACTGGTCACTCCTA-3′
CCR2	5′-CCTTGGGAATGAGTAACTGTGTGA-3′	5′-TGGAGAGATACCTTCGGAACTTCT-3′
36B4	5′-GGACCCGAGAAGACCTCCTT-3′	5′-GCACATCACTCAGAATTTCAATGG-3′

VACCINATION AND THE INDUCTION OF ATHEROSCLEROSIS

Mice were vaccinated prior to the induction of atherosclerosis with 10^8 cfu *S. typhimurium* transformed with empty pcDNA3.1 (control) or pcDNA3.1-IL-15 as previously described.[19] Male LDLr$^{-/-}$ mice 10-12 weeks of age were fed a Western-type diet containing 15% cocoa butter and 0.25% cholesterol two weeks prior to collar placement. Atherosclerosis was induced by placement of collars (0.3 mm, Dow Corning, Midland, Michigan) around the carotid arteries as previously described.[20] Hereafter, the mice were fed a Western-type diet for 8 more weeks. Total cholesterol levels during the experiment were quantified spectrophotometrically using an enzymatic procedure (Roche Diagnostics, Germany). Precipath standardized serum (Boehringer, Germany) was used as an internal standard.

CYTOTOXICITY ASSAY

The murine fibroblast cells were used as target cells and were co-transfected with pcDNA3.1-IL-15 and pcDNA3.1-eGFP using ExGen500 (Fermentas, Germany) according to the manufacturer's protocol. 24 hours after transfection, 10^6 spleen cells isolated from IL-15 vaccinated or control vaccinated mice were added to the target cells. 24 hours later, cells were fixed using FormalFixx (3.7%, Thermo Shandon, Pittsburgh, PA), and the number of GFP-fluorescent cells per well was determined.

HISTOLOGY AND IMMUNOHISTOCHEMISTRY

Carotid arteries were removed for analysis as described by Von der Thüsen *et al*.[20] The arteries were embedded in OCT compound (TissueTek; Sakura Finetek, The Netherlands). Cryosections of 5 µm were made proximally of the collar occlusion and stained with hematoxylin (Sigma Diagnostics, MO) and eosin (Merck Diagnostica, Germany). Corresponding sections on separate slides were stained immunohistochemically for macrophages using an antibody against a macrophage-specific antigen (MoMa-2, Research Diagnostics Inc.). Quantification of the staining was performed by using a Leica DM-RE microscope and Leica Qwin Imaging software (Leica Ltd., Germany).

FACS ANALYSIS OF LEUKOCYTES

Peripheral Blood Mononuclear Cells (PBMC) were isolated after orbital bleeding using Lympholyte (Cedarlane, Canada) as described in the manufacturer's protocol. Spleens were dissected and single cell suspension was obtained by passing the spleen through a 70 µm cell strainer (Falcon, The Netherlands). Leukocytes were purified using Lympholyte. Cells were stained with FITC-conjugated anti-mouse CD8 (0.125 µg/sample, Pharmingen) and PE-conjugated anti-mouse CD69 (0.125 µg/sample, eBioscience). For the staining of surface bound IL-15, the leukocytes were stained with biotinylated anti-mouse IL-15 (R&D systems) and PE-conjugated streptavidin (BD Pharmingen) and analyzed by flowcytometry on a FACSCalibur. All data was analyzed with CELLQuest software (BD Bioscience, The Netherlands).

STATISTICAL ANALYSIS

All data are expressed as means ± SEM. The two-tailed student's t-test was used to compare individual groups of mice or cells. When indicated, a Mann-Whitney test was used to analyze not normally distributed data. *P* values of <0.05 were considered significant.

RESULTS

IL-15 IS UPREGULATED IN HYPERCHOLESTEROLEMIC MICE

The spleens of LDLr-/- mice were collected at different time points after the start of the Western-type diet feeding and mRNA expression of IL-15 was quantified. The expression of IL-15 mRNA was significantly elevated in the spleen at 6 weeks after the start of the diet (Figure 2.1A). Since IL-15 expression is also regulated at a post-translational level and is mainly membrane bound[5], we also determined the cell surface expression of IL-15. Spleen cells and PBMCs were isolated from LDLr-/- mice which were fed a Western diet or a normal Chow diet for 10 weeks. FACS analysis showed that the percentage of IL-15 expressing cells within the spleen and PBMCs was elevated after 10 weeks of Western type diet. (Figure 2.1B; 12.59±0.65%versus 26.07±3.44%, *P*<0.05 and 0.28±0.06% versus 4.95±0.98%, *P*<0.05, respectively).

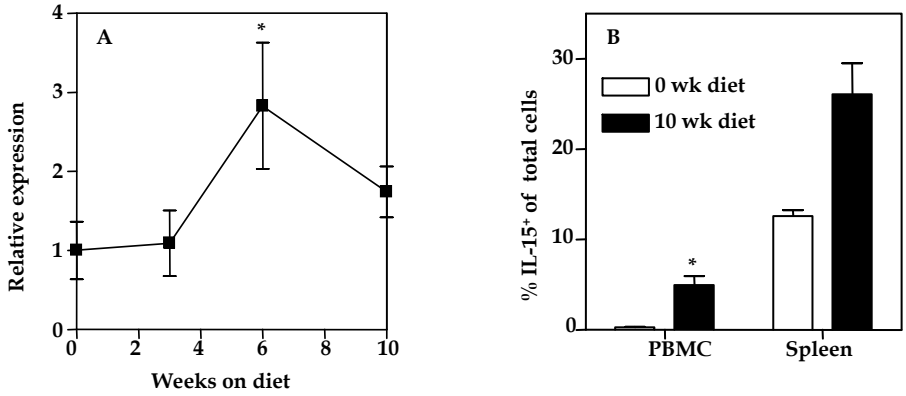

FIGURE 2.1: EXPRESSION OF IL-15 IN HYPERCHOLESTEROLEMIC MICE.
IL-15 mRNA expression level was determined in spleen cells of LDLr-/- mice at different time points after Western-type diet feeding(A). PBMCs and spleen cells were isolated and stained for surface bound IL-15(B). The percentages of IL-15 positive cells are determined by FACS analysis after 0 weeks of Western type diet (white bars, N=5) and after 10 weeks of Western type diet (black bars, N=5). * *P*<0.05

IL-15 PREDOMINATELY AFFECTS MACROPHAGES IN VITRO

We determined the effect of IL-15 on cell lines that represent the main cell types in the atherosclerotic lesion; macrophages (RAW cells), vascular smooth muscle cells (vSMCs) and endothelial cells (H5V cells). The relative expression of IL-15 is highest for macrophages (Figure 2.2A), while also for vSMCs and endothelial cells a distinct expression is found. Addition of recombinant IL-15 to the various cell types leads only for macrophages to increased expression of tumor necrosis factor (TNF)-α on protein level (Figure 2.2B). We observed in macrophages a distinct increase in the pro-inflammatory cytokine IL-1β, whereas there was no significant effect seen on mRNA encoding IL-10 (Figure 2.2C), IFN-γ or IL-12 (p40) (data not shown). In addition, IL-15 significantly induced the expression of CXCL1, CCL2 and CCR2 in macrophages (Figure 2.2D).

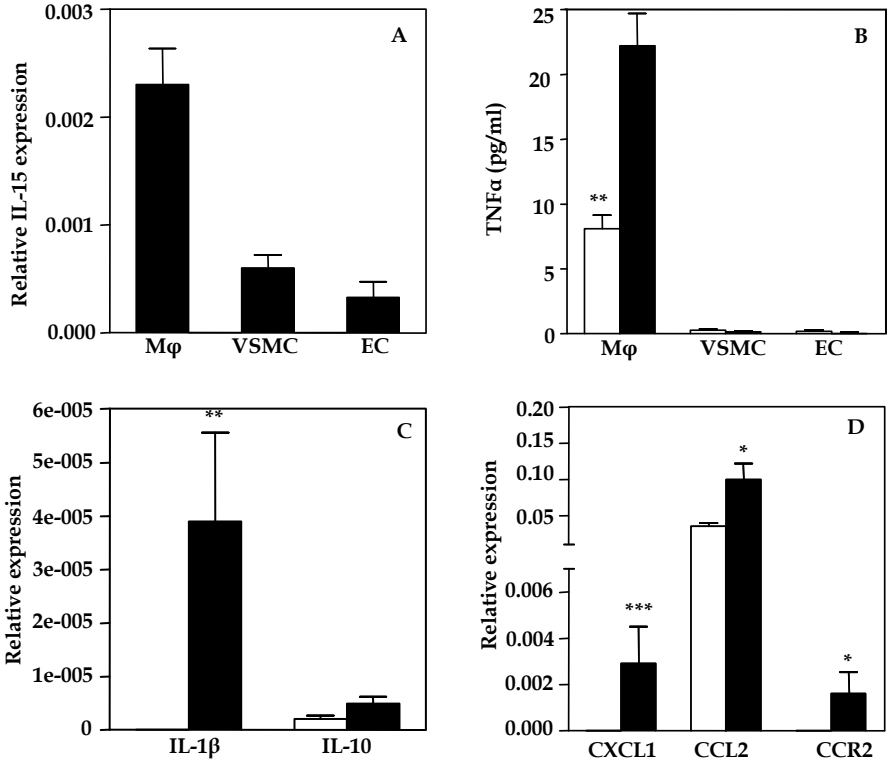

FIGURE 2.2: EFFECT OF IL-15 ON GENE EXPRESSION IN PLAQUE RELATED CELL TYPES.
IL-15 expression was determined in RAW cells, H5V cells and vSMC (A). IL-15 (black) or no IL-15 (control; white) was added to different cell types. Cellular activation was measured by TNF-α release (B). The expression of several genes was measured in RAW cells after addition of 100 ng/mL IL-15 (black), or without IL-15 (white) for inflammatory genes (C) and chemotaxic associated genes (D). **P*<0.05, ***P*<0.01 and ****P*<0.001

These results indicate that IL-15 may affect the chemokines induced migration of macrophages.[21] Endothelial cells did not respond to IL-15 by upregulation of CXCL1, CCL2 or CCR2 on mRNA levels. In addition, IL-15 did not affect the expression of adhesion molecules such as VCAM-1, ICAM-1, PECAM and P-selectin in endothelial cells (data not shown).

VACCINATION AGAINST IL-15 INDUCES AN IL-15 SPECIFIC CYTOTOXIC T CELL RESPONSE

The Western-type diet induced IL-15 expression on spleen cells and PBMCs and the IL-15 mediated activation of macrophages stimulated us to analyze the effect of IL-15 blockade on atherosclerosis via vaccination. To this end, LDLr$^{-/-}$ mice were vaccinated against IL-15 by oral delivery using an attenuated strain of *S. typhimurium* transformed with an IL-15 expression vector (pcDNA3.1-IL-15) or with *S. typhimurium* transformed with an empty vector (pcDNA3.1) as a control. This vaccination strategy leads to the induction of CD8$^+$ cytotoxic T cells that specifically lyse those cells that overexpress IL-15 and present IL-15 peptides via MHC class I. [19] This protocol was recently used to study the role of VEGFR2 and CD99 in atherosclerosis.[19, 22, 23] Following vaccination, mice were fed a Western-type diet for 2 weeks and collars were placed around the carotid arteries which results in flow-induced atherosclerotic lesion formation.[20] We established the activation state of the CD8$^+$ T cell population. Spleen cells were isolated and stained for CD8 and CD69, an early T cell activation marker, and analyzed by FACS analysis. The percentage of CD8$^+$CD69$^+$ double positive cells was increased significantly upon vaccination against IL-15 compared to the control vaccination (Figure 2.3A; 16.0±2.1% versus 10.4±0.1%, P<0.05). In order to study the specificity of CD8$^+$ cytotoxic T cells, spleen cells from vaccinated and control mice were co-cultured with murine fibroblasts that were co-transfected with pcDNA3.1-IL-15 and pcDNA3.1-GFP. The number of surviving IL-15 expressing target cells was determined by counting GFP positive cells. The number of IL-15 expressing target cells was reduced by 50% after incubation with spleen cells from IL-15 vaccinated mice, whereas spleen cells from control vaccinated mice, did not significantly lyse IL-15 expressing cells (Figure 2.3B; 49±1% in vaccinated group versus 81±4% in control group, P<0.01).

VACCINATION AGAINST IL-15 REDUCES ATHEROSCLEROTIC LESION SIZE IN HYPERCHOLESTEROLEMIC LDLR$^{-/-}$ MICE

Atherosclerosis was determined 6 weeks after collar placement. During the experiment blood samples were obtained and plasma cholesterol concentration

was determined. IL-15 vaccination did not affect plasma cholesterol levels during the experiment (Figure 2.3C). Hematoxylin-Eosin (HE) staining of the atherosclerotic plaque was performed and plaque sizes were quantified. Vaccination against IL-15 resulted in a 75% decrease in lesion size as compared to the control group (Figure 2.4A, B and C; 13722±3116 μm^2 versus 53977±15332 μm^2, $P<0.05$). Immunohistochemical staining for macrophages showed a significant change in plaque composition (Figure 2.4F). The relative number of macrophages per plaque area was 2-fold higher in IL-15 vaccinated mice (Figure 2.4E) than that in control vaccinated mice (Figure 2.4D), indicative for a less advanced state of the lesions in the vaccinated mice.

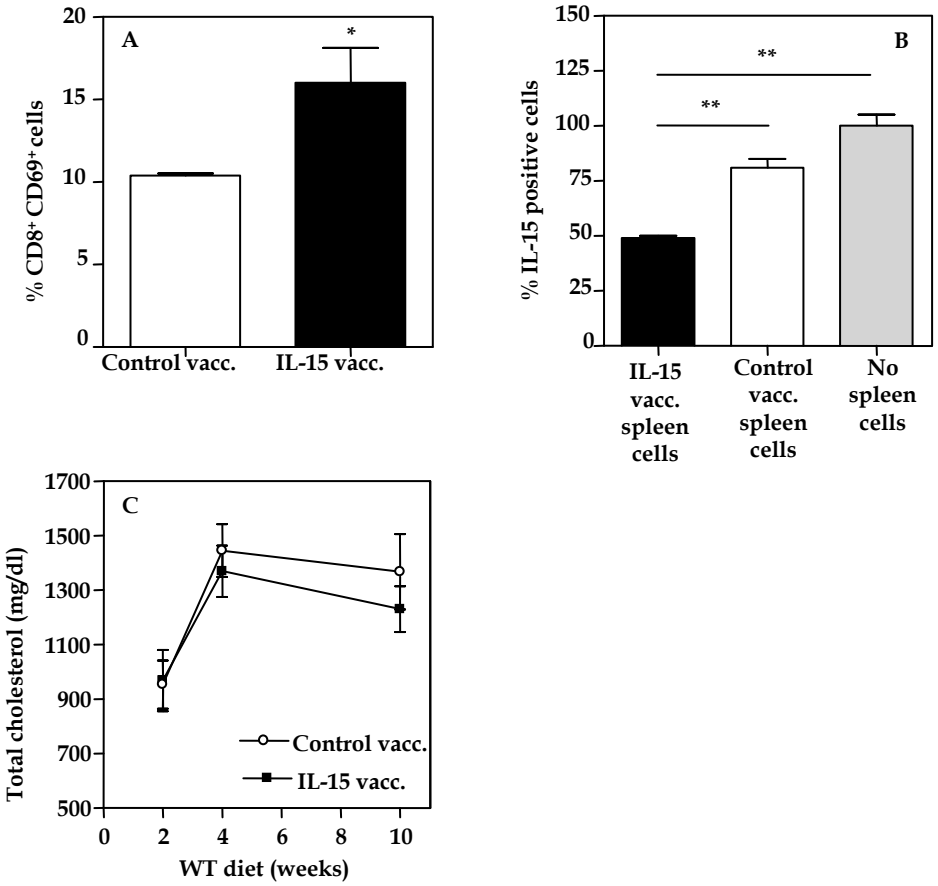

FIGURE 2.3: ***IN VIVO*** **EFFECT OF THE VACCINATION AGAINST IL-15.**
The percentage of double positive cells ($CD8^+CD69^+$ cells) was determined in a single cell suspension of the spleen cells in vaccinated and control mice (A, N=5). The induction of specific cytotoxic $CD8^+$ T cells against IL-15 expressing cells was determined by incubating spleen cells from vaccinated mice (black), control mice (white) or without spleen cells (grey) with fibroblast transfected with an IL-15 and GFP expression plasmid (B, N=5). Blood was taken from vaccinated and control mice at the indicated times and total cholesterol level was determined in serum (C, N=9). *$P<0.05$ and **$P<0.01$

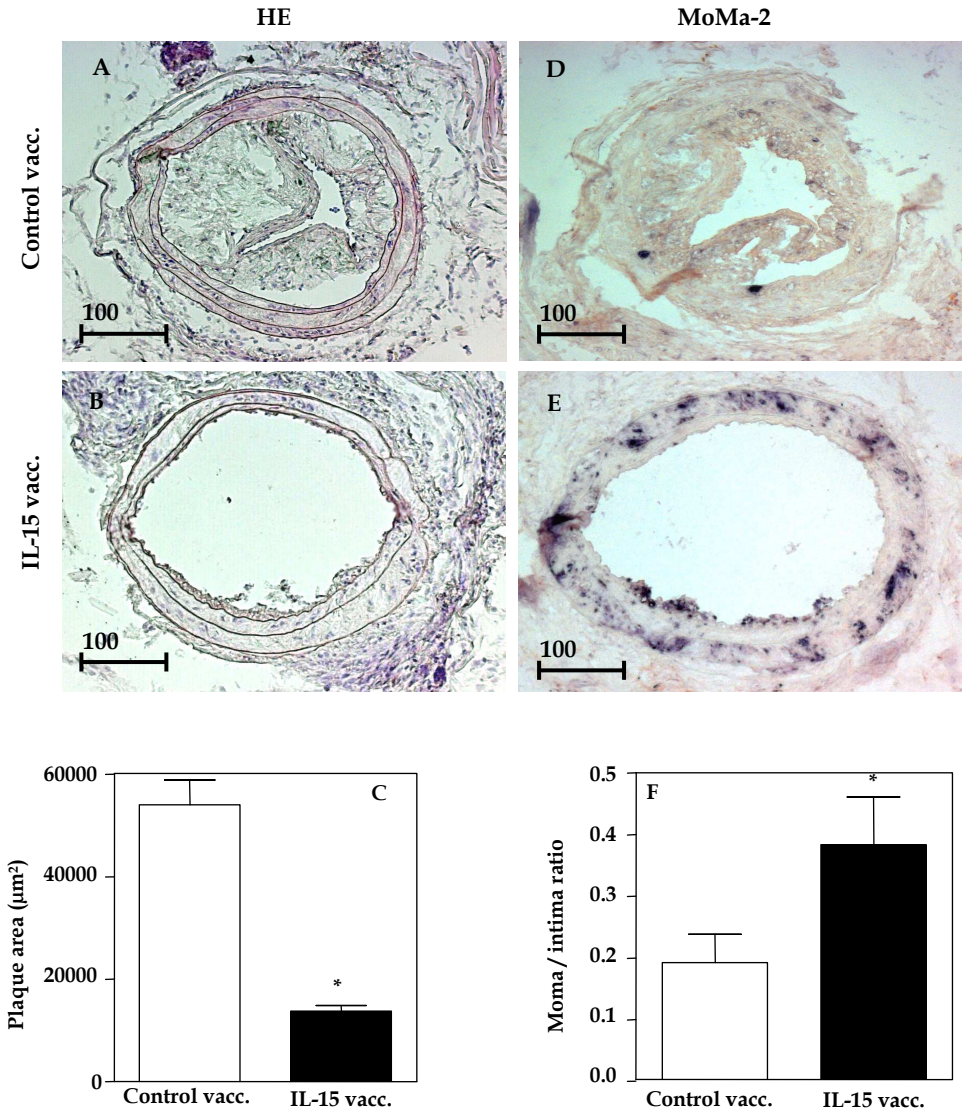

FIGURE 2.4: THE EFFECTS OF VACCINATION ON ATHEROSCLEROTIC LESION DEVELOPMENT IN LDLR-/- MICE.

5 µm cross sections of the carotid artery of the control group (A) and the vaccinated group (B) were made and subsequently stained for HE and quantified (C, N=8). To determine macrophage content, cross sections were made and stained with MoMa-2 (D and E) and the ratio of macrophage count and plaque area was determined (F, N=8). *P<0.05

SURFACE EXPRESSION OF IL-15 ON SPLEEN CELLS AND PBMC IS REDUCED AFTER VACCINATION

As hypercholesterolemia induced surface expression of IL-15 on PBMCs and spleen cells (Figure 2.1B), we evaluated the effect of IL-15 vaccination on IL-15 positive cells within the spleen and PBMCs. Spleen cells and PBMCs were stained for IL-15 and analyzed by FACS. Upon IL-15 vaccination, the surface expression of IL-15 on spleen cells was almost completely reduced to a similar level as found in mice before the start of the Western-type diet (Figure 2.5A, $P<0.05$). Within the PBMC population IL-15 surface expression was also decreased (Figure 2.5A, $P<0.05$). This indicates a systemic reduction of IL-15 expressing cells upon vaccination.

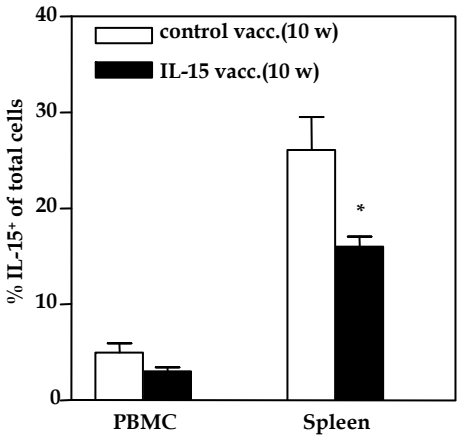

FIGURE 2.5: LEUKOCYTE IL-15 EXPRESSION IN VACCINATED AND CONTROL LDLR$^{-/-}$ MICE.
After vaccination and collar placement, PBMCs and spleen cells were isolated and stained for IL-15. The percentage of IL-15 positive cells is determined by FACS analysis, after control vaccination and 10 weeks of diet (white, N=6) and after IL-15 vaccination and 10 weeks of diet (black, N=6) *$P<0.05$

DISCUSSION

Atherosclerosis is considered a dyslipidemic induced chronic inflammatory disease of the arterial wall. During atherosclerotic lesion formation, monocytes and subsequently T cells infiltrate the arterial wall.[1] DNA vaccination against IL-15 leads in LDLr$^{-/-}$ mice to a greatly blocked atherosclerotic lesion development, indicating that IL-15 accelerates lesion formation.

Upon the start of a hypercholesterolemic diet in LDLr$^{-/-}$ mice the mRNA expression of IL-15 is increased within the spleen. Furthermore, hypercholesterolemia does increase the percentage of IL-15 expressing cells in both blood and in spleen. These findings point to a possible relation between IL-15 expression and the induction of atherosclerosis. IL-15 appears to be highly

expressed by macrophages and to a lesser extend by endothelial cells and vSMCs. After stimulation of macrophages with IL-15, the mRNA levels of several pro-inflammatory cytokines, such as TNF-α and IL-1β are upregulated, while the secretion of TNF-α is also increased by IL-15. Important proteins in the chemoattraction of macrophages, CXCL1, CCL2 and CCR2, are also upregulated after incubation with IL-15. These latter effects are also seen on human monocytes when stimulated with IL-15.[24]

Vaccination against IL-15 was accomplished by oral administration of a live attenuated *Salmonella typhimurium* bacteria, transformed with an eukaryotic expression vector encoding IL-15. This vaccination method induces a strong, IL-15 specific, cytotoxic immune response, resulting in the killing of cells overexpressing IL-15. This is a similar mechanism as achieved by the oral vaccination against FLK-1 as described by Niethammer *et al*.[19] and by Hauer *et al*.[22] and vaccination against CD99 described by van Wanrooij *et al*.[23] These vaccination procedures resulted in a cytotoxic T cell-mediated killing of cells expressing FLK-1 and CD99, respectively. The reduction in IL-15 expressing cells within the spleen and blood upon vaccination was accompanied by a 75% reduction in atherosclerotic lesion size. During the experiment no difference was detected in total cholesterol levels in the serum between the groups, indicating that IL-15 does not affect lipid-metabolism and the reduction in plaque is more likely due to changes in the inflammatory status of the mice. The reduced plaque size was accompanied by a two-fold increase in the relative amount of macrophages. As macrophage infiltration is a feature of early vascular lesion formation[25], it may be speculated that plaque formation and progression is strongly retarded but not prevented due to the blocking of IL-15.

Although IL-15 is involved in the expression of important chemoattractants for macrophages, it is likely that there are additional sources for these chemokines within the plaque, for example endothelial cells or vSMCs. We can also speculate that the recruited macrophages within the plaque do not, or to a lesser extent, express IL-15/IL15Rα as is demonstrated by the reduction of the surface expression of IL-15 on cells within spleen and PBMCs. Macrophages express IL-15/IL15Rα complexes on their surface upon activation and are able to activate T cells in an antigen-independent way. Membrane bound IL-15 is not only 5-times more effective in inducing T cell proliferation than soluble IL-15, it also signals through different effectors and can therefore exert distinct biological responses. Membrane bound IL-15, expressed on macrophages can participate in reverse signaling between the IL-15Rα on T cells, whereas soluble IL-15 modulates cellular function in both a paracrine and autocrine fashion.[17, 26] Macrophages which lack IL-15/IL15Rα complex on the surface are not able to sustain a full immune

response within the plaque and thereby are less capable to recruit inflammatory cells into the plaque.

We suggest that the development of the lesion is arrested in the fatty streak stadium. This may provide an explanation for the increased number of macrophages in the vessel wall and the smaller lesion size, since mainly the innate immune response is activated and adaptive immune response is likely impaired. However, IL-15 expressing cells are activated inflammatory cells, which are also able to express other inflammatory mediators. Therefore it should be taken into account that the effect we observe may also be due to the absence of other mediators. The vaccination method used in this study may lead to the initiation of new therapies, which block the action of IL-15. There are some promising results with phase I/II clinical trails with an anti-IL-15 antibody treatment in patients with rheumatoid arthritis,[27] which might be extended to cardiovascular patients.

The vaccination strategy used in this study successfully evoked a cytotoxic response targeting IL-15 expressing cells. This resulted in a vast reduction in atherosclerosis, thereby providing new insights in the process of atherosclerosis and the contribution of IL-15 in this process. These new insights may contribute to a future immunomodulating treatment of patients with cardiovascular diseases.

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Chapter 3

Vaccination against interleukin-17 attenuates atherosclerosis in LDL receptor deficient mice

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Submitted for publication

ABSTRACT

Interleukin-17 (IL-17) is a T cell-derived pro-inflammatory cytokine that is linked to autoimmune diseases. IL-17 exhibits pleiotropic effects on atheroma-associated cell types and induces the secretion of pro-inflammatory cytokines and chemokines.

In this study, we investigated the effect of IL-17 blockade on the initiation of atherosclerosis by vaccination against IL-17. A plasmid (pcDNA3.1) encoding IL-17 and the dominant T helper cell HEL epitope was used to vaccinate LDL receptor deficient ($LDLr^{-/-}$) mice prior to induction of atherosclerosis by feeding a Western-type diet and collar placement.

Functional blockade of IL-17 upon vaccination was demonstrated by a reduced induction of serum IL-6 levels after administration of IL-17. DNA vaccination with the HEL-IL-17 plasmid resulted in a 90.2% reduction in lesion size in the carotid artery ($P<0.01$) and 59% reduction in the aortic root ($P<0.05$). This reduction was dependent on the HEL sequence to break tolerance against endogenous IL-17 during vaccination.

Neutralizing the IL-17 production by vaccination forms a promising approach to inhibit atherosclerotic lesion development.

INTRODUCTION

Cardiovascular disease (CVD), with atherosclerosis as the main underlying pathology is the leading cause of death in the Western world and atherosclerotic lesion initiation and progression has been shown to be associated with a chronic inflammatory response.[1] Macrophages and T cells are present within the intima in early stages of atherogenesis and play a crucial role in the initiation and subsequently in the progression of the atherosclerotic plaque.[1, 2] $CD4^+$ effector T cells are the major T cell subset in atherosclerotic lesions and T cells are involved in atherosclerotic lesion formation.[3]

Traditionally, it was postulated that activated T cells differentiate into either T helper 1 (Th1) or Th2 cells. Th1 cells produce mainly pro-inflammatory cytokines, such as interferon-γ (IFN-γ) and tumor necrosis factor (TNF)-α, whereas Th2 cells predominantly produce anti-inflammatory cytokines, such as IL-4, IL-5 and IL-10.[4] A disturbed balance between Th1 and Th2 cells is thought to be responsible for the immunopathological conditions in several autoimmune diseases, such as atherosclerosis.[5]

More recently, a new subset of T helper cells with a T memory cell phenotype has been identified, the T helper 17 (Th17) cell and this cell type is associated with several (auto)immune diseases.[6] Although the loss of IFN-γ receptor appears to be protective in atherosclerosis[7], this newly identified Th cell may also be involved in the development of atherosclerosis.

The differentiation of Th17 cells is subject to debate. TGF-β, IL-1β and IL-6 in the presence of the appropriate antigen are ascribed to participate in the differentiation and initiation of Th17 cells.[8, 9] Th1 and Th2 cytokines, such as IFN-γ and IL-4 may antagonize the Th17 development, but fully differentiated Th17 are unresponsive to these cytokines.[10] IL-17 production is the hallmark of the Th17 cell and IL-17 has been identified in several autoimmune diseases, such as Experimental Autoimmune Encephalomyelitis (EAE) and rheumatoid arthritis to have a negative effect on disease progression.[11, 12] IL-17 exhibits pleiotropic biological effects on various cell types , such as endothelial cells, vascular smooth muscle cells, and macrophages that are associated with atherosclerosis.[13, 14] In these cells IL-17 can induce a number of pro-inflammatory cytokines, chemokines and matrix metalloproteinases (MMPs), including IL-6, IL-8, monocyte chemoattractant protein 1 (MCP-1) and MMP-9.[15, 16]

However, the role of IL-17 is not yet established in atherosclerosis. Therefore, we vaccinated mice against endogenous IL-17 via DNA vaccination in order to neutralize endogenous IL-17. To break T cell tolerance for IL-17, a specific T helper cell epitope (HEL) was coupled to IL-17.[17] Vaccination against HEL-IL-17 established a strong protection against atherosclerosis, which may indicate that IL-

17 is important in atherosclerosis and therapies to neutralize the action of IL-17 may provide a new approach to treat atherosclerosis.

METHODS

ANIMALS

All animal work was approved by Leiden University and was in compliance with the Dutch government guidelines. LDLr$^{-/-}$ mice on a C57/Bl6 background were from Jacksons Laboratory and were kept under standard laboratory conditions and food and water were administered *ad libitum*.

VACCINE PREPARATION

Genes coding for the antigen were cloned into pcDNA3.1(-) (Invitrogen, The Netherlands). cDNA coding for murine IL-17 was obtained by PCR on stimulated murine spleen cells. The following primers were used: IL-17, 5'- GAT CAG GAC GCG CAA ACA- 3' (forward) and IL-17, 5'- GGG TTT CTT AGG GGT CAG- 3' (reverse). The IL-17 PCR product was cloned into a pcDNA3.1(-) plasmid. The HEL sequence was derived from the amino acid sequence 81-95 of hen egg-white lysozyme and was cloned upstream of IL-17 with *XbaI*.[21] The following HEL sequence was used: 5'- CTA GAA TGT CAG CCC TGC TGA GCT CAG ACA TAA CAG CGA GCG TGA ACT GCG CGC CT- 3'.

TESTING THE CONSTRUCT

Expression of constructs was tested by transient transfection of COS7 cells with the plasmid. COS7 cells were transfected with pcDNA3.1-HEL-IL-17 and pcDNA3.1 using Exgen500 according to manufacturer's protocol (Fermentas, Germany). Supernatant was collected 24 and 48 hours after transfection. Expression of IL-17 was determined with a specific murine IL-17 ELISA according to manufacturer's protocol (BD Bioscience, The Netherlands). To confirm the functional blockade of IL-17 after vaccination we performed an experiment in which we made use of the fact that IL-17 is able to induce IL-6 production. In a separate experiment LDLr$^{-/-}$ mice were vaccinated against IL-17 or control vaccinated. Three days after the last vaccination, 0.2 µg of murine IL-17 (1 µg/ml) was injected intravenously. Four hours after the injection of IL-17, blood was collected and serum IL-6 levels were quantified with a mouse IL-6 ELISA (eBioscience, Belgium).

CELL CULTURE

The murine monocyte/macrophage cell line (RAW 246.7) and an endothelial cell line (H5V) were cultured in DMEM supplemented with 10% FCS, 2 mmol/L glutamin, 0.1 U/L penicillin, and 100 mg/L streptomycin. Primary vascular smooth muscle cells were isolated from murine aorta and cultured as described previously.[22]

IN VITRO ASSESSMENT OF IL-17

0.5 ml of cells were added to a 24-wells plate ($2.5x10^5$ RAW cells/ml, $1.0x10^5$ cells for H5V and vSMC). Unless stated otherwise, 100 ng/ml recombinant IL-17 was added to the culture medium and culture medium alone served as a control. Cells were incubated for 24 hours, and thereafter the supernatant was used for ELISA. All experiments were performed in triplicate. Where indicated cells were used for qPCR.

QUANTATIVE PCR ASSAYS

Total RNA was isolated using guanidium isothiocyanate (GTC) method and reverse transcribed to cDNA (RevertAid™ M-MuLV reverse transcriptase, Fermentas). Gene expression was analyzed with an ABI PRISM 7700 (Applied Biosystems, Foster city, CA) using SYBR Green technology. Primer pairs as described in table 3.1 were used to quantify IL-17, ADAM-15 and MMP-9 gene expression. As a reference gene hypoxanthine phosphoribosyl transferase (HPRT) was used. The relative gene expression was calculated by subtracting the threshold of the target gene from the reference gene and raising 2 to the power of this difference.

TABLE 3.1: SEQUENCE OF PRIMERS. Primer sequences for the genes analyzed with qPCR.

GENE	FORWARD PRIMER	REVERSE PRIMER
IL-17	5′-CCAGGGAGAGCTTCATCTGTGT-3′	5′-AAGTCCTTGGCCTCAGTGTTTG-3′
ADAM-15	5′-TGTGGCTTCCCAGATGAATG-3′	5′-GTTTTGACAACAGGGTCCATCA-3′
MMP-9	5′-CTGGCGTGTGAGTTTCCAAAAT-3′	5′-TGCACGGTTGAAGCAAAGAA-3′
HPRT	5′-TTG CTCGAGATGTCATGAAGGA-3′	5′-AGCAGGTCAGCAAAGAACTTATAG-3′

VACCINATION AND INDUCTION OF ATHEROSCLEROSIS

The DNA vaccine was isolated with an Endofree plasmid Giga kit (Qiagen, The Netherlands). Male low-density lipoprotein receptor deficient ($LDLr^{-/-}$) mice, 10-12 weeks old, were used for the *i.m.* vaccination. Three days prior to the first vaccination, mice received an *i.m.* bilateral Cardiotoxin I (Sigma, USA) injection, 10 µM, 50 µl per muscle. Mice were vaccinated by a total of three bilateral *i.m.* injections of 100 µg plasmid in 100 µl PBS, 50 µl per muscle, with two-week intervals (N=15 each group). Immediately after the last vaccination, mice were put on a Western type diet, containing 0.25% cholesterol and 15% cocoa butter (Special Diet Services, UK). After two weeks of Western type diet feeding, atherosclerosis was induced within the carotid arteries by bilateral perivascular collar placement, as described previously.[23] During the experiment, plasma samples were obtained by tail vein bleeding. Total cholesterol levels were quantified during the experiment using an enzymatic procedure (Roche Diagnostics, Germany) using Precipath as an internal standard.

TISSUE HARVESTING

Six weeks after collar placement, carotid arteries were obtained after *in situ* perfusion for 15 minutes with Formalfixx. Carotids were embedded in OCT compound (Sakura Finetek, The Netherlands), snap-frozen in liquid nitrogen and stored at -20°C until further use. Transverse 5 µm cryosections were prepared in a proximal direction from the carotid bifurcation and were mounted on a parallel series of slides. For analysis of atherosclerosis at the site of the aortic semilunar valves, 10 µm transverse cryosections were made of the aortic root as previously described.[23, 24]

HISTOLOGICAL ANALYSIS AND MORPHOMETRY

Cryosections were routinely stained with hematoxylin (Sigma Aldrich, Zwijndrecht, The Netherlands) and eosin (Merck Diagnostica, Germany). Corresponding sections were stained for lipids by Oil red O staining. Hematoxylin-eosin stained sections of carotid arteries were used for morphometric analysis of atherosclerotic lesions. Each vessel was assessed ~0.5 mm proximal to the collar, and the site of maximal stenosis was used for morphometric assessment. Atherosclerosis in the aortic root was quantified with Oil red O stained sections of plaques developed in the region of the aortic semilunar valves, as previously described.[24]

STATISTICAL ANALYSIS

All data are expressed as mean ± SEM. The two-tailed student's t-test was used to compare individual groups of mice or cells. Mann-Witney test was applied to compare not normally distributed values. *P* values of <0.05 were considered significant.

RESULTS

EVALUATING THE EXPRESSION OF IL-17 IN ATHEROSCLEROSIS

To define the role of IL-17 in atherosclerosis we determined the expression of IL-17 in atherosclerosis prone mice, fed a Western type diet (WTD, 0.25% cholesterol). Starting from three weeks after the start of the Western type diet we observed a significant 2-fold increase in the IL-17 gene expression in the spleen (Figure 3.1; 1 ± 0.22 versus 2.20 ± 0.32, *P*<0.05). Subsequently, the expression of IL-17 steadily increased, leading to a more than 3-fold increase at week 6 of Western type diet feeding (Figure 3.1; 3.18 ± 0.30, *P*<0.01).

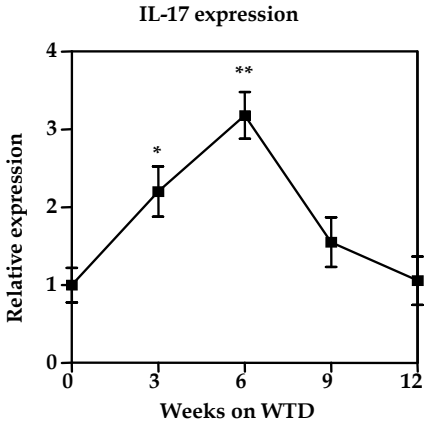

FIGURE 3.1: MRNA EXPRESSION OF IL-17 IN RESPONSE TO A WESTERN-TYPE DIET.
At different time points spleen samples were obtained and mRNA was isolated using the guanidium-isothiocyanate method and IL-17 expression was determined. Expression of IL-17 is expressed relative to HPRT and subsequently related to the expression of mice on chow diet. * *P*< 0.05, ** *P*<0.001

Thereafter, the expression of IL-17 rapidly decreased and declined to basal levels at 12 weeks of Western-type diet, indicating specifically the induction of IL-17 during the initiation of atherosclerosis. Next, we studied the effect of IL-17 on different athero-associated cell types in vitro. A macrophage cell line (RAW264.7), an endothelial cell line (H5V) and primary mouse vascular smooth muscle cells were stimulated with IL-17 and the production of IL-6 and TNF-α was determined.

RAW cells responded with a strong increase in TNF-α production (Figure 3.2A; 81 ± 10 pg/ml versus 372 ± 49 pg/ml, $P<0.01$), whereas H5V did not respond to IL-17 (data not shown). Isolated VSMC responded to IL-17 with a significant increase in IL-6 production (Figure 3.2B; 65.6 ± 4.1 pg/ml versus 293.4 ± 7.9 pg/ml, $P<0.001$).

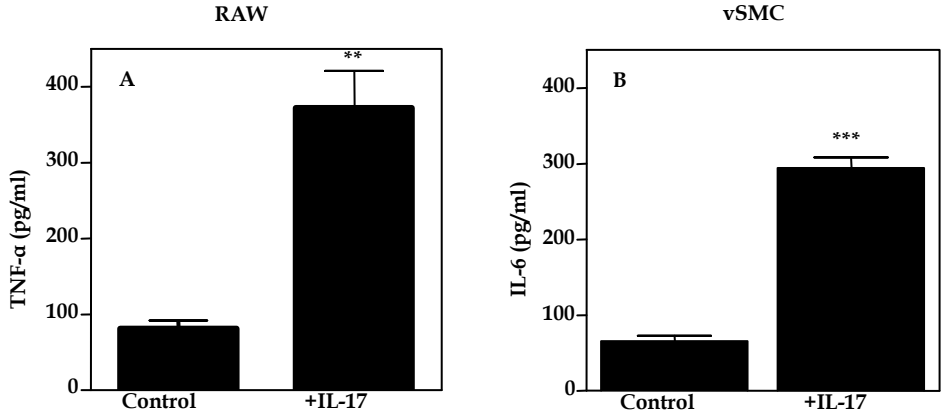

FIGURE 3.2: STIMULATION OF RAW AND VSMCS WITH IL-17.
RAW and vSMCs were stimulated with 100 ng/ml IL-17 and after 24 hours the supernatant was collected and analyzed with a TNF-α (A) or IL-6 (B) ELISA. $^{**}P<0.01$, $^{***}P<0.001$

Since, VSMC play an important role in the production of MMPs,[25, 26] thereby contributing to remodeling and stability of the plaque, we measured the expression of MMP-9 and ADAM metallopeptidase domain 15 (ADAM-15) by VSMCs. IL-17 specifically enhanced MMP-9 expression in vSMCs significantly but did not affect ADAM15 expression (Figure 3.3).

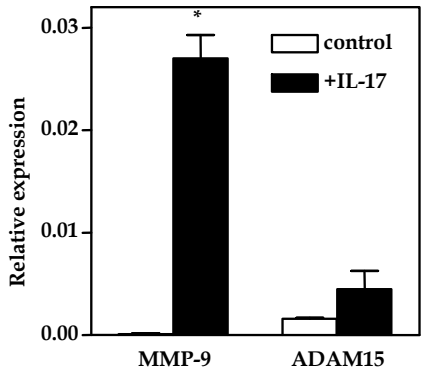

FIGURE 3.3: EFFECT OF IL-17 ON METALLOPROTEINASE EXPRESSION IN VSMCS.
vSMCs were stimulated with 100 ng/ml IL-17 and incubated for 24 hours. mRNA was isolated using the guanidium-isothiocyanate method. Expression of MMP-9 and ADAM15 are shown relative to HPRT. * $P< 0.05$

CONSTRUCTION OF A HEL-IL-17 DNA VACCINE

We cloned the IL-17 coding sequence of murine IL-17A into the eukaryotic expression vector, pcDNA3.1. The coding sequence is preceded by the Th cell epitope HEL, to enhance the breaking of tolerance against endogenous IL-17 as has been shown before for vaccination against several self antigens such as TNF-alpha.[17] *In vitro* experiments were performed to determine the expression and functionality of the used construct. COS7 cells were transfected with the HEL-IL-17 plasmid and the supernatant was collected after 24 and 48 hours of incubation. We assessed the supernatant for IL-17 expression with a mouse specific IL-17 ELISA. 24 hours after transfection the HEL-IL-17 protein was detectable in the supernatant of the COS7 cells using an IL-17 ELISA. At 48 hours the concentration of HEL-IL-17 protein in the supernatant was almost doubled (Figure 3.4), indicating that IL-17 together with the HEL epitope is translated and excreted into the medium. Cells transfected with the empty pcDNA3.1 plasmid or non-transfected cells showed no expression of IL-17 (Figure 3.4).

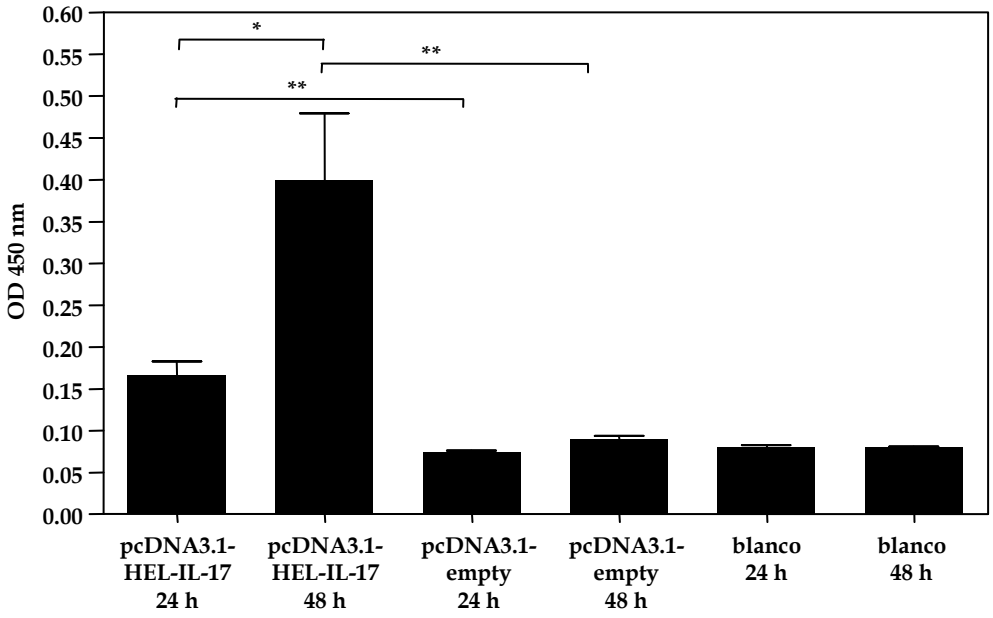

FIGURE 3.4: EXPRESSION OF THE VACCINE IN AN EUKARYOTIC EXPRESSION SYSTEM.
Expression of IL-17 was determined by transfection the plasmid to COS7 cells. Culture supernatant was collected 24 or 48 hours after transfecting with either HEL-IL-17 construct, an empty plasmid or PBS. IL-17 production was determined using a mouse IL-17 specific ELISA. The optical density was measured at a wavelength of 450 nm. *P< 0.05; **P<0.01

IMPAIRED IL-17 SIGNALING IN IL-17 VACCINATED MICE

To determine whether a functional blockade of IL-17 was established by vaccination, male LDLr$^{-/-}$ mice received a triple vaccination either with a plasmid encoding HEL-IL-17 or with an empty plasmid, both after pre-treatment of the mice with cardiotoxin I (CTX-I) at the site of vaccination to enhance the vaccination efficacy. Three days after the last *i.m.* vaccination, IL-17 was injected intravenously and 4 hours thereafter blood was collected to quantify the IL-17 response in terms of IL-6 production. Injection of IL-17 led to a significant 31% reduction in serum concentration of IL-6 in mice vaccinated against IL-17 as compared to control vaccinated mice (Figure 3.5; 7.87 pg/ml versus 11.44 pg/ml, $P<0.05$), indicating that the anti-IL-17 vaccination induced at least a partially blockade of the function of IL-17.

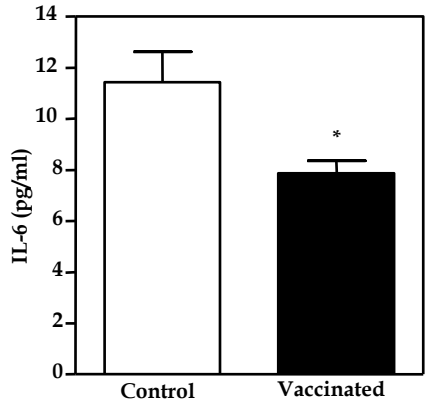

FIGURE 3.5: DETERMINATION OF NEUTRALIZING ACTIVITY OF THE VACCINE. Serum IL-6 concentration was determined with an ELISA 4 hours after administration of IL-17 to mice that were either vaccinated against IL-17 or control vaccinated. *$P<0.05$

EFFECT OF IL-17 VACCINATION ON ATHEROGENESIS

As the vaccine proved to effectively reduce IL-17 signaling, we studied the effect of anti-IL-17 vaccination on *de novo* plaque formation in male LDLr$^{-/-}$ mice using the *i.m.* IL-17-HEL vaccine. Following the last DNA vaccination LDLr$^{-/-}$ mice were put on a Western-type diet to induce hypercholesterolemia. Two weeks later, perivascular carotid collars were placed to induce atherosclerosis within the carotid arteries. During the experiment, we did not detect any difference in cholesterol levels between the anti-IL-17 vaccinated group and the control vaccinated group (Figure 3.6A). Six weeks after collar placement mice were sacrificed and the plaque size proximal to the collar was quantified. Vaccination with the HEL-IL-17 plasmid significantly reduced the formation of atherosclerotic lesions by 90.2% (Figure 3.6 B-D; 5654 ± 2099 μm^2 versus 57702 ± 14120 μm^2,

P<0.01), indicating that the initiation of atherosclerosis was largely blocked in the IL-17 vaccinated group. Furthermore, a beneficial 87.5% reduction in intima/media ratio (Figure 3.6E; 0.13 ± 0.033 versus 1.00 ± 0.27, *P*<0.01) and a 79.6% reduction in intima/lumen ratio (Figure 3.6F; 0.14 ± 0.043 versus 0.70 ± 0.087, *P*<0.05) were observed, which indicates reduced stenosis in mice vaccinated with HEL-IL-17. We determined plaque development also at another site in the vascular bed, the aortic valve region. In mice vaccinated with HEL-IL-17 (Figure 3.7A) a significant 59% reduction in plaque size (Figure 3.7C; 126691 ± 20739 μm2 versus 310170 ± 78706 μm2 *P*<0.05) was observed compared to the control group (Figure 3.7B). We assessed plaque composition in the lesions in terms of collagen and macrophage composition but detected no differences between the control group and vaccinated groups (data not shown).

DISCUSSION

Already in 1986 Mosmann *et al.* described two distinct Th subsets, Th1 and Th2 cells, with their own specific production of cytokines.[4] An exaggerated pro-inflammatory response due to an overexpression of Th1 associated cytokines such as IL-12 and IFN-γ enhances atherosclerosis.[24] Blockade of pro-inflammatory cytokines or cytokines involved in Th1 differentiation may introduce a new therapy for atherosclerosis. We previously demonstrated that vaccination against IL-12, a prominent Th1 cytokine, reduces atherosclerosis in LDLr$^{-/-}$ mice.[25] However, some experimental results with respect to the role of IL-4 in atherosclerosis can not be explained by the classical Th1/Th2 model.[26-28] These observations may indicate that other T helper subsets are involved in atherosclerosis and we focused in our present work on the role of IL-17 a main product of the pro-inflammatory T helper cell subset, the Th17 cells.[6, 10, 27, 29, 30]

Th17 cells and IL-17 have been identified in various autoimmune disease[29, 31] and may also play a role in atherosclerosis, which is also recognized as an autoimmune disease.[32-34] IL-17 may have pleiotropic effects on the various cell types within the atherosclerotic lesion and may thereby stimulate a pro-inflammatory environment, which aggravates atherosclerosis.[14, 15, 29, 35-37]

To address the role of IL-17 in atherosclerosis we first examined the effect of hypercholesterolemia on the expression of IL-17 within the spleen by feeding LDLr$^{-/-}$ mice a Western-type diet. 6 weeks after starting Western-type diet feeding, the IL-17 expression was more than 3-fold increased compared to the level before the diet. This may indicate a relation between the initiation of the inflammatory response during atherosclerosis and IL-17 expression.

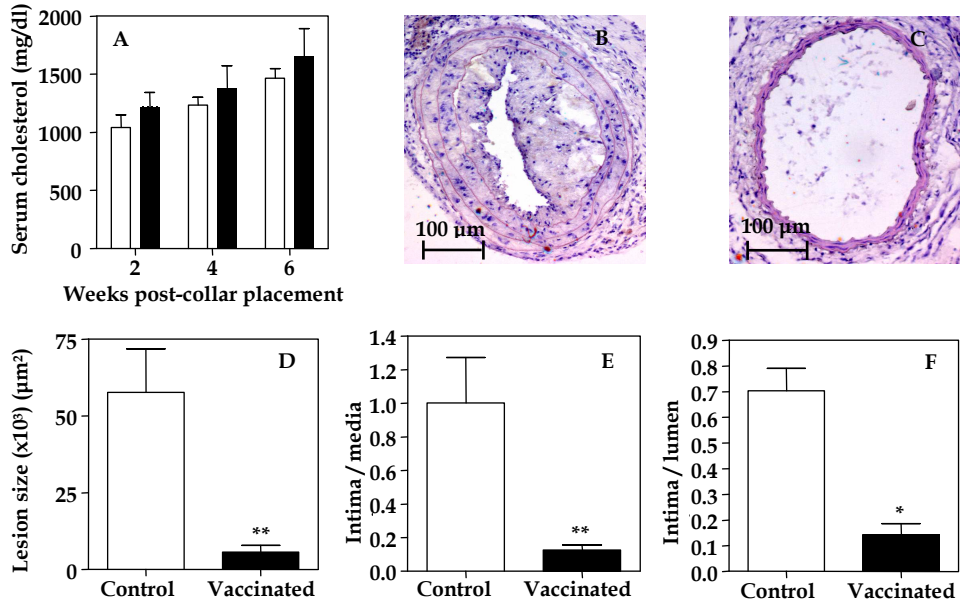

FIGURE 3.6: INITIAL ATHEROSCLEROTIC LESION FORMATION IN VACCINATED MICE. Mice were vaccinated by intramuscular (i.m.) injection of HEL-IL-17 plasmid or empty plasmid. Subsequently mice were put on a Western type diet and two weeks later equipped with a perivascular collar. At the indicated time points, cholesterol levels were determined in the various groups of mice (A). Six weeks after collar placement mice were sacrificed and the carotid arteries of control mice (B) and vaccinated mice (C) were sectioned and stained with hematoxylin-eaosin. Lesion size from all mice(D) as well as intima/media ratio (E) and intima/lumen ratio (F) were determined by computer-assisted morphometric analysis to asses the degree of atherosclerosis. *P<0.05, **P<0.01

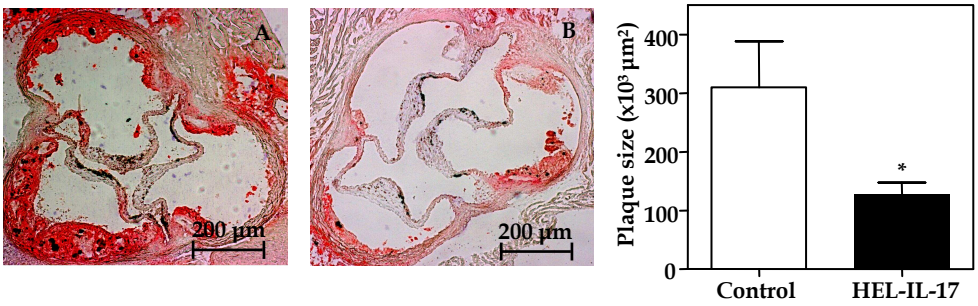

FIGURE 3.7: EFFECT OF IL-17 VACCINATION ON PLAQUE FORMATION IN THE AORTIC ROOT. Mice were *i.m.* vaccinated with an empty plasmid (A) or with a HEL-IL-17 plasmid (B). Subsequently mice were put on a Western type diet and 8 weeks later the mice were scarified and sections of the aortic root were stained with Oil-red-O and hematoxylin. Plaque size was determined by computer assisted analysis (C). *P<0.05

For the vaccination against IL-17, murine IL-17 was cloned into a pcDNA3.1 plasmid preceded by the specific immunodominant T-helper epitope HEL to break T cell tolerance. In this study we used a specific part of the HEL sequence which binds with high affinity to MHC class II and is able to activate T cells, which can subsequently provide the necessary stimuli to break the tolerance against self-antigens and help B cells to produce antibodies against IL-17.[17, 38] This approach is in line with data from Dalum *et al.* who used a HEL-TNF-α to vaccinate atherosclerosis prone apoE$^{-/-}$ mice against TNF-α.[17, 38] We detected high concentrations of IL-17 in the supernatant of cells transfected with HEL-IL-17, which indicates that the HEL peptide did not alter the conformation of IL-17 and did not interfere with the excretion of the protein into the medium, which is important for successful vaccination. To study the role of IL-17 in the process of atherogenesis we vaccinated LDLr$^{-/-}$ mice against HEL-IL-17 and vaccination was preceded by treatment of the hind leg muscles with Cardiotoxin I, which improves the outcome of DNA vaccinations.[39]

In the present study, we show that the function of IL-17 is partially blocked by vaccination against IL-17. Intravenous administration of recombinant IL-17 after anti-IL-17 vaccination resulted in a significant reduction in serum IL-6 levels. This indicates that vaccination against IL-17 induced a partial and functional blockade of IL-17 *in vivo*. In the HEL-IL-17 vaccinated mice we observed a highly significant reduction of 90.2% in plaque size in the carotid artery and a 59.0% reduction in the aortic valve region. Furthermore, the beneficial effect on lesion initiation is illustrated by an 87.5% reduction in intima/media ratio and a 79.6% reduction in intima/lumen ratio. Interestingly, unpublished data from our lab show that *i.m.* vaccination with an IL-17 plasmid lacking the HEL sequence did not have any effect on atherosclerosis, which clearly indicates that the HEL sequence in the vaccine is essential for a functional blockade of IL-17 (data not shown). In line with the findings on vaccination against Il-17, we have observed that transplantation of IL-17 receptor deficient bone marrow into LDLr$^{-/-}$ mice inhibits atherosclerosis by almost 50% (Van Es *et al.* unpublished results, chapter 4). It should be kept in mind that Th17 cells do not form the only source of IL-17, since more cell types such as γδT cells and NKT-like cells are able to produce IL-17.[40] Additional research is therefore needed to assess the role of these different cell types to IL-17 production and their individual role in atherosclerosis.

In conclusion, in this study we describe a prominent role for IL-17 in atherosclerosis: IL-17 is upregulated in hypercholesterolemic LDLr$^{-/-}$ mice upon Western-type diet feeding and neutralization of IL-17 by vaccination, using a novel DNA vaccination strategy, attenuates atherosclerotic lesion formation. Therefore interfering in the IL-17 pathway will be an interesting target for therapeutic intervention in cardiovascular disease.

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Chapter 4

Attenuated atherosclerosis upon interleukin-17 receptor signaling disruption in LDL receptor deficient mice

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Submitted for publication

ABSTRACT

Atherosclerosis is an inflammatory disease, which is illustrated by the influx of macrophages and T cells in the sub-endothelial layer. IL-17 is an important cytokine, which bridges the innate and adaptive immune response and moreover IL-17 is involved in the transition from an acute into a chronic inflammation.

In this study we investigated the role of IL-17 receptor signaling in atherosclerosis. We therefore performed a bone marrow transplantation with IL-17 receptor deficient donor bone marrow into LDL receptor deficient recipient mice. After full bone marrow reconstitution a Western-type diet feeding was started and atherosclerotic lesions were quantified after 12 weeks.

A 46% reduction in lesion size in the aortic root was observed ($P<0.05$). Furthermore, a decrease in auto-antibodies against oxLDL was detected. The inflammatory status of IL-17 deficient bone marrow recipients was changed as indicated by the reduced IL-6 production by the spleen and increased IL-10 production within the HLN and PBMCs.

In conclusion, the IL-17 signaling is involved in the aggravation of atherosclerosis. This is probably mediated by a decrease in IgG anti-oxLDL antibodies and a change in the inflammatory status. Therefore, interfering in the IL-17 receptor pathway could provide a new therapeutic approach for inhibiting atherosclerosis lesion development.

INTRODUCTION

Atherosclerosis is an inflammatory disease, which involves both components of the immune system, the adaptive and innate immune system[1]. The inflammatory response is tightly regulated by several mechanisms for example via interleukins (IL).

IL-17 is involved in the early activation of the immune system and plays an important role in bridging the innate immune response with the adaptive immune response.[2] IL-17 is mainly produced by T cells, especially $CD4^+$ effector memory T cells and has more recently been linked to a new class of T helper cells, the Th17 cells.[3, 4] IL-17 has a protective role, since IL-17 protects against infectious microorganisms such as *Klebsiella pneumoniae* , *Candida albicans* and *Toxoplasma gondii*.[5,6] On the other hand an elevated concentration of IL-17 is associated with different autoimmune diseases such as, rheumatoid arthritis and multiple sclerosis, where IL-17 plays a pathogenic role. [7, 8]

The IL-17 family comprises six members (IL-17A, B, C, D, E and F) and the best characterized member is IL-17A, which is also designated as IL-17 as it is the founding member of the IL-17 family. The receptor for IL-17 (IL-17R) is a type I transmembrane protein consisting of a 293 amino acids long extracellular domain and a relatively long intra cellular domain consisting of 525 amino acids.[9, 10] The IL-17 receptor is widely expressed with a prominent mRNA expression in lung, kidney, liver, spleen and also in isolated fibroblasts, endothelial cells, mesothelial cells and myeloid cells from mice.[9] This wide expression is also seen in humans, were the IL-17R for example is found on peripheral blood T cells and vascular endothelial cells. [11,12] The pathogenic role of IL-17 in autoimmunity and the parallel in function with proinflammatory cytokines, such as tumor necrosis factor (TNF)-α and IL-1β makes IL-17 an interesting target for studying its role in atherosclerosis.[13]

IL-17 induces the expression of a wide range of proinflammatory cytokines and chemokines in various cell types as a consequence of the broad expression of IL-17R.[14] IL-17 stimulates the expression of IL-6 and CXCL8 (IL-8) by stromal cells and ICAM-1 by fibroblasts and keratinocytes.[9, 10, 15] Even more interesting is the effect of IL-17 on macrophages, which produce IL-1β, IL-1Ra, IL-6, IL-10, TNF-α and prostaglandin E_2 (PGE_2) in response to IL-17. Matrix metalloproteinase (MMP)-3 and MMP-9 are also induced by IL-17.[16, 17] These proteinases and proinflammatory interleukins and chemokines have already been implicated in atherosclerotic lesion growth and destabilization of the plaque.[18-20]

The role of the IL-17R signaling pathway, although extensively studied in other autoimmune diseases, is not yet established in the process of atherosclerosis. Therefore we transplanted bone marrow of IL-17R$^{-/-}$ mice to LDLr$^{-/-}$ mice, which

resulted in a 46% reduction in atherosclerotic lesion size. These data indicate an aggravating role of IL-17 in the process of atherosclerosis and establish a new target to beneficially influence atherosclerosis lesion development.

METHODS

ANIMALS

All animal work was approved by Leiden University and was in compliance with the Dutch government guidelines. LDL receptor deficient ($LDLr^{-/-}$) mice were purchased from Jackson Laboratories. IL-17 receptor knockout mice were a kind gift from J. Peschon (Amgen, Seattle, WA) and created as described by Ye *et al.*[6] The mice were kept under standard laboratory conditions and food and water were provided *ad libitum*.

BONE MARROW TRANSPLANTATION (BMT) AND INDUCTION OF HYPER-CHOLESTEROLEMIA

To induce bone marrow aplasia, male $LDLr^{-/-}$ mice were exposed to a single dose of 9 Gy (0.19 Gy/min, 200 kV, 4 mA) total body irradiation, using an Andrex Smart 225 Röntgen source (YXLON Int, Copenhagen, Denmark) with a 6-mm aluminum filter. Bone marrow was isolated by flushing the femurs and tibias from mice with phosphate-buffered saline (PBS). Single-cell suspensions were prepared by passing the cells through a 30 µm nylon gauze. Irradiated recipients received $0.5x10^7$ bone marrow cells by intravenous injection into the tail vein. After a recovery of 8 weeks animals received a Western-type diet *ad libitum* containing 15% cocoa butter and 0.25% cholesterol (Special Diet Services, Witham, Essex, UK) for 12 weeks. During the experiment the mice were weighted every week and checked for well-being.

SERUM LIPID LEVELS

Every 3 weeks the serum cholesterol levels were determined to assess the effect of the Western-type diet. Blood samples were collected by tail bleeding from non-fasting animals. Total cholesterol levels were quantified spectrophotometrically using an enzymatic procedure (Roche Diagnostics, Germany). Precipath standardized serum (Boehringer, Germany) was used as an internal standard.

HISTOLOGY AND IMMUNOHISTOCHEMISTRY

Mice were anaesthetized with ketamine-hypnorm and perfused with PBS and subsequently with FormalFixx. The heart and complete aorta were removed. The heart was embedded in OCT compound (TissueTek; Sakura Finetek, The Netherlands) and cryosections of 10 µm were made of the aortic root containing the aortic valves. Cryosections were routinely stained with Oil-Red-O and hematoxylin (Sigma Diagnostics, MO). Corresponding sections on separate slides were stained immunohistochemically for macrophages using an antibody against a macrophage-specific antigen (MoMa-2, Research Diagnostics Inc.) and for collagen using Masson trichrome staining according to manufacturer's protocol (Sigma Diagnostics). Neutrophils were stained by specific esterase staining (Naphthol AS-D chloroacetate, Sigma). Mast cells were stained by Toluidin Blue Staining (Sigma). The different histological stains were quantified using a Leica DM-RE microscope and Leica Qwin Imaging software (Leica Ltd., Germany).

FACS ANALYSIS OF LEUKOCYTES

Peripheral Blood Mononuclear Cells (PBMC) were isolated via orbital bleeding and erythrocytes were removed by incubating the cells with erythrocyte lysis buffer (0.15 M NH_4Cl, 10 mN $NaHCO_3$, 0.1 mM EDTA, pH 7.3). Spleens, Heart lymph nodes (HLN) and Mesenteric lymph nodes (MNL) were dissected from the mice and single cell suspension was obtained by passing the organs through a 70 µm cell strainer (Falcon, The Netherlands). Cells were stained with surface markers (0.20 µg antibody/300.000 cells) and subsequently analyzed by flow cytometric analysis. The F4/80-FITC and CD19-FITC antibody were used for the detection of macrophages and B cells, respectively (Immunosource, Belgium). The unlabeled antibody for IL-17R was purchased from R & D systems and as a secondary antibody anti goat-IgG-PE (Abcam, UK) was used according manufacturers protocol. All data were acquired on a FACScalibur and analyzed with CELLQuest software (BD Biosciences, The Netherlands).

OXLDL ANTIBODY DETECTION

Cu-oxLDL was synthesized as described previously [21, 22]. Antibodies against Cu-oxLDL were determined according to Damoiseaux et al.[23] MaxiSorp 96 wells plates (Nunc, Roskilde, Denmark) were coated overnight with 100 µg oxLDL in coating buffer (50mM $NaHCO_3$, 50mM Na_2CO_3, pH=9.6) at 4 ^{0}C. IgM, IgG2a and IgG1 antibodies directed against oxLDL were detected with an isotype Ig detection kit according manufacturer's protocol (Zymed lab. Inc., CA).

CYTOKINE PRODUCTION

Peripheral Blood Mononuclear Cells (PBMC), spleens, heart lymph nodes (HLN) and mesenteric lymph nodes (MNL) were dissected and a single cell suspension was made as described above. Subsequently the cells were cultured in a 96-wells round bottom plate which was coated with α-CD28 and α-CD3 (0.25 µg/well) at a cell density of 2.10^5 cells per well. Cells were cultured in RPMI 1640 (with L-Glutamine) supplemented with 10 % FCS, 100 U/ml penicillin and 100 µg/ml streptomycin (all from BioWhittaker Europe). Supernatant was used for IL-10 and IL-6 ELISA's according to manufacturer's protocol (both from eBioscience, Belgium).

STATISTICAL ANALYSIS

All data are expressed as mean ± SEM. The two-tailed student's t-test was used to compare individual groups of mice or cells. When indicated, a Mann-Whitney test was used to analyze not normally distributed data. *P* values of <0.05 were considered significant.

RESULTS

EFFICACY OF IL-17R$^{-/-}$ BONE MARROW TRANSPLANTATION

To asses the efficacy of IL-17R$^{-/-}$ BMT, we determined the IL-17R expression by FACS analysis. 20 weeks after BMT the IL-17R expressing cells in different organs were analyzed. The number of IL-17R expressing cells was significantly reduced with 85% within the blood (Figure 4.1A, $P<0.01$). Furthermore, the IL-17R expression on circulating macrophages was analyzed. We observed a strong reduction of 84% in IL-17R expressing macrophages in IL-17R$^{-/-}$ transplanted mice (Figure 4.1B). Within the spleen of IL-17R$^{-/-}$ transplanted mice the expression of IL-17R on mRNA level was also stongely reduced (Figure 4.1C). These data demonstrate an effective replacement of acceptor bone marrow by IL-17R$^{-/-}$ donor bone marrow.

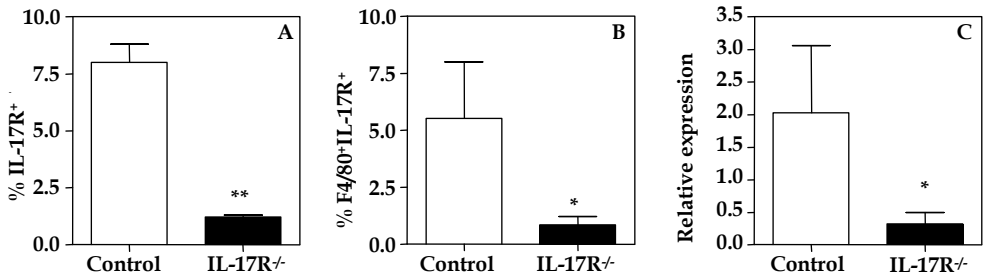

FIGURE 4.1: IL-17 RECEPTOR EXPRESSING CELLS OF PBMCS IN IL17R-/- BM RECIPIENT AND CONTROL MICE. After sacrificing the mice, PBMCs were stained with an antibody directed against IL-17R and F4/80 or IL-17R alone and analyzed with a FACS machine. The expression of the IL-17R was significant lower within IL-17R$^{-/-}$ transplanted mice (A). Within the macrophage population of the PBMCs IL-17R was also significant lower in IL-17R$^{-/-}$ transplanted mice (B). Total RNA was isolated from the spleen cells and the expression of the IL-17R was assessed by qPCR analysis, illustrating almost complete abolishment of IL-17R expression IL-17R$^{-/-}$ transplanted mice (C). Control mice (N=5) and IL-17R$^{-/-}$ BM recipients (N=5). *P< 0.05, **P<0.01

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EFFECT OF IL-17R$^{-/-}$ BMT IN ATHEROSCLEROSIS

Next we determined the effect on IL-17R$^{-/-}$ BMT in atherosclerosis whereby the mice were fed a Western-type diet for 12 weeks. The mice were subsequently sacrificed and the aorta and the aortic root were analyzed for the atherosclerotic burden. Atherosclerotic lesions were quantified in the aortic root of IL-17R$^{-/-}$ BM recipients (Figure 4.2B) and control transplanted mice (Figure 4.2A). In the IL-17R$^{-/-}$ BM recipients a significant reduction of 46% in plaque size was observed compared to the control group (Figure 4.2C; 245,000 ± 43,700 versus 454,000 ± 91,200; P<0.05).

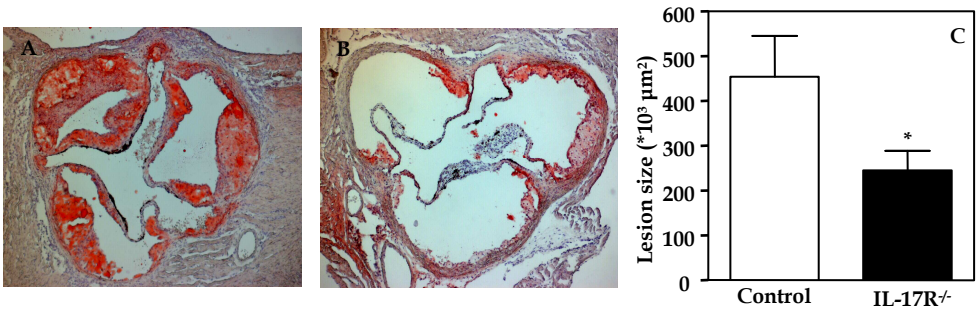

FIGURE 4.2: IL-17R$^{-/-}$ BM RECIPIENTS DEMONSTRATE REDUCED LESION SIZE AT THE AORTIC ROOT. After BMT the mice were fed a Western-type diet for 12 weeks and were sacrificed. Cryosections of the aortic root of the control group (A) and the IL-17R$^{-/-}$ BM recipients (B) were made and subsequently stained for lipid with Oil-red-O. Within the IL-17R$^{-/-}$ BM recipients (open bar, N=10) there is a reduction of 46% in lesion size when compared to the control mice (closed bar, N=8) (C). *P<0.05

EFFECT OF IL-17R DEFICIENCY ON CHOLESTEROL LEVELS

After the BMT we observed no difference in bodyweight between the IL-17R$^{-/-}$ BM recipients and control group (Figure 4.4A). The drop in weight during the first week is characteristic for bone marrow transplantation. In week 9 both groups of mice were put on a Western-type diet (0.25% cholesterol) to initiate atherosclerosis. During Western-type diet feeding serum cholesterol levels were determined and no significant difference was observed between mice which received IL-17R$^{-/-}$ and wild-type bone marrow (Figure 4.4B).

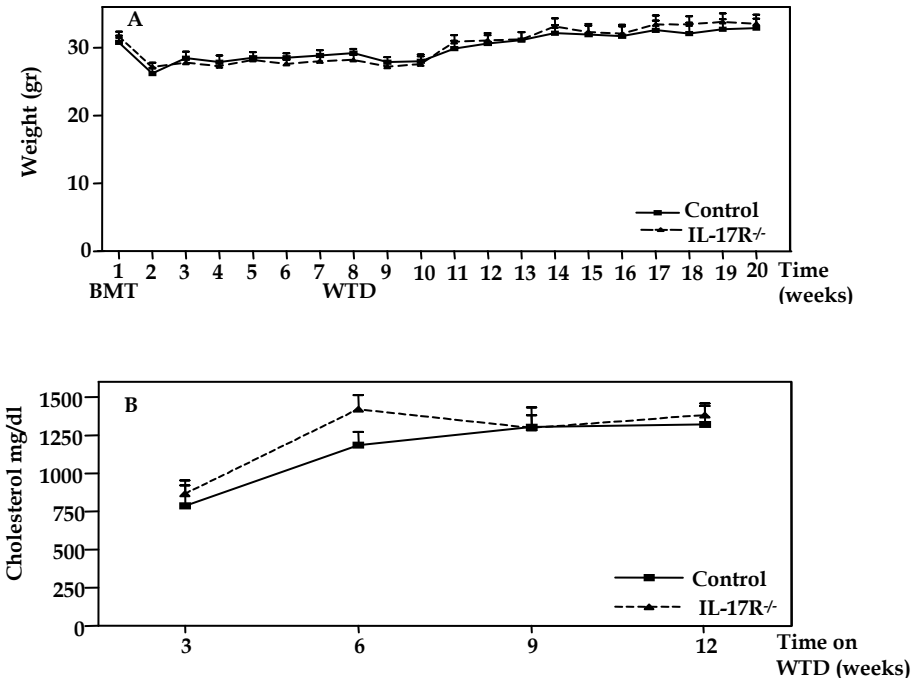

FIGURE 4.4: BODYWEIGHT AND CHOLESTEROL LEVELS IN IL-17R$^{-/-}$ AND CONTROL BMT MICE. During the experiment the animal were weighted to study well being (A). The mice were fed a Western-type diet and every three weeks blood samples were taken and cholesterol levels in the serum were determined (B).

PLAQUE COMPOSITION IS ALTERED AFTER IL-17R$^{-/-}$ BMT

IL-17R signaling is involved in the regulation of different MMP's[24] and recruitment of polymorphonuclear leukocytes.[25] Therefore we assessed whether a bone marrow transplantation with IL-17R$^{-/-}$ BM affected morphological parameters and composition within the lesions in the aortic root. To assess the collagen content and thus stability of the plaque, a Masson trichrome staining was performed. The collagen content within the lesion was not altered in IL-17R$^{-/-}$ BM recipient mice compared to control mice, indicating that the plaque stability is not affected

(Figure 4.5A; 0.12 ± 0.04 versus 0.14 ± 0.03, *P*=0.63). To examine the number of macrophages in the lesion, a MoMa-2 staining was performed. Interestingly, we observed a 25 % increase in macrophage content within the plaque of mice which received IL-17R$^{-/-}$ BM compared to control mice (Figure 4.5B; 0.32 ± 0.02 versus 0.43 ± 0.03, *P*=0.01). There was no significant difference in neutrophils content between the control group and the IL-17R$^{-/-}$ BM recipients (Figure 4.5C; 2.14 ± 0.63 versus 1.14 ± 0.36, *P*=0.29). Interestingly, IL-17R$^{-/-}$ BM recipients demonstrated a 43.8% reduction in the number of mast cells as determined by toluidin blue staining in the aortic root section when compared to controls (Figure 4.5D; 4.18 ± 0.96 versus 7.45 ± 1.20, *P*<0.05)

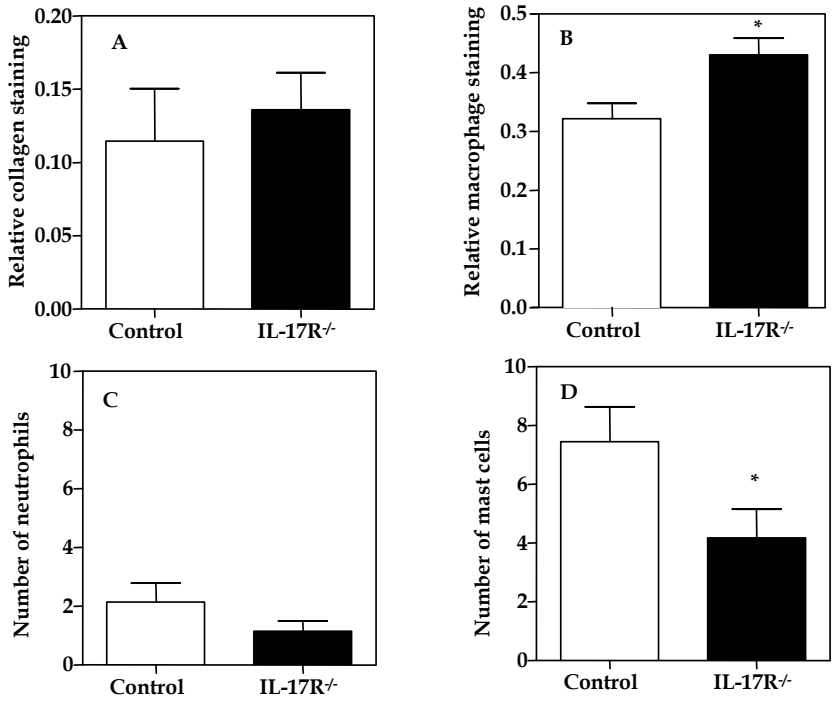

FIGURE 4.5: COMPOSITION OF PLAQUE IN THE AORTIC ROOT IN IL-17R$^{-/-}$ BM RECIPIENT AND CONTROL MICE. Cryosections of the aortic root of control mice (open bars) and IL-17R$^{-/-}$ BM recipients (closed bars) were stained. There is no difference in collagen content within the plaque (A). The relative macrophage within the intima is significantly increased in the IL-17R$^{-/-}$ BM recipients (B). The amount of neutrophils within the plaque does not change in the IL-17R$^{-/-}$ BM recipient mice (C). Mast cells were significantly reduced in the IL-17R$^{-/-}$ BM recipient mice (D). **P*<0.05

REDUCTION OF IL-17R^{+} B CELLS AND REDUCED LEVELS OF AUTOANTIBODIES IN IL-17R$^{-/-}$ BM RECIPIENTS

Recently, the role of IL-17R expressing B cells is described in relation to germinal center (GC) activity and spontaneous development of antibody mediated autoimmunity.[26] As the IL-17R$^{-/-}$ BM recipients have reduced IL-17R expressing B

cells in the lymphoid organs (Figure 4.6A-C), we studied the effect thereof on the formation of oxLDL specific autoantibodies. We observed a significant reduction of 33% in IgG antibodies directed against oxLDL in serum of mice that had received bone marrow from IL-17R deficient donors (Figure 4.6B, $P<0.05$).

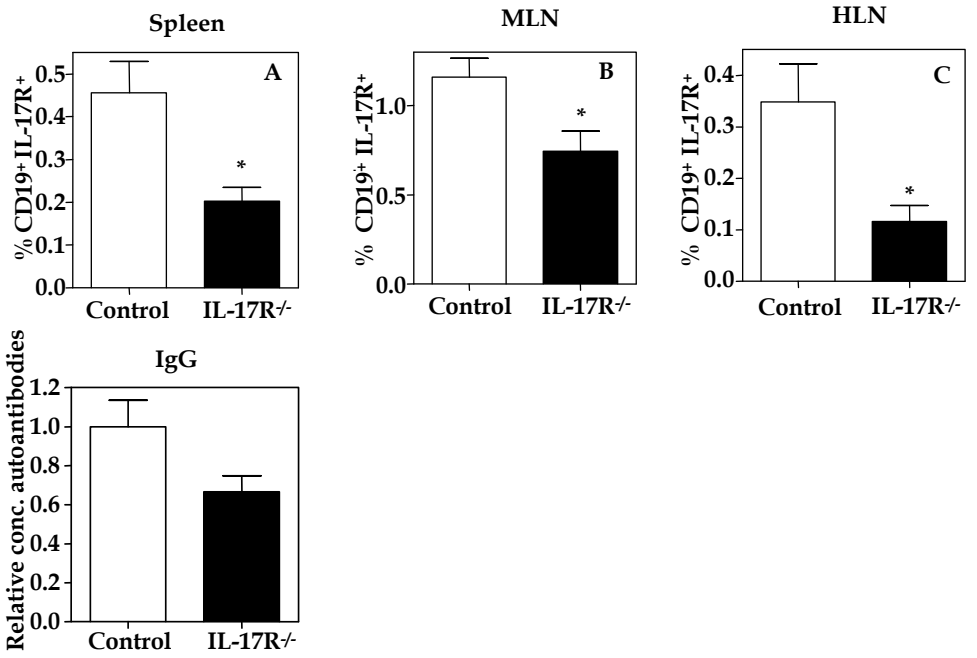

FIGURE 4.6: REDUCED IL-17R+ B CELLS AND REDUCED AUTOANTIBODIES AGAINST OXLDL IN IL-17R-/- BM RECIPIENT MICE. Single cell suspensions of lymphoid organs of control mice (white bars) and IL-17R-/- BM recipient mice (black bars) was obtained and stained for CD19 and IL17R and subsequent analyzed with a FACS machine. Within the spleen (A), MLN (B) and HLN (C) there is a significant decrease in IL-17R expressing B cells. The serum of control mice (open bars) and IL-17R-/- BM recipient mice (closed bars) was used for detection of antibodies directed against oxLDL (D). The level of total IgG autoantigens was significant lower in IL-17R-/- BM recipient mice. *$P<0.05$

DOWNSTREAM SIGNALING OF IL-17 IS IMPAIRED IN IL-17R-/- BM

IL-17 is involved in the activation of the immune system and therefore we wanted to study the effect of IL-17R deficiency on the production of interleukins by lymphoid cells. First we studied IL-6, a prominent downstream effector product of IL-17 signaling.[9] We assessed IL-6 production with an ELISA on supernatant of α-CD28 and α-CD3 activated cells from several lymphoid organs. The IL-6 production dropped 66% in the spleen of IL-17R-/- BM recipients compared to control (Figure 4.7A; 215.17 ± 114.60 versus 635.5 ± 148.22 pg/ml, $P<0.05$), whereas there was no significant change detected in the MLN, HLN and PBMCs.

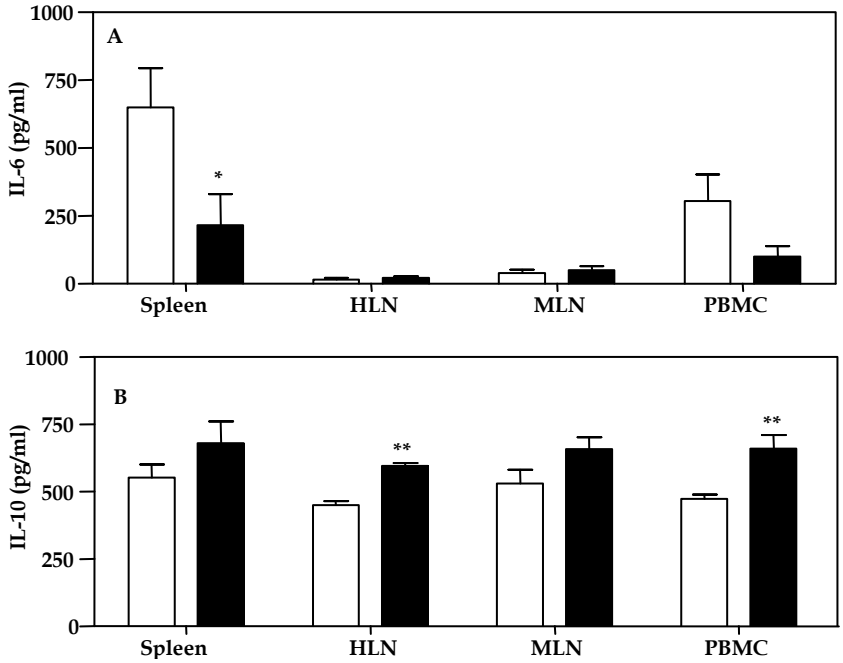

FIGURE 4.7: CYTOKINE PROFILE IS CHANGED IN IL-17R$^{-/-}$ RECIPIENT MICE. The spleen, HLN, MLN and PBMCs of control mice (open bars) and IL-17R$^{-/-}$ BM recipient mice (closed bars) were removed. *Ex vivo* stimulated lymphoid cells were used to analyze the cytokine production with an ELISA. The IL-6 expression is decreased in spleen cells of IL-17R$^{-/-}$ recipient mice (A). IL-10 was significant induced in HLN and PBMCs of IL-17R$^{-/-}$ recipient mice(B) **P*<0.05, ***P*<0.01

Furthermore, we determined IL-10 production with an ELISA on activated lymphoid cells from different lymphoid organs. Interestingly, we observed a very significant increase in IL-10 expression of 24.61% in HLN and an increase of 28.28% in PBMCs in IL-17R$^{-/-}$ BM recipients (Figure 4.7B: HLN, 449.33 ± 15.20 versus 596.00 ± 10.54, *P*<0.01; PBMCs, 473.33 ± 16.14 versus 660.00 ± 50.71, *P*<0.01). However, within the spleen and MLN IL-10 production was not changed.

DISCUSSION

The receptor for IL-17 (IL-17R) is a type I transmembrane protein that is ubiquitously expressed in the body.[9] IL-17 exhibits pleiotropic biological actions on various atheroma-associated cell types, such as endothelial cells, vascular smooth muscle cells and macrophages.[16, 17, 24] Upon activation by IL-17 these cells produce pro-inflammatory cytokines, chemokines and matrix metalloproteinases (MMPs), including TNF-α, IL-1β, IL-6, CXCL8, CCL2 and MMP-9.[18-20, 24] Although, IL-17 is

considered to be an important interleukin in several autoimmune diseases, research on the role of IL-17 in atherosclerosis is limited.

To investigate the role of IL-17 signaling in atherosclerosis, we performed a BMT with IL-17R$^{-/-}$ donor bone marrow into LDLr$^{-/-}$ recipients and evaluated the effect thereof on atherosclerosis after 12 weeks of Western-type diet. First, we verified whether the BMT was successful in replacing BM cells. We therefore analyzed the expression of IL-17R in the spleen with qPCR and observed a large reduction in IL-17R expression in IL-17R$^{-/-}$ transplanted mice. Furthermore, we analysed IL-17R expression with FACS analysis in PBMCs. We observed a large reduction of 85% in IL-17R expressing cells in the PBMCs, indicating that the BMT with IL-17R$^{-/-}$ bone marrow was successful and cells were effectively replaced by donor IL-17R$^{-/-}$ bone marrow. The reduction in IL-17R was further specified to macrophages, were we observed a reduction of 84% in the expression of the IL-17R. This finding is in line with previous bone marrow transplantation experiments in our laboratory, for example with CCR2 deficient bone marrow. [27]

Next, we studied the effect of IL-17R deficiency on atherosclerosis. We observed a striking reduction in plaque size within the aortic valve region. This effect was independent of cholesterol levels and bodyweight as these parameters were unchanged between the IL-17R$^{-/-}$ transplanted and control transplanted mice. IL-17 signaling is involved in the regulation of different MMPs.[24] Therefore we studied plaque stability, since MMPs, such as MMP-9, are well-known for their plaque destabilizing potential.[19] We did however not detect any changes in collagen content, a marker for plaque stability, within the lesion. It should be noted that MMPs are also produced by smooth muscle cells and these cells are not (effectively) replaced by IL-17R$^{-/-}$ deficient bone marrow cells. Therefore, smooth muscle cells still expres the IL-17R and are thus responsive to IL-17 within the lesion.

Interestingly, we observed an increase in relative macrophage content within the plaque of IL-17R$^{-/-}$ transplanted mice, which is in agreement with the decreased lesion size, as more initial plaques show a higher proportion of macrophages.[28] The macrophages within the plaque are likely to be IL-17R deficient and thus not, or to a lesser extent, responding to IL-17 within the plaque. This impaired IL-17 signaling pathway may lead to a diminished production of pro-inflammatory interleukins, chemokines and proteinases, which have been implicated in lesion growth and destabilization. Furthermore, we observed a significant reduction in mast cells within the lesion of the IL-17R$^{-/-}$ BM recipients. Mast cells are more prominent in advanced stages of atherosclerotic plaque development, so their reduced presence do agree with the less advanced stage of lesion formation in the IL-17R$^{-/-}$ BM recipients.[29,30, 31] IL-17 is able to induce the production of eotoxin-1 (CCL11), which is an important chemoattractant for mast

cells and is detected in atherosclerotic lesions.[32, 33] IL-17R$^{-/-}$ BM recipients have an impaired IL-17 signaling and thus the eotoxin signaling may also be impaired, leading to the decreased number of mast cells within the atherosclerotic plaque in the IL-17R$^{-/-}$ transplanted mice. In contrast to the mast cells, we did not observe any change in neutrophil count within the plaque of IL-17R$^{-/-}$ BM recipients. This is surprising as IL-17 is involved in CXCL-1 mediated neutrophil recruitment.[34] However, neutrophils are normally observed in very low numbers within the atherosclerotic lesion in the aortic valve.

Impairment in IL-17 signaling may also affect the general inflammatory status, since IL-17 is involved in bridging the innate and adaptive immune response.[2, 35] We determined the expression of IL-6 and IL-10. IL-6 is a proinflammatory cytokine that provokes a broad range of cellular and physiological responses and was one of the earliest defined IL-17 induced target genes.[3, 9] Indeed, within the spleens of IL-17R$^{-/-}$ BM recipients we observed a lower expression of IL-6. Interestingly, IL-10 expression is significantly increased in IL-17R$^{-/-}$ BM recipients. IL-10 is known to reduce atherosclerosis in LDLr$^{-/-}$ mice upon overexpression.[36-38]

Recently, Hsu *et al* suggested that IL-17 may result in spontaneous generation of autoreactive GCs as IL-17 increases the retention of B cells within the GCs through modulation of the activity of the Regulators of G-protein signaling (RGS) genes.[26] In the spleens of mice transplanted with IL-17R$^{-/-}$ bone marrow, a reduction in IL-17R ecpressing B cells paralleled a reduction in anti-oxLDL IgG antibodies which is in line with the hypothesis of Hsu *et al.* Increased IgG autoantibodies against oxLDL are associated with a larger atherosclerotic burden, therefore this may provide an additional explanation for the reduced lesion size in the IL-17R$^{-/-}$ BM recipients.

The IL-17R signaling is an interesting target for clinical applications to modulate the immune response in atherosclerosis. IL-17 forms a bridge between the innate and adaptive immune response and plays a crucial role in the progression from acute to chronic inflammation.[2] In our laboratory we blocked IL-17 via DNA vaccination, which resulted in a decreased atherogenesis in LDLr$^{-/-}$ mice.[39] Furthermore, Th17 cells, profound producers of IL-17, have been identified in patients with acute coronary syndrome, underlining the potential role of IL-17 in atherosclerosis.[40]

In conclusion, we demonstrate that an impaired IL-17R signaling results in less atherosclerosis, indicating an aggravating role for IL-17 in this disease. Therapies interfering in the IL-17 pathway may provide a newly explored treatment against atherosclerosis.

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Chapter 5

Bone marrow transplantation with p19 deficient bone marrow does not alter the atherosclerotic burden in LDL receptor deficient mice

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ABSTRACT

Interleukin 23 (IL-23) has been associated with several autoimmune diseases. IL-23 is a heterodimeric interleukin consisting of a p19 subunit and a p40 subunit, which is shared with IL-12. Previous studies in which p40 was neutralized indicate a possible role for IL-23 in atherosclerosis.

To study the role of IL-23 in atherosclerosis, a bone marrow transplantation with p19 deficient bone marrow into LDL receptor deficient mice was performed and atherosclerosis was assessed after feeding a cholesterol rich diet.

The degree of atherosclerosis was similar in mice transplanted with p19 deficient bone marrow compared to mice receiving wild-type bone marrow. Furthermore, no change in IL-17 producing T cells and Tregs was observed. The expression of p40, p35 and p19 was not changed in the spleen of p19 deficient bone marrow transplanted mice, although bone marrow transplantation was successful.

A bone marrow transplantation with p19 deficient bone marrow in LDL receptor deficient mice does not affect atherosclerotic lesion formation, possibly because of the contribution of non-bone marrow derived cells to the production of IL-23.

INTRODUCTION

Since atherosclerosis is considered as an inflammatory disease, much research has been done to illuminate the contribution of different components of the immune system in atherosclerosis. In atherosclerosis, T cells and macrophages play an important role in bridging the innate and adaptive immune response and are able to exert pro- and anti-atherosclerotic properties.[1-3] Within the atherosclerotic lesion and lymph nodes that drain from the lesions, macrophages and dendritic cells are capable to present antigens to T cells, thereby inducing an adaptive immune response. These antigen presenting cells have a pivotal role in immune regulation as they produce several cytokines and chemokines to "direct" the immune system to the appropriate immune response. An important cytokine in this process is interleukin (IL)-23, which is a heterodimeric interleukin consisting of a p40 and a p19 subunit. IL-23 is a close family member of IL-12 as they both share the p40 subunit.[4]

The receptor of IL-23 consists of a heterodimeric complex consisting of IL-12Rβ1, which is shared with the IL-12 receptor and a unique IL-23R. The IL-23R shares many features with IL-12Rβ2, which is the other part of the IL-12 receptor. However, IL-23 induces a strong phosphorylation of Stat3 and a weak activation of Stat4, as for IL-12 the opposite is true, thereby explaining the different effect of these cytokines.[4, 5] Furthermore, the IL-23R is mainly expressed on effector T cells and not on naive T cells, suggesting an important role of IL-23 in ongoing inflammation.[4, 6] The expression of the IL-23R is also detected in human NK cells, murine bone marrow derived DCs and activated macrophages.[4, 6]

IL-23 is mainly expressed by macrophages and dendritic cells and p19 is dependent on the expression of p40 in the same cell in order to be functionally excreted as IL-23.[4] An important function for IL-23 was recently discovered by Langrish *et al.*, where IL-23 is associated with the expansion of the IL-17-producing Th17 cells.[7] Furthermore, p19-deficient animals do not develop IL-17-producing T cells and are protected from EAE.[8] Moreover, in patients with Crohn's disease a single nucleotide polymorphism (SNP) in the coding sequence of IL-23R results in a strong protection against this disease, indicating a pathogenic role of IL-23 in chronic inflammation.[9] Besides the effect of IL-23 on Th17 cells, IL-23 also induces the production of IL-1β and TNFα by peritoneal macrophages, which occurred even in the presence of neutralizing antibodies to IFN-γ, thereby suggesting that these effects are independent from Th1 cells.[8]

In our laboratory, we already demonstrated that vaccination against p40 (IL-12/IL-23) results in a reduction of almost 70% in atherosclerotic lesion formation.[10] However, p40 is shared by both IL-12 and IL-23 and consequently, it is not possible to elucidate the relative contribution of IL-12 and IL-23 in

atherosclerosis. Since IL-23 is involved in the development of Th17 cells and IL-17 has been associated with aggravated atherosclerosis (van Es *et al.*, unpublished results), studies into the role of IL-23 in atherosclerosis are of even more interest.

We now show that a bone marrow transplantation with p19 deficient bone marrow into LDL receptor deficient mice did not affect lesion formation. More research should be performed with LDLr and p19 double knock outs to exclude the p19 expression by radiant resistant cells to further elucidate the role of IL-23 in atherosclerosis.

METHODS

ANIMALS

All animal work was approved by Leiden University and was in compliance with the Dutch government guidelines. LDL receptor deficient ($LDLr^{-/-}$) mice were purchased from Jackson Laboratories. IL-23-p19 knockout mice were a kind gift from E. Lubberts and were created by Ghilardi *et al*. [11] The mice were kept under standard laboratory conditions and food and water were provided *ad libitum*.

BONE MARROW TRANSPLANTATION AND INDUCTION OF HYPERCHOLESTEROLEMIA

Male $LDLr^{-/-}$ mice were lethally irradiated by a single dose of 9 Gy (0.19 Gy/min, 200 kV, 4 mA) total body irradiation, using an Andrex Smart 225 Röntgen source (YXLON Int, Copenhagen, Denmark) with a 6-mm aluminium filter. Bone marrow was isolated by flushing the femurs and tibias from mice with phosphate-buffered saline (PBS). Single-cell suspensions were prepared by passing the cells through a 30-μm nylon gauze. Irradiated recipients received $0.5x10^7$ bone marrow cells by intravenous injection into the tail vein. Some bone marrow was stored at -80 ^oC, which was used for genotyping as described later on. After a recovery of 8 weeks animals received a Western-type diet *ad libitum* containing 15% cocoa butter and 0.25% cholesterol (Special Diet Services, Witham, Essex, UK) for 12 weeks. During the experiment the mice were weighted every week and checked for well-being and every 3 weeks the serum cholesterol levels were determined to assess the effect of the Western-type diet. Blood samples were collected by tail bleeding from non-fasting animals. Total cholesterol levels were quantified spectrophotometrically using an enzymatic procedure (Roche Diagnostics, Germany). Precipath standardized serum (Boehringer, Germany) was used as an internal standard.

GENOTYPING THE BONE MARROW AFTER BONE MARROW TRANSPLANTATION

After sacrificing the donor animals the bone marrow was isolated by flushing the femurs from mice with PBS. Genomic DNA was isolated by incubating with lysis buffer (25 mm NaOH, 0.2 mM EDTA, pH=12) for 20 minutes at 99°C. Subsequently 100 µl neutralization buffer (40 mM TRIS HCl, pH=5) was added. A PCR was performed with the following primers: antisense primer (5'-GCC TGG GCT CAC TTT TTC TG-3'), wild type-specific (5'-GCG TGA AGG GCA AGG ACA CC-3') and knockout-specific (5'-AGG GGG AGG ATT GGG AAG AC-3') sense primers. This primer-triplet amplifies a 210-bp fragment for the wild-type allele and a 289-bp fragment for the mutant allele.

HISTOLOGY AND IMMUNOHISTOCHEMISTRY

Mice were anaesthetized with ketamine-hypnorm and perfused with PBS and subsequently with FormalFixx. The heart and complete aorta were removed. The heart was embedded in OCT compound (TissueTek, Sakura Finetek, The Netherlands) and cryosections of 10 µm were made of the aortic root containing the aortic valves. Cryosections were routinely stained with Oil-Red-O and hematoxylin (Sigma Diagnostics, MO). Corresponding sections on separate slides were stained immunohistochemically for macrophages using an antibody against a macrophage-specific antigen (MoMa-2, Research Diagnostics Inc.) and for collagen using Masson trichrome staining according to manufacturers protocol (Sigma Diagnostics. The different histological stainings were quantified using a Leica DM-RE microscope and Leica Qwin Imaging software (Leica Ltd., Germany). The complete aorta was longitudinally cut open and subsequently stained for lipids with Oil-Red-O. The aortas were *en face* fixated and high resolution pictures were taken and the plaque was quantified using Leica Qwin imaging software (Leica Ltd., Germany).

QPCR ON SPLEEN SAMPLES

Total RNA was isolated using guanidium isothiocyanate (GTC) method and reverse transcribed to cDNA (RevertAid™ M-MuLV reverse transcriptase, Fermentas). Gene expression was analyzed with an ABI PRISM 7700 (Applied Biosystems, Foster city, CA) using SYBR Green technology and a final primer concentration of 300 nM. Primer pairs as described in table 5.1 were used to quantify IL-17, IL-10, IL-6 and IL-4 gene expression. As an internal standard Hypoxanthine-guanine phosphoribosyltransferase (HPRT) was used. The samples were analyzed on an ABI Prism® 7700 sequence detector (Applied Biosystems).

TABLE 5.1: SEQUENCE OF PRIMERS. Primer sequences for the genes analyzed with qPCR.

GENE	FORWARD PRIMER	REVERSE PRIMER
IL-17	5′-GAAACATGGTTGATGACTCCAAA-3′	5′-CTCCACAGAGGGGGTGGT-3′
IL-10	5′-TCCCCTGTGAAAATAAGAGCA-3′	5′-ATGCAGTTGATGAAGATGTCAAA-3′
IL-6	5′-GAAGAATTTCTAAAAGTCACTTTGAGATCTAC-3′	5′-CACAGTGAGGAATGTCCACAAAC-3′
IL-4	5′-ACTTGAGAGAGATCATCGGCATTT-3′	5′-AGCACCTTGGAAGCCCTACAG-3′
HPRT	5′-TTGCTCGAGATGTCATGAAGGA-3′	5′-AGCAGGTCAGCAAAGAACTTATAG-3′

STATISTICAL ANALYSIS

All data are expressed as mean ± SEM. The two-tailed student's t-test was used to compare individual groups of mice or cells. When indicated the data was analyzed with linear mixed model analysis. *P* values of <0.05 were considered significant.

RESULTS

BONE MARROW OF RECIPIENTS IS REPLACED WITH DONOR BONE MARROW

The mice were sacrificed after 12 weeks of feeding a Western-type diet and a PCR on genomic DNA of the BM of the recipients was performed. We also analyzed the donor BM, which served as a positive control (Figure 5.1, lane 6 and 12). In the $p19^{-/-}$ BM recipients we observed a PCR fragment corresponding with the knock out genotype (Figure 5.1 , lane 7-11). PCR on the mice that received wild-type BM resulted in a smaller fragment, corresponding with the wild-type p19 genotype (Figure 5.1 A, lane 1-5), indicating that the BMT was successful.

SERUM CHOLESTEROL LEVELS ARE INCREASED IN $p19^{-/-}$ BM RECIPIENTS

During the experiment the mice were weighted. There was no difference observed between the $p19^{-/-}$ transplanted mice and control (Figure 5.2A). During Western-type diet feeding serum cholesterol was determined and we observed a significant higher level in total serum cholesterol level in $p19^{-/-}$ BM recipients during the experiment (Figure 5.2B; 48% on week 3, 23% on week 6, 33% on week 9 and 44 % on week 12 on Western-type diet. P<0.01).

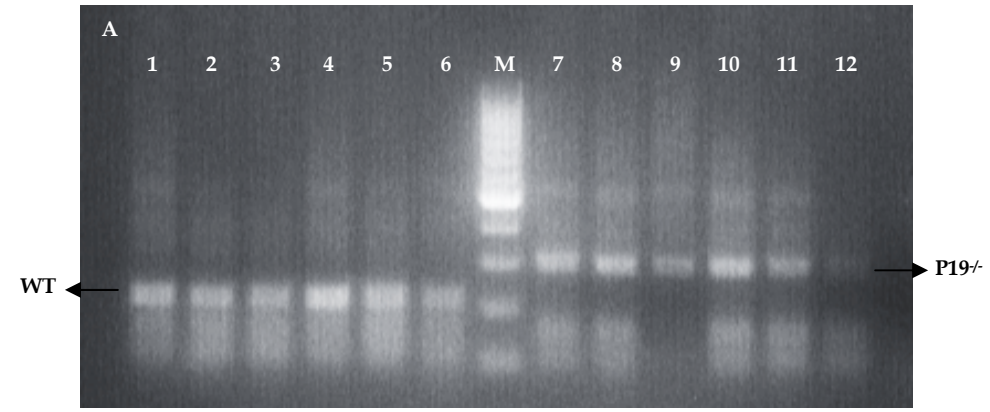

FIGURE 5.1: BONE MARROW OF RECIPIENTS IS REPLACED WITH DONOR BONE MARROW. After the mice were sacrificed, the bone marrow was flushed out of the femurs and genomic DNA of control mice (lanes 1-5) and p19-/- BM recipients (lanes 7-11) was isolated and a PCR was performed to amplify the wild type or p19-/- gene. In lane 6 and 12 Genomic DNA was isolated from BM of wild type (lane 6) and p19-/- donor mice (lane 12) and served as a positive control.

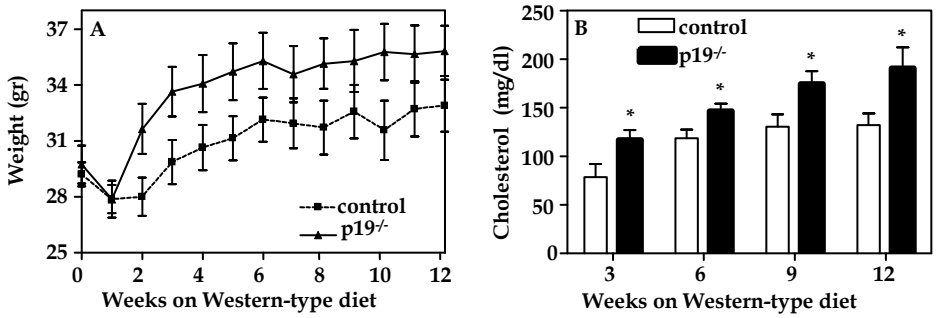

Figure 5.2: Bodyweight and serum cholesterol levels in p19-/- BM recipients. During the experiment the mice were weighted every week to determine the well-being of the mice (A, N=13-15). Every three weeks, blood was taken via tail vein bleeding and the serum cholesterol levels were determined using an enzymatic procedure (B, N=13-15). A linear mixed model analysis was used to analyze the results. **P*<0.05

PLAQUE SIZE AND STABILITY IS NOT CHANGED IN P19-/- BM RECIPIENT MICE

To determine the effect of p19-/- BMT in atherosclerosis, the mice were fed a Western-type diet for 12 weeks after recovery from BMT. The level of atherosclerosis in the aortic root within the control group was not significantly different from the p19-/- BM recipients (Figure 5.3 A-C, P=0.28). In addition, we observed no difference in plaque size in the entire aorta between wild type and p19-/- BM transplanted mice (Figure 5.3 D-F, P=0.11). Furthermore, we analyzed plaque stability in the aortic root by determining the collagen content of the

plaque. However, we did not observe any difference between the control mice and p19$^{-/-}$ BM recipients (Figure 5.4 A-C, *P*=0.55).

EXPRESSION OF P40, P35 AND P19 IS NOT CHANGED WITHIN THE SPLEEN

To study whether the expression of the subunits of IL-12 and IL-23 was affected by the bone marrow transplantation, a qPCR analysis was performed on mRNA for the various subunits in spleen cells. The expression of the IL-23 subunit p19 was determined (Figure 5.5A) and surprisingly, we did not observe any difference in p19 expression between the groups. Furthermore, when we analyzed the expression of p40, a subunit shared with IL-12, and p35, the other subunit of IL-12, there were no differences observed. The mRNA expression of IL-10, IL-17, IL-6 and IL-4 was also determined in the spleen (Figure 5.5B). The expression of IL-10 was 76% decreased, IL-17 was 66% decreased, IL-6 was 87% decreased and IL-4 was 64% decreased in the p19$^{-/-}$ transplanted mice, indicating a change in the inflammatory status in these mice.

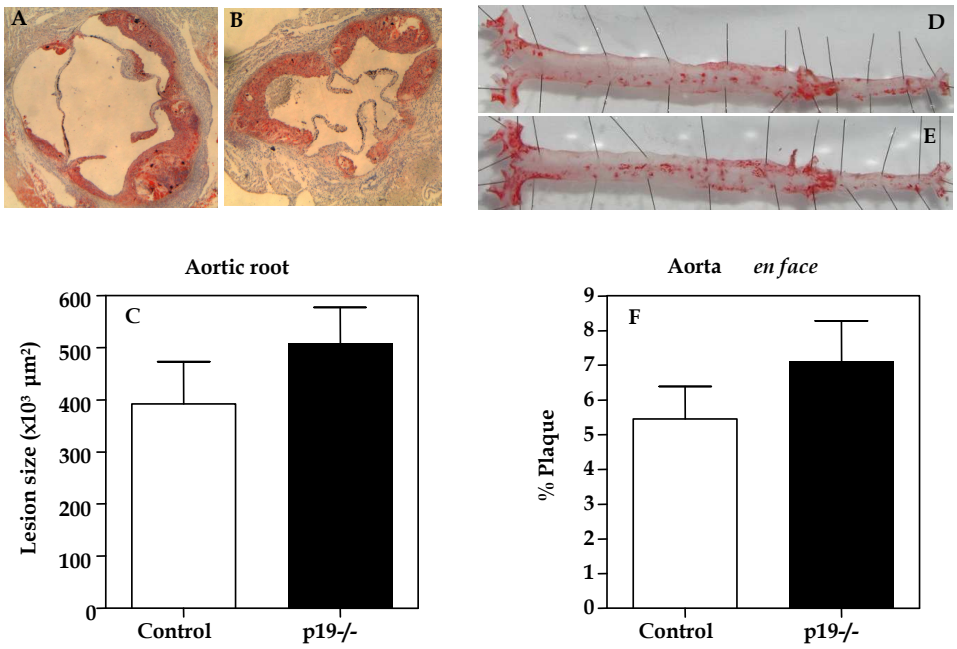

Figure 5.3: Plaque size is not changed in p19$^{-/-}$ BM recipient mice. After the mice were sacrificed hearts were removed and cryosections of the aortic root were made of the control mice (A, N=13) and the p19$^{-/-}$ BM recipients (B, N=12) and subsequently stained for Oil-red-O and the lesions were quantified (C). The aorta was also removed from control mice (D, N=13) and p19$^{-/-}$ BM recipients (E, N=12) and stained with Oil-Red-O staining and quantified (F).

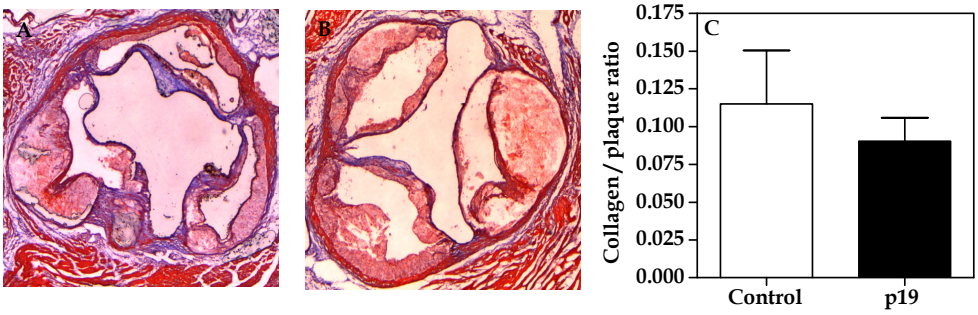

Figure 5.4: Collagen content of the atherosclerotic lesion is not changed. After the mice were sacrificed hearts were removed and cryosections of the aortic root were made. For collagen detection, cryosections of the aortic root of the control mice (A, N=12) and p19-/- BM recipient mice (B, N=12) were stained with a Masson's trichrome staining and the relative collagen amount in the plaque was quantified (C).

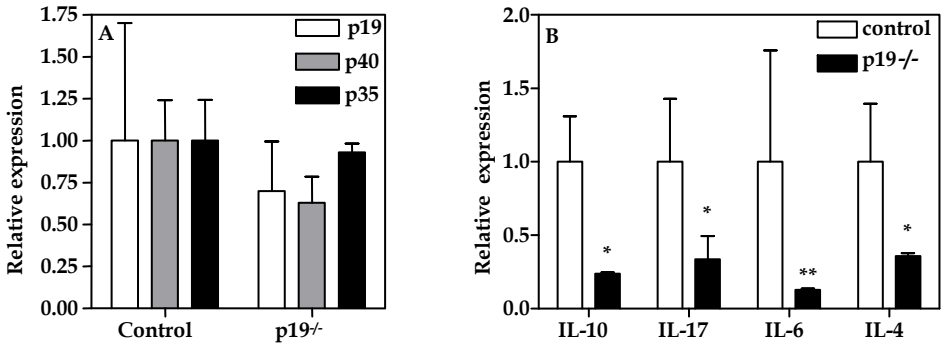

Figure 5.5: Effect of p19-/- BMT on the expression of interleukins in the spleen. Spleens were dissected and mRNA was isolated and subsequently reverse transcribed into cDNA. Expression of p40, p35 and p19 was determined with qPCR (A, N=9-13). Furthermore, the expression of IL-10, IL-17, IL-6 and IL-4 was also assed within the spleen with qPCR and are depicted relative to the expression in the control mice. (B, N=9-13) *P<0.05, **P<0.01

DISCUSSION

IL-23 has been associated with several autoimmune diseases.[7, 8, 12] Immunization against p40, which is shared by both IL-12 and IL-23, resulted in a reduction of almost 70% in atherosclerotic lesion formation.[10] Together with the relation between IL-23 and the development of Th17 cells, this interleukin is an interesting subject to study in relation to autoimmune diseases, such as atherosclerosis.[7] Therefore we performed a BMT with BM of p19 deficient mice into LDLr deficient mice to study the role of IL-23 in atherosclerosis.

To determine the efficacy of the BMT, a genotyping was performed on the BM in p19-/- BM and wild type BM transplanted mice. Within the wild type

recipients we detected the wild type p19 gene and the p19$^{-/-}$ recipients demonstrated a knockout fragment. This indicates after irradiation and subsequent BMT, the BM is replaced with the donor BM.

Surprisingly, already after 3 weeks of Western-type (11 weeks after the BMT) diet the serum cholesterol levels were 48% increased in the p19$^{-/-}$ recipient group and remained significantly higher throughout the experiment. However, little is known about IL-23 in relation with cholesterol metabolism and this remains to be investigated.

Since the dramatic reduction in lesion size upon IL-12 vaccination we expected a reduction in lesion size upon p19$^{-/-}$ BMT. However, we did not observe any difference in atherosclerotic lesion size between p19$^{-/-}$ BM recipient and control mice. Dubin *et al*. demonstrated that p19$^{-/-}$ mice have reduced matrix metalloproteinase-9 (MMP-9).[13] MMP-9 is associated with atherosclerotic plaque stability[14] and therefore the collagen content was assessed as a marker for plaque stability. However there was no difference in collagen content detected between the groups, which indicate that p19 deficiency does not affect plaque stability. These data are surprising, since Hauer *et al*. demonstrated that vaccination against p40 (IL-12/IL-23) resulted in a reduction of almost 70% in atherosclerotic lesion formation.[10] Furthermore, Hauer *et al*. also demonstrated an increase in plaque stability.[10] Neither of these events is observed in p19$^{-/-}$ BM recipients. This may suggest that IL-23 is not involved in initial atherosclerosis and that the reduction seen with p40 vaccination is mediated via a reduction in IL-12.

Within the spleen we determined the expression of the subunits p40, p35 and p19. Interestingly, none of the subunits displayed an altered expression level after p19$^{-/-}$ BMT, indicating that the spleen is still capable of expressing IL-12 and IL-23. This may suggest another source of p19, besides bone marrow derived cells. An unpublished report of S. Pflanz and R.A. Kastelein demonstrates a heterodimer consisting of p19 and EBI3 in vitro.[6] Whether these heterodimers may be formed in vivo and what function this new interleukin has and by which cell type(s) it is expressed remains to be elucidated.

Together with the observation that T cells are able to express p19 (E. Lubberts, personal communication) it may be suggested that not only bone marrow derived antigen presenting cells express p19 to form IL-23. Furthermore p19 may also be expressed by radiant resistant cells (including T cells) within the spleen. Whether this resulted in functional IL-23 remains to be determined using intracellular FACS analysis. These data may explain the fact that we do not observe any change in atherosclerotic parameters.

However, when we assessed the expression of IL-6, IL-17, IL-10 and IL-4 expression we observed a reduction in gene expression in p19$^{-/-}$ BM recipient mice. This indicated that there is a change in the inflammatory status of p19$^{-/-}$ BM

recipient mice. A decrease in IL-10 may be causative for the elevated serum cholesterol level in p19$^{-/-}$ BM recipients, since it has been demonstrated that IL-10 may reduce cholesterol levels.[15]

Although atherosclerosis is considered an autoimmune disease with many similarities with other autoimmune diseases, there may be differences in the involvement of the immune system in the pathogenesis of different autoimmune diseases. Recently, Mangino *et al.* demonstrated that there is no relation between several polymorphisms of p19 and IL-23R genes and a myocardial infarction.[16] This study indicates that the role of IL-23 in atherosclerosis may be distinct from other autoimmune diseases.

In conclusion, we demonstrate that a BMT with p19$^{-/-}$ BM in LDLr deficient mice does not result in an alteration in atherosclerosis. Conclusions on the role of IL-23 are however hard to draw since we still observed p19 expression in the spleens of mice receiving p19$^{-/-}$ bone marrow, indicating that non-bone marrow derived cells contribute in vivo to the production of IL-23. To definitively determine the role of IL-23 in atherosclerosis, we will have to generate p19 and LDL receptor double knockout mice and investigate the effects on atherosclerosis.

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Chapter 6

Vaccination against the p28 subunit of interleukin-27 aggravates atherosclerosis in LDL receptor deficient mice

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Manuscript in preparation

ABSTRACT

Interleukin 27 (IL-27) is a heterodimeric cytokine consisting of p28 and EBI3. It has opposing effects on a number of lymphoid and non-lymphoid cell types and is related to the IL-12, IL-23 and IL-6 family, which are associated with atherosclerosis. We now studied the expression of both the subunits of IL-27, p28 and EBI3, during atherosclerosis and studied the role of IL-27 in atherosclerosis by vaccinating $LDLr^{-/-}$ mice against the p28 subunit of IL-27 using a DNA vaccine.

During atherosclerosis, we observed an increase in the expression of both subunits of IL-27 in the atherosclerotic lesion. Vaccination against the p28 subunit of IL-27 led to a significant increase in lesions size in the carotid artery and in the aorta as assessed by *en face* staining, but did not significantly affect lesion size in the aortic root. Atherosclerotic plaque composition within carotid artery and the aortic root was not affected by vaccination against p28. FACS analysis of the spleen demonstrated an increase in the Th17 population and a decreased Treg population. Furthermore, a significant reduction in B cells was observed within the spleen.

We conclude that both subunits of IL-27 are upregulated in initial atherosclerosis and the results on vaccination against IL-27 suggest that IL-27 has anti-atherosclerotic properties. From this data we propose that IL-27 may be used as therapeuticum to treat atherosclerosis.

INTRODUCTION

Atherosclerosis is an inflammatory disease of the vessel wall, which progresses in the context of high plasma cholesterol levels.[1] Accumulation of inflammatory cells within the lesion results in a complex auto-immune like disease, which results in a destabilized and ultimately ruptured lesion, leading to cardiovascular complications.[2] The innate immune response and the subsequent adaptive immune response, illustrated by the differentiation of naïve T cells into effector T cells plays an important role in the process of atherosclerosis.[2]

The recently discovered interleukin IL-27 is a cytokine that shows structural resemblance with IL-12 and IL-23 and IL-27 affects T cell function and cytokine production.[3] IL-27 is a heterodimeric cytokine composed of Epstein-Barr virus induced gene 3 (EBI3) and p28. IL-27 is produced by activated antigen presenting cells (APCs), as well as resident macrophages, early in the immune response against pathogens such as, *Mycobacteria tuberculosis*, *Trichuris muris*, and *Toxoplasma gondi*.[4-6] The IL-27 subunit p28 is poorly secreted unless it is co-expressed with its partner EBI3, thus creating a situation where expression of IL-27 can be tightly controlled during an immune response by regulating the transcription of both subunits.[4] IL-27 exerts its effects via the IL-27 receptor, a complex of gp130 and the novel IL-27R (also designated as WSX-1 or TCCR) and the heterodimeric IL-27 receptor is mainly expressed by lymphocytes but expression on monocytes is also reported.[7]

Initially, IL-27 was assigned to have proinflammatory properties, illustrated by studies performed in the group of Goldberg *et al.* They demonstrate that vaccination against p28 suppresses experimental autoimmune encephalomyelitis (EAE) and adjuvant induced arthritis, suggesting a proinflammatory role for IL-27 in these autoimmune diseases.[8, 9] However, recent studies show a more complex role for IL-27. For example, IL-27 suppresses the development of the pro-inflammatory T helper cell, the Th17 cell, in EAE models and thereby attenuates the disease.[4, 10, 11] Furthermore, IL-27 also inhibits the development of regulatory T (Treg) cells and Th2 cells.[12, 13] This divergent effect of IL-27 on T cell populations was explained by Yoshimura *et al*., who proposed that IL-27 stimulates Signal Transducers and Activator of Transcription (STAT)1 and STAT3 in naive Th cells and only STAT3 in activated Th cells, thereby enabling IL-27 to exert a stimulating effect on naive cells and an inhibiting effect on effector T cells.[14]

The role of IL-27 in atherosclerosis is to our knowledge not yet investigated. Since IL-27 affects T cell development and may specifically affect regulatory T cell function, we vaccinated LDLr$^{-/-}$ mice against the p28 subunit of IL-27. We observed a strong increase in atherosclerosis, which may suggest a protective role for IL-27 in the context of atherosclerosis.

MATERIAL AND METHODS

ANIMALS

All animal work was approved by Leiden University and was in compliance with the Dutch government guidelines. Low-density lipoprotein receptor deficient (LDLr$^{-/-}$) mice were purchased from Jackson Laboratories. The mice were kept under standard laboratory conditions and food and water were provided *ad libitum*.

IN VITRO CYTOKINE PROFILE

A spleen was dissected from a LDLr$^{-/-}$ mouse and a single cell suspension was obtained by passing the spleen through a 70 µm cell strainer (Falcon, The Netherlands). Spleen cells were subsequently incubated with erythrocyte lysis buffer (0.15 M NH_4Cl, 10 mM $NaHCO_3$, 0.1 mM EDTA, pH 7.3). Spleen cells were cultured in a 96-wells round bottom plate ($2x10^5$ cells per well), coated with anti-CD28 and anti-CD3 (0.25 µg/well). Cells were cultured in RPMI supplemented with 10% FCS, 100 U/ml penicillin and 100 µg/ml streptomycin (all from BioWhittaker Europe). Where indicated cells were incubated with either: 0, 2.5 or 5 ng/ml IL-27 (R&D sytems) for 48 hours. Supernatant of the cells was used for an IL-17 (OptEIA kit, PharMingen), IL-6 (eBioscience, Belgium), TGF-β1 (eBioscience, Belgium) and an IL-2 ELISA (ebioscience, Belgium) all according to manufacturer's protocol.

EXPRESSION OF P28 AND EBI3 IN ATHEROSCLEROSIS

LDLr$^{-/-}$ mice were fed a Western-type diet, containing 0.25% cholesterol and 15% cocoa butter (Special Diet Services, UK) and after two weeks collars were placed around both carotid arteries and continued for 10 more weeks on Western-type diet. Mice were sacrificed at several time points on Western-type diet and at several times after collar placement. The carotid arteries were removed and total RNA was isolated and reversed transcribed as is described later on. Subsequently,

the expression of EBI3, p28 and CD86 was determined with qPCR relative to HPRT as described later on. Sequences of used primers are described in table 6.1.

TABLE 6.1: SEQUENCE OF PRIMERS. Primer sequences for the genes analyzed with qPCR.

Gene	Forward primer	Reverse primer
P28	5′-CACAGGCACCTCCGCTTT-3′	5′-TTGGGATGACACCTGATTGG-3′
EBI3	5′-CCCGGACATCTTCTCTCTCA-3′	5′-CAATACTTGGCATGGGGTTT-3′
CD68	5′-CCTCCACCCTCGCCTAGTC-3′	5′-TTGGGTATAGGATTCGGATTTGA-3′
HPRT	5′-TTGCTCGAGATGTCATGAAGGA-3′	5′-AGCAGGTCAGCAAAGAACTTATAG-3′

CLONING OF THE PADRE-P28 VACCINE

The amino acid sequence of PADRE was derived from the protein sequence: aKXVAAWTLKAAC (X=cyclohexylamin, a=R-Alanin) Cyclohexylamin was replaced with phenylalanine, which has the most resemblance. The R-Alanine was replaced with L-Alanine. The following nucleotide sequence was used for PADRE: 5′-ATG GCT AAA TTT GTG GCT GCT TGG ACA CTT AAA GCT GCT GCT-3′ and subsequently cloned into the pcDNA3.1 with *Nhe I* and *BamH I*. The p28 subunit of IL-27 (NM_145636) was amplified with a primer set with an additional *BamH I* recognition site at the N-terminus (5′-ATA TAT *GGA TCC* GGC CAG GTG ACA GGA GAC CT-3′) and an *Acc65I* recognition site at the C-terminus (5′-ATA TAT *GGT ACC* TTA GGA ATC CCA GGC TGA GC-3′, Genomic, Oligo-eurogentech). The construct was used for transfection into DH5α (Invitrogen) according to the manufacturers protocol. The amplified plasmid was isolated with a Quickclean miniprep kit (Genescript Corp., USA) and sequenced. All sequences were analyzed with a BigDye terminator v3.1 cycle sequencing kit (Applied Bioscience) according to the manufacturer's protocol and analyzed on an ABI-Prism® 3100 Avant Genetic Analyzer (Applied Biosystems).

Subsequently, COS7 cells were transfected with pcDNA3.1-PADRE-p28 construct and pcDNA3.1-PADRE using Exgen500 according to manufacturer's protocol (Fermentas, Germany). Total RNA was isolated using guanidium isothiocyanate (GTC) method and reverse transcribed to cDNA (RevertAid™ M-MuLV reverse transcriptase, Fermentas). P28 expression was analyzed with an ABI

PRISM 7700 (Applied Biosystems, Foster city, CA) using SYBR Green technology and a final primer concentration of 300 nM.

VACCINATION AND INDUCTION OF ATHEROSCLEROSIS

The DNA vaccine was isolated with an Endofree plasmid Giga kit (Qiagen, The Netherlands) from transfected DH5-α. Three days prior to the first vaccination, male LDLr$^{-/-}$ mice, 10-12 weeks old, received an *intra muscular* (*i.m.*) bilateral Cardiotoxin I (Sigma, USA) injection (10 μM, 50 μl per muscle). Mice were vaccinated by a total of three bilateral *i.m.* injections of 100 μg plasmid in 100 μl PBS, 50 μl per muscle, with two-week intervals. After the last vaccination mice were fed a Western-type diet. After two weeks of diet feeding, local atherosclerosis was induced within the carotid arteries by bilateral perivascular collar placement, as described previously.[15] During the experiment plasma samples were obtained by tail vein bleeding for cholesterol measurements. Total cholesterol levels were quantified during the experiment using an enzymatic procedure (Roche Diagnostics, Germany) using Precipath as an internal standard.

FACS ANALYSIS OF SPLEEN CELLS

Spleens were dissected from the mice and single cell suspension was obtained by passing the spleen through a 70 μm cell strainer (Falcon, The Netherlands). Cells were stained with surface markers (0.20 μg antibody/300.000 cells) and subsequently analyzed by flow cytometric analysis. The following antibodies were used: CD4-FITC, CD25-APC, Foxp3-PE, CD19-FITC, CD62L-APC and IL-17-PE. All antibodies were purchased from eBioscience (Immunosource, Belgium). For the Th17 cell FACS analysis, the cells were incubated with Golgiplug (BD Bioscience) for 4 hours before the staining. All data were acquired on a FACScalibur and analyzed with CELLQuest software (BD Biosciences, The Netherlands).

TISSUE HARVESTING

Eight weeks after collar placement mice were sacrificed and carotid arteries were obtained after *in situ* perfusion for 15 minutes with Formalfixx. Carotid arteries were embedded in OCT compound (TissueTek, Sakura Finetek, The Netherlands), snap-frozen in liquid nitrogen and stored at -20 °C until further use. Transverse 5 μm cryosections were prepared in a proximal direction from the carotid bifurcation and were mounted on a parallel series of slides. The heart was embedded in OCT compound (TissueTek; Sakura Finetek, The Netherlands) and cryosection of 10 μm

were made of the aortic root containing the aortic valves. The complete aorta was removed for *en face* analysis.

HISTOLOGICAL ANALYSIS AND MORPHOMETRY

Cryosections were routinely stained with hematoxylin (Sigma Aldrich, Zwijndrecht, The Netherlands) and eosin (Merck Diagnostica, Germany). Corresponding sections were stained for lipids by Oil-red-O staining. Hematoxylin-eosin. Corresponding sections on separate slides were stained immunohistochemically for macrophages using a macrophage-specific antigen (MoMa-2, Research Diagnostics Inc.) Collagen was stained with Masson's trichrome staining according to manufacturer's protocol (Sigma Diagnostics). Atherosclerosis in the aortic root was quantified with Oil-red-O stained sections of plaques developed in the region of the aortic semilunar valves. The complete aorta was longitudinally cut open and subsequently stained for lipids with Oil-Red-O. The aortas were *en face* fixated and high resolution pictures were taken and the lipid rich areas were quantified. All sections and images were analyzed with Leica Qwin software using a Leica DM-RE microscope.

OXLDL ANTIBODY DETECTION

Cu-oxLDL was synthesized as described previousely [16, 17]. Antibodies against Cu-oxLDL were determined according to Damoiseaux et al.[18] MaxiSorp 96 wells plates (Nunc, Roskilde, Denmark) were coated overnight with 100 µg oxLDL in coating buffer (50 mM $NaHCO_3$, 50 mM Na_2CO_3, pH=9.6) at 4 ^{0}C. IgM, IgG2a, IgG2b and IgG1 antibodies directed against oxLDL were detected with an isotype Ig detection kit according manufacturer's protocol (Zymed lab. Inc., CA).

STATISTICAL ANALYSIS

All data are expressed as mean ± SEM. The two-tailed student's t-test was used to compare individual groups of mice or cells. When indicated, a Mann-Whitney test was used to analyze not normally distributed data. *P* values of <0.05 were considered significant.

RESULTS

IL-27 INHIBITS THE EXPRESSION OF SEVERAL CYTOKINES IN ACTIVATED SPLEEN CELLS.

We determined the effect of IL-27 on the cytokine production in spleen cell cultures from LDLr$^{-/-}$ mice. A commonly used bioassay for IL-27 is its ability to inhibit IL-2 production by activated T cells within the spleen.[19] To that end, we used anti-CD3 and anti-CD28 activated T cells from the spleen. We observed a rapid decline in IL-2 expression when IL-27 was added and a low dose of IL-27 already optimally inhibited IL-2 production, confirming the results of Villarino *et al.*[19] (Figure 6.1 A).

IL-27 is recently associated with the regulation of Treg and Th17 cells.[11, 12] To study these T cell populations indirectly, we measured the production of IL-6 (involved in Th17 proliferation, figure 6.1 B), IL-17 (the product of Th17 cells, figure 6.1 C) and TGF-β (a product of Tregs, figure 6.1 D). IL-17 and TGF-β showed a similar pattern as IL-2, as they were already effectively inhibited by low doses of IL-27. IL-6 production displays a different kinetic response to IL-27, as only the highest concentration IL-27 (5 ng/ml) resulted in a significant decline in IL-6 production.

EXPRESSION OF P28 AND EBI3 IS UPREGULATED IN ATHEROSCLEROTIC PLAQUES

To determine whether the two subunits of IL-27 are induced in atherosclerotic lesions, we assessed the mRNA expression of the IL-27 subunits in the carotid artery. CD68 mRNA expression was performed to determine the macrophage content of the lesion.

First, the effect of Western-type diet feeding alone was studied to determine diet-induced effects on IL-27 expression in the arterial wall without atherosclerotic lesions. No effect of the Western-type diet on the expression of both subunits of IL-27 in the arterial wall was observed, indicating that IL-27 is not induced upon hypercholesterolemia in the healthy vessel wall (Figure 6.2 A). Secondly, we studied the expression of IL-27 in carotid artery lesions. CD68 expression increased after 2 weeks of collar placement, indicating the presence of macrophages in the carotid artery and thus of initial lesion formation (Figure 6.2 B). The expression of p28 and EBI3 was significantly increased at 2 and 4 weeks after the induction of lesion formation, indicating a possible role for IL-27 in the initiation of atherosclerosis (Figure 6.2 B). These data demonstrate that p28 and EBI3 were both upregulated at the same time, indicating that EBI3 can form a hetero dimer with p28 leading to secretion of functional IL-27.

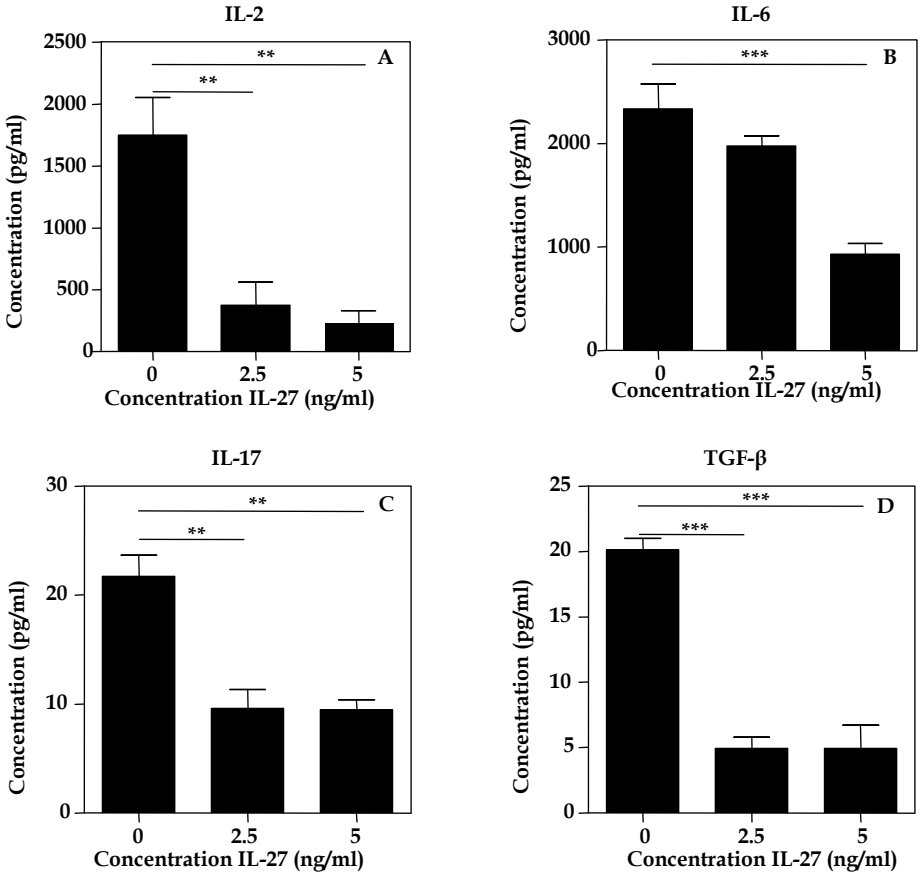

FIGURE 6.1: EFFECTS OF IL-27 ON CYTOKINE PRODUCTION OF ACTIVATED SPLEEN CELLS. Spleen cells were activated by plate-bound anti-CD3 and anti-CD28. Spleen cells were exposed to increasing levels of IL-27 and incubated for 48 hours. Supernatant was analyzed for the expression of IL-2 (A, N=3), IL-6 (B, N=4), IL-17 (C, N=4) and TGF-β (D, N=3). ***P*<0.01, ****P*<0.001

CONSTRUCTION OF THE PADRE-P28 VACCINE

Since both subunits of IL-27 are upregulated in early phases of atherosclerosis, we aimed to study the effect of IL-27 *in vivo*. Therefore we constructed a DNA vaccine against the p28 subunit in order to block the function of IL-27. The coding sequence of the p28 subunit was cloned into pcDNA3.1, an eukaryotic expression vector. The coding sequence was preceded by the sequence coding for a dominant Th cell epitope, PADRE, to break tolerance against the self-antigen p28.

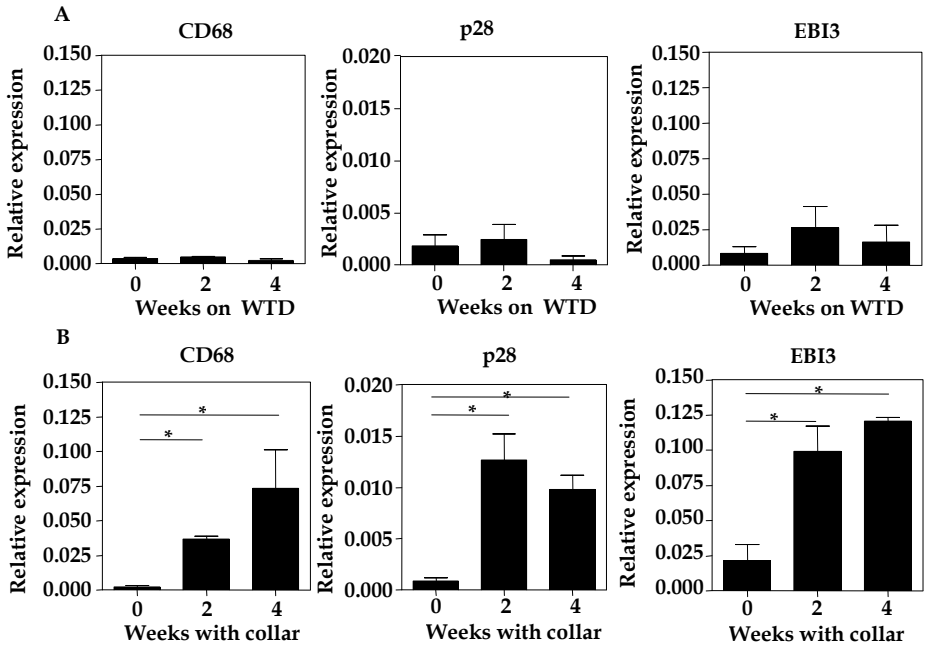

FIGURE 6.2: EXPRESSION OF P28 AND EBI3 IN COLLAR INDUCED ATHEROSCLEROSIS. LDLr$^{-/-}$ mice were fed a Western type diet (WTD) and at the indicate time points carotid artery was removed and analyzed for gene expression of the indicated genes (A, N=2-4). After two weeks of Western-type diet, collars were placed around both carotid arteries and diet feeding was continued. Mice were sacrificed at the indicated time points as indicated on x-axis. mRNA was isolated and subsequently reverse transcribed into cDNA and the expression of the indicated genes was analyzed by qPCR (B, N=2-4). All genes are depicted relative to HPRT. **P*<0.05

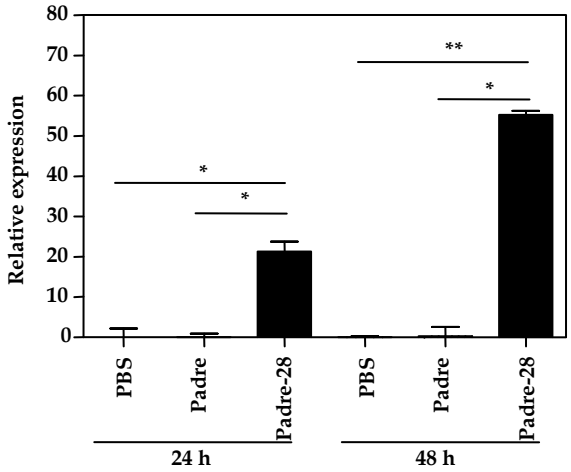

FIGURE 6.3: PADRE-P28 EXPRESSION IN COS7 CELLS. COS7 cells were transfected with PBS, pcDNA3.1-PADRE or pcDNA3.1-PADRE-p28 (N=3) and incubated 24 or 48 hour. mRNA was isolated and reverse transcribed into cDNA, subsequently, the expression of p28 was determined relative to HPRT. **P*<0.05, ***P*<0.01.

Expression of the vaccine was assessed *in vitro* by transfecting COS7 cells with the PADRE-p28 plasmid. Cells were harvested after 24 or 48 hours of incubation and total RNA was isolated. Expression was determined using qPCR. At 24 hours a significant increase in p28 expression is observed, which continued to augment until 48 hours after transfection, whereas the PBS and control transfected cells do not express p28 at the indicated time points (Figure 6.3)

VACCINATION AGAINST P28 AGGRAVATES ATHEROSCLEROSIS

We studied the effect of p28 vaccination on *de novo* plaque formation in male LDLr$^{-/-}$ mice using a DNA vaccination strategy. Mice were vaccinated three times *i.m.* at a two-week interval with the PADRE-p28 or control (same plasmid without the p28 encoding region). After the third vaccination, the LDLr$^{-/-}$ mice were put on a Western type diet (0.25% cholesterol) to induce hypercholesterolemia. Two weeks later this was followed by collar placement around the carotid arteries. To study the consequence of p28 vaccination on serum cholesterol levels, we determined serum cholesterol levels after vaccination, but we did not observe any difference between PADRE-p28 vaccinated mice and control vaccinated mice (data not shown).

Six weeks after collar placement mice were sacrificed and the plaque size in the carotid artery of control mice (Figure 6.4A) and p28 vaccinated mice (Figure 6.4B) was quantified. Vaccination against p28 resulted in a significant 74.57% increase in atherosclerotic lesion size compared to control vaccination (Figure 6.4C; 36,610±8,568 μm^2 (control vaccinated) versus 63,910±8,541 μm^2 (p28 vaccinated), P<0.05).

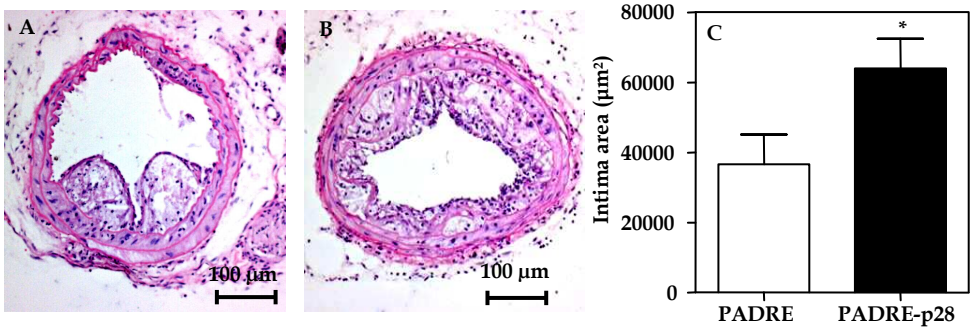

FIGURE 6.4: EFFECT OF P28 VACCINATION ON PLAQUE SIZE IN THE CAROTID ARTERY. LDLr$^{-/-}$ mice were vaccinated and 6 weeks after collar placement (8 weeks Western-type diet) the carotid arteries were removed. Plaque size was quantified in HE stained cryosections of the carotid artery in control mice (A, N=8) and in PADRE-p28 vaccinated mice (B, N=9) (C). *P<0.05

To study atherosclerotic lesion formation along the aorta, the aortas from control mice (Figure 6.5A) and vaccinated mice (Figure 6.5B) were dissected and stained *en face* for lipids. We observed a significant 69.18% increase in the atherosclerotic burden in the PADRE-p28 vaccinated mice (Figure 6.5 C: 8.89±1.19% (control vaccinated) versus 15.04±1.78% (p28 vaccinated); $P<0.05$). Furthermore, we studied the atherosclerotic lesion formation in the aortic root, and observed a non-significant 30% increase in lesion size after vaccination against p28 (Figure 6.6A-C, $P=0.08$).

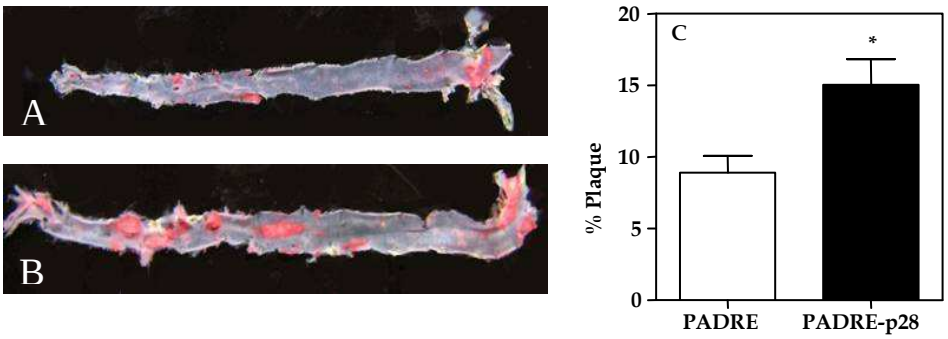

FIGURE 6.5: INCREASED ATHEROSCLEROTIC BURDEN IN THE AORTA IN P28 VACCINATED MICE. The aorta was removed after 8 weeks of Western type diet and stained with O-Red-O staining. The plaque (red staining) of PADRE vaccinated mice (A, N=7) and PADRE-p28 vaccinated mice (B, N=9) was quantified, subsequently the percentage of plaque was calculated(C). *$P<0.05$

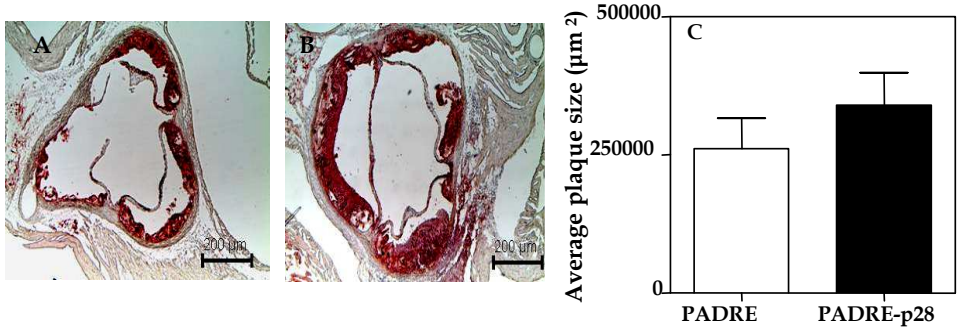

FIGURE 6.6: PLAQUE SIZE IN THE AORTIC ROOT UPON P28 VACCINATION. After 8 weeks of Western type diet the hearts were dissected and cryosections of the aortic root were made of the PADRE vaccinated mice (A, N=9) and the PADRE-p28 vaccinated mice (B, N=9) and subsequently stained for Oil-red-O and the lesions were quantified (C).

EFFECT OF VACCINATION AGAINST PADRE-P28 ON PLAQUE COMPOSITION

To study the effect of IL-27 blockade on plaque composition, we analyzed the collar induced plaques in the carotid artery for macrophage content. Vaccination against IL-27 did not result in a significant difference in relative macrophage

content in the p28 vaccinated group compared to the control group (Figure 6.7A-C). To assess whether IL-27 is involved in plaque stability, we studied the collagen content within the intima. Blocking of IL-27 did not result in a difference in relative collagen staining between the p28 vaccinated mice and control vaccinated mice (Figure 6.7D-F).

VACCINATION AGAINST P28 ALTERS THE BALANCE BETWEEN TH17 CELLS AND TREGS

Since IL-27 has an inhibiting effect on Treg development[12] and is also able to suppress the development of Th17 cells,[4, 10, 11] we analyzed these T cell populations within the spleen of p28 and control vaccinated mice. We observed a significant increase of 28.6% in IL-17 expressing cells within the $CD4^+CD62L^{high}$ cells in the spleen upon vaccination against p28 (Figure 6.8A: 21.51±1.58% versus 29.81±1.39% ; P<0.01). Next we studied the Tregs and in the PADRE-p28 vaccinated mice we observed a significant reduction of 18.5% in $Foxp3^+$ $CD4^+CD25^+$ cells within the spleen compared to control vaccinated mice (Figure 6.8A: 54.49±3.019% (control) versus 44.39±2.739% (p28 vaccinated); P<0.05).

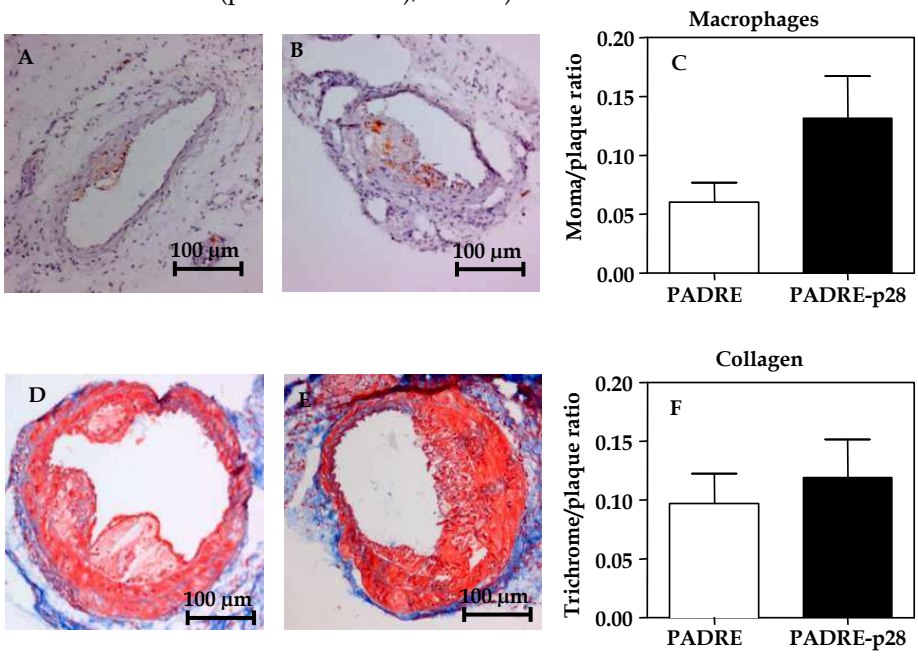

FIGURE 6.7: PLAQUE COMPOSITION IN THE CAROTID ARTERY IS NOT ALTERED UPON VACCINATION AGAINST P28. Cryosections were made of the carotid arteries after 8 weeks of Western type diet. PADRE vaccinated mice (A, N=8) and PADRE-p28 vaccinated mice (B, N=9) were stained with MoMa-2 and quantified (C). For the collagen detection the cryosections of PADRE vaccinated mice (D, N=8) and PADRE-p28 vaccinated mice (E, N=9) were stained with a Masson's trichrome staining and the relative collagen amount in the plaque was quantified(C).

B CELL POPULATION DECLINED WITHIN THE SPLEEN OF P28 VACCINATED MICE

Little is known about the effect of IL-27 on B cells, however there is some evidence that IL-27 plays a role in different stages of B cell development.[20] To study the effect of p28 vaccination on the B cell population, we stained the spleen cells for CD19. Vaccination against p28 resulted in a highly significant 20.1% decrease in the number of $CD19^+$ cells within the spleen compared to the control mice (Figure 6.8C: 37.55±0.28% versus 47.02±0.95%; $P<0.001$). Since the B cell population is declined in p28 vaccinated mice we determined the level of IgG auto-antibodies against oxLDL in the circulation. Despite the decline in B cells, there was no difference in the level of anti-OxLDL auto-antibodies (Figure 6.8D).

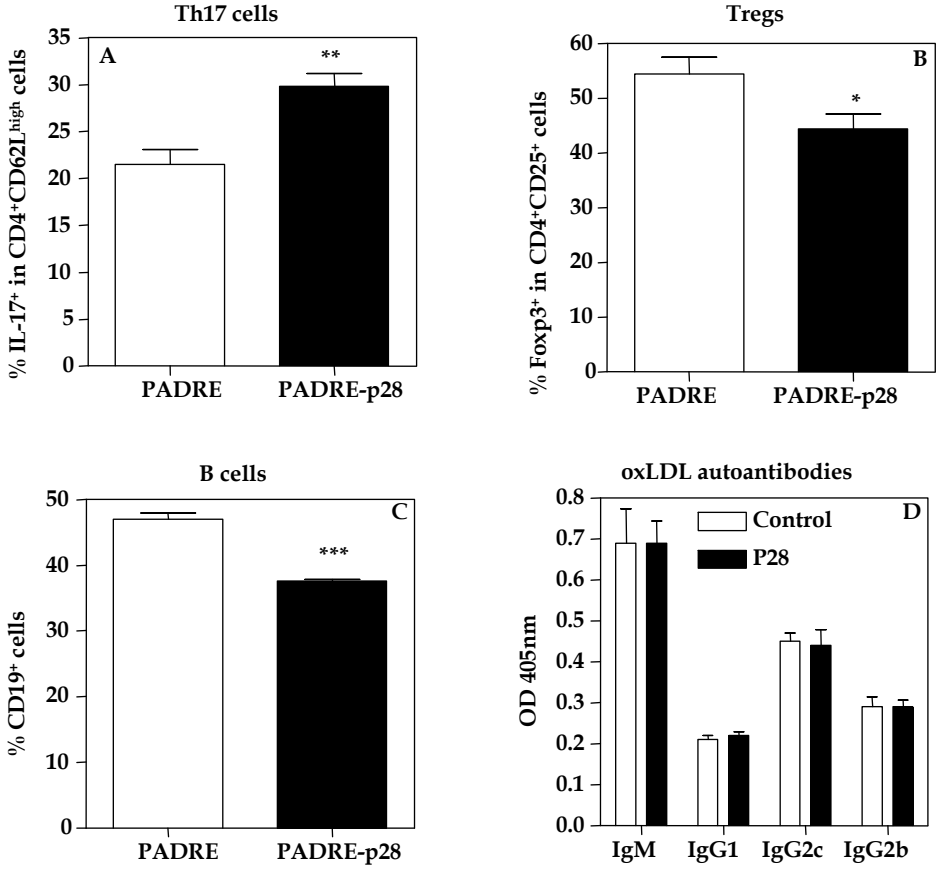

FIGURE 6.8: EFFECT OF P28 VACCINATION ON TH17 CELLS, REGULATORY T CELLS AND B CELLS. Spleens were removed after 8 weeks of Western type diet and a single cell suspension was made and subsequently stained for Th17 cells (A, N=6) , Treg cells (B, N=6) and B cells (C, N=6) and analyzed with a FACScalibur. The serum of control mice and p28 vaccinated mice was used for detection of antibodies directed against oxLDL. The autoantibody levels of IgM, IgG1, IgG2a and IgG2b were not changed in p28 vaccinated mice (D, N=9). *$P<0.05$, **$P<0.01$, ***$P<0.001$

DISCUSSION

In this study, we demonstrate that IL-27 acts as an anti-atherogenic cytokine during the initiation of atherosclerosis. Vaccination against the p28 subunit of IL-27 aggravates atherosclerosis and this effect may be explained by an altered balance between Th17 cells and Treg cells favoring the Th17 cells.

IL-27 has pleiotropic effects on the various T cell subsets. Therefore we assessed the effect of IL-27 on activated T cells within the spleen of LDLr$^{-/-}$ mice and studied the cytokine profile. A decrease in IL-2, IL-6, TGF-β and IL-17 production by activated spleen cells was observed *in vitro,* when exposed to IL-27. Decreased levels of IL-6, IL-2 and IL-17 may result in attenuated atherosclerosis as they are associated with aggravation of atherosclerosis [21,22] (van Es *et al.*, unpublished results) and this suggests an anti-atherosclerotic role for IL-27. On the other hand a reduced level of TGF-β as induced by IL-27 may suggest that IL-27 has a pro-atherosclerotic role as TGF-β has shown to be atheroprotective.[23-25] We therefore wanted to know what the *in vivo* effect of IL-27 on atherosclerosis was since, to our knowledge, IL-27 has not been studied in the context of atherosclerosis.

To determine whether IL-27 plays a role in atherosclerotic lesion development, expression of the separate sub-units of IL-27 was determined in the carotid artery. We observed an increase in p28 and EBI3 gene expression in initial atherosclerotic lesion in the carotid artery. This induction of both subunits at the same time indicates that EBI3 can form a heterodimer with p28 to facilitate its secretion.[4] The upregulation of p28 and EBI3 expression was accompanied by an increase in the expression of the macrophage marker (CD68), indicating an influx of macrophages into the intima and this suggests that macrophages may be responsible for the IL-27 expression.

The fact that IL-27 is upregulated in atherosclerotic lesions, led to an investigation of the role of IL-27 on atherosclerotic lesion development *in vivo.* Therefore, we developed a vaccine against p28 and vaccinated LDLr$^{-/-}$ mice. The DNA sequence of p28 was preceded by the DNA sequence of a specific immunodominant T-helper epitope to overcome T cell tolerance. In this study we used PADRE, which binds with high affinity to MHC class II and is able to activate helper T cells, thereby providing the necessary stimuli to break tolerance.[26]

During the experiment we analyzed blood samples for the presence of antibodies against p28. However we were not able to obtain a positive read-out. This may be due to the low concentrations of anti-p28 antibodies that may not exceed the threshold of the developed assay. Despite the lack of detection of antibodies directed towards p28, the vaccination against p28 resulted in a clear change in T cell populations. Since IL-27 has been shown to affect Th17 cells and Tregs[11, 12], we investigated whether these T cell populations were altered upon p28

vaccination. Vaccination against p28 resulted in a reduction in Treg cells within the spleen. Furthermore, we studied the Th17 cell population within the spleen of vaccinated mice and observed an increase in the Th17 cell population compared to the control group. An explanation may be that IL-27 is a strong inhibitor of TGF-β and IL-6, as we have demonstrated in this study *in vitro.* TGF-β is needed for both Th17 cells and Tregs, whereas IL-6 has a pivotal role in shifting the balance towards Th17 cells.[27, 28] Another explanation for the increased Th17 cell population may be that IL-27 is able to stimulate the expression of suppressor of cytokine signaling (SOCS) and thereby inhibit STAT3 activation, which is crucial for Th17 cells development.[29] More so, IL-27 can also directly inhibit STAT3 as described by Huber *et al.*[30, 31] Therefore it is likely that vaccination against p28 results in more Th17 cells and reduced numbers of Treg cells by increased STAT3 signaling and thereby shifting the balance towards Th17 cells.

Next we studied the effect of p28 vaccination in atherosclerosis. We observed a 74.5% increase in plaque size in the carotid artery of p28 vaccinated mice. Furthermore, we observed an increase of 70% in plaque burden in the aorta after p28 vaccination. The serum cholesterol level did not change in p28 vaccinated mice versus control treated mice. The increased plaque size is therefore likely due to changes in the inflammatory status of the vaccinated mice and is not related to serum cholesterol levels. These results demonstrate an anti-atherosclerotic function of IL-27.

This anti-atherosclerotic function of IL-27 is probably mediated by the altered balance between Treg and Th17 cells, as mentioned before. Tregs are associated with the protection against atherosclerosis[23,25,32-34] and therefore, the reduction in Treg cells within the spleen of p28 vaccinated mice may be an explanation for the aggravated atherosclerotic lesions.

Although the role of Th17 cells is not directly associated with atherosclerosis yet, the main product, IL-17, is associated with aggravated atherosclerosis (van Es *et al.* unpublished results). The suppressing effect of IL-27 on Th17 cells is further supported by observation of Stumhofer *et al.*, where they observed enhanced inflammation during chronic inflammation of the central nervous system in IL-27 receptor deficient mice, which was a result of increased Th17 cell population.[39]

The effect of vaccination against p28 on plaque composition was studied by determining the macrophage and collagen content. However, we did not observe any effect on relative macrophage content, indicating that IL-27 is not involved in the influx of macrophages into the lesion. Furthermore, the collagen content remained the same in the p28 vaccinated and control mice. This suggests that IL-27 does not affect plaque stability in this stage of atherosclerosis.

IL-27 also has an effect on B cells by promoting proliferation of naive cells, which express the IL-27 receptor.[35] Interestingly, we observe a 20% reduction in B cell content in the p28 vaccinated mice. IL-27 plays a role in the induction of T-bet expression and IgG2a class switching.[35] IgG2a (IgG2c in the case of C57BI/6 mice) class auto-antibodies are associated with aggravating atherosclerosis.[36] However, we did not detect any difference in auto-antibodies directed against oxLDL between the vaccinated and control mice, which indicates that p28 vaccination did not interfere with antibody class switching.

Recently, a novel property of IL-27 is described by several groups. They demonstrated that IL-27 is able to induce IL-10 in $CD4^+$ and $CD8^+$ T cells.[37-39] These new insights provide a new pathway that leads to the production of this key inhibitor of inflammation, which is demonstrated to be athero-protective.[40-43]

In conclusion, both subunits of IL-27 are upregulated in atherosclerotic lesions. We demonstrated, via vaccination against the p28 subunit of IL-27, that IL-27 has a protective role in atherosclerosis. Vaccination resulted in an increase of Th17 cells and a decrease in Treg cells, thereby, partly, explaining the aggravated atherosclerosis. Based on these results, overexpression or administration of IL-27 may be a potential therapeutic application to treat atherosclerosis in the future. However, based on the pleiotropic nature of IL-27, more research is necessary to unravel the IL-27 signaling mechanism and effects.

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Chapter 7

Induction of oral tolerance to HSP60 or an HSP60-peptide activates T cell regulation and reduces atherosclerosis

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ABSTRACT

HSP60-specific T cells contribute to the development of the immune responses in atherosclerosis. This can be dampened by regulatory T cells activated via oral tolerance induction and we explored the effect of oral tolerance induction to HSP60 and the peptide HSP60(253-268) on atherosclerosis.

HSP60 and HSP60(253-268) were administered orally to $LDLr^{-/-}$ mice prior to induction of atherosclerosis and resulted in a significant 80% reduction in plaque size in the carotid arteries and in a 27% reduction in plaque size at the aortic root. Reduction in plaque size correlated with an increase in $CD4^+CD25^+Foxp3^+$ regulatory T cells in several organs and in an increased expression of Foxp3, CD25 and CTLA-4 in atherosclerotic lesions of HSP60-treated mice. The production of IL-10 and TGF-β by lymph node cells in response to HSP60 was observed after tolerance induction.

Oral tolerance induction to HSP60 and a small HSP60-peptide leads to an increase in the number of $CD4^+CD25^+Foxp3^+$ regulatory T cells, resulting in a decrease in plaque size as a consequence of increased production of IL-10 and TGF-β. We conclude that these beneficial results of oral tolerance induction to HSP60 and HSP60(253-268) may provide new therapeutic approaches for the treatment of atherosclerosis.

INTRODUCTION

Heat shock proteins (HSPs) are a family of highly conserved proteins with various functions in normal and stressful situations. Expression of HSPs on endothelial cells and macrophages[1, 2] can be induced by fluid shear stress,[3] oxidized lipoproteins[4] and cytokines.[2] Under these circumstances, HSPs repair or prevent degradation of denaturated proteins and increase the cell's ability to survive stressful stimuli.[5, 6] HSPs such as HSP60 are also involved in inflammatory diseases, probably resulting from their raised expression in cells exposed to pro-inflammatory mediators.[7, 8] In human atherosclerotic lesions,[9] enhanced HSP60 expression has been detected. In addition, patients with atherosclerosis show an elevated concentration of HSP60-specific antibodies in serum,[2] and T cell clones with self-HSP60 reactivity have been detected within the atherosclerotic plaques.[10] This may be related to initial immune responses against bacterial HSPs which are highly homologous to HSPs in various other species including men, rats and mice.[11] HSP60-specific antibodies may contribute to endothelial damage and the inflammatory response in the vessel wall accelerating atherosclerosis.[12]

The autoimmune process in atherosclerosis is characterized by a T cell response to different autoantigens, e.g. oxidized LDL[13], glycoproteins[14] and HSPs[15]. HSP60-specific T cells are mainly of a Th1 phenotype, producing pro-atherogenic cytokines, such as IFN-γ, IL-12 and TNF-α and causing a disturbed balance between Th1 and Th2 cytokines.[16, 17] For a long time, this disturbed balance was regarded as the cause of the ongoing inflammation in atherosclerosis. Recent publications however suggest that regulatory T cells (Tregs) play an important role in prevention of Th1 mediated autoimmune diseases such as multiple sclerosis,[18] diabetes mellitus[19] and atherosclerosis.[20] Mallat et al. hypothesized that in atherosclerosis an imbalance exists between pathogenic T cells (Th1 and Th2) and Tregs specific for 'altered' self and non-self antigens (e.g. oxidized phospholipids, heat shock proteins).[21]

One way to increase the number of antigen specific Tregs is "low dose" oral tolerance induction. This method is already used as a treatment in animal models for Th1 mediated autoimmune diseases such as multiple sclerosis,[22] rheumatoid arthritis[23] and type I diabetes.[24] Initial studies also show that oral tolerance induction to β2-glycoprotein I[25] and HSP65[26, 27] results in the suppression of early atherosclerosis. However, these studies do not show the involvement of Tregs. We describe in a recent study an increase in the number of $CD4^+CD25^+Foxp3^+$ cells after oral tolerance induction to oxidized LDL (oxLDL)[28] and a subsequent reduction in plaque size. These $CD4^+CD25^+Foxp3^+$ cells form a class of Tregs that may either be natural Tregs which act via cell-cell contact via

surface-bound TGF-β[29] or cytotoxic T lymphocyte-associated antigen-4 (CTLA-4)[30] or adaptive Tregs operating via the secretion of TGF-β.[31]

The present study shows that induction of oral tolerance to HSP60 and a highly conserved sequence of HSP60 (HSP60(253-268)) attenuates atherosclerosis. The effect on atherosclerosis is explained by an increased number of $CD4^+CD25^+Foxp3^+$ Tregs in both lymphoid organs and the atherosclerotic lesion. This is accompanied by an increase in HSP60-specific TGF-β and IL-10 production in mesenteric lymph node cells.

METHODS

ANIMALS

All animal work was approved by the regulatory authority of Leiden University and carried out in compliance with the Dutch government guidelines. Male $LDLr^{-/-}$ mice were obtained from the Jacksons Laboratory. Mice were kept under standard laboratory conditions and were fed a normal chow diet or a 'Western-type' diet containing 0.25% cholesterol and 15% cocoa butter (Special Diet Services, Witham, Essex, UK). All mice used were 10-12 weeks of age. Diet and water were administered *ad libitum*.

ANTIGENS AND ADJUVANT

Dimethyl dioctadecyl ammonium bromide (DDA; Sigma Diagnostics, MO), used as adjuvant, was dissolved in phosphate buffered saline (PBS) and 100 μg was mixed with 100 μg of the antigen (HSP60, HSP60(253-268) or HSP70(111-125)) before immunization. Purified recombinant HSP60 of *Mycobacterium bovis* bacillus Calmette-Guérin was kindly provided by J.D.A. van Embden (National Institute of Public Health and Environmental Hygiene, Bilthoven, The Netherlands). HSP60(253-268) based on the sequence of mycobacterial HSP60 aa 253-268 (NH_2-EGEALSTLVVNKIRGT-amide), was made by regular peptide synthesis (FMOC protection). Another peptide HSP70(111-125) was based on a partially conserved (human, rat, mouse) sequence of the HSP70 peptide aa 111-125 (NH_2-ITDAVITTPAYFNDA-amide).[32]

IMMUNIZATIONS

LDLr$^{-/-}$ mice were immunized via one *i.p.* injection with PBS or 100 µg of HSP60, HSP60(253-268) or HSP70(111-125). The antigens were dissolved in 200 µl of PBS containing 100 µg DDA. After 14 days the spleens were dissected and used in the proliferation assay described below.

SPLEEN CELL PROLIFERATION ASSAY

Spleens from either naive (n=3), immunized (n=3) or oral treated mice which were immunized subsequently (n=12 per group) were dissected and squeezed through a 70 µm cell strainer (Falcon, The Netherlands). The erythrocytes were eliminated by incubating the cells with erythrocyte lysis buffer (0.15 M NH_4Cl, 10 mM $NaHCO_3$, 0.1 mM EDTA, pH 7.3). The splenocytes were cultured for 48 hours in triplicate at $2{\cdot}10^5$ cells per well of a 96-wells round-bottom plate in the presence or absence of different concentrations of HSP60, HSP60(253-268) or HSP70(111-125). RPMI 1640 (with L-Glutamine, 10% fetal calf serum (FCS), 100 U/ml penicillin, and 100 µg/ml streptomycin (all from BioWhittaker Europe)) was used as culture medium. Concanavalin A (Con A; Sigma-Diagnostics, MO) (2 µg/ml) was used as a positive control. Cultures were pulsed for an additional 16 hours with [6-^{3}H]-thymidine (1 µCi/well, sp. act. 24 Ci/mmol; Amersham Biosciences, The Netherlands). The amount of [6-^{3}H]-thymidine incorporation was measured using a liquid scintillation analyzer (Tri-Carb 2900R). The magnitude of the proliferative response is expressed as stimulation index (SI) defined as the ratio of the mean counts per minute of triplicate cultures with antigen to the mean counts per minute in culture medium without antigen.

INDUCTION OF ATHEROSCLEROSIS

To determine the effect of oral tolerance induction on the initiation of atherosclerosis, atherosclerosis was induced in LDLr$^{-/-}$ mice. The mice were put on a Western-type diet three weeks prior to surgery. Atherosclerosis was induced by placement of perivascular collars, prepared from elastic tubing (0.3 mm inside diameter; Dow Corning, Midland, Michigan), around both carotid arteries (method described by von der Thüsen et al.[33]). During the experiment, the diet response was followed by measuring the cholesterol and triglyceride levels in serum of these mice. Total cholesterol levels were quantified spectrophotometrically using an enzymatic procedure (Roche Diagnostics, Germany). Precipath standardized serum (Boehringer, Germany) was used as an internal standard.

ORAL TOLERANCE INDUCTION

After one week of Western-type diet and two weeks prior to collar placement, the LDLr$^{-/-}$ mice were treated 4 times over a period of 8 days with intragastrically administered antigens. Before each intragastrical administration, the animals were deprived of food but not water for 16 hours. To prevent degradation of the administered antigen, 2 mg of soybean trypsin inhibitor (STI, Sigma-Diagnostics, MO) was administered intragastrically. Ten minutes after the STI administration, the control group received 100 µl of PBS (n=7). The other mice received 30 µg of HSP70(111-125) (n=6), HSP60 (n=6) or HSP60(253-268) (n=7). All antigens and STI were diluted and dissolved in physiological saline (0.9% NaCl) prior to injection. After administering the antigens intragastrically, the mice were kept on Western-type diet for another week before collars were placed.

PLAQUE ANALYSIS

Six weeks after collar placement the mice were euthanized and exsanguinated by femoral artery transsection. The mice were perfused and fixated through the left cardiac ventricle with PBS and FormalFixx (Thermo Shandon, Pittsburgh, PA) for 30 min. Common carotid arteries and the heart with the aortic root were removed for analysis as described by von der Thüsen et al.[33] The arteries were embedded in OCT compound (TissueTek; Sakura Finetek, The Netherlands) and proximally of the place of collar occlusion 5 µm sections were made on a Leica CM 3050S Cryostat (Leica Instruments, UK). These cryosections were stained with hematoxylin (Sigma Diagnostics, MO) and eosin (Merck Diagnostica, Germany). 10 µm section were made of the aortic root and these sections were stained with Oil-red-O and hematoxylin. Plaque areas and intima/lumen ratios were measured using a Leica DM-RE microscope and LeicaQwin software (Leica Imaging Systems, UK).

SPLENOCYTE PROLIFERATION AFTER TOLERANCE INDUCTION

To test the effect of tolerance induction to HSP60 on the proliferation of splenocytes, mice were treated orally with PBS or HSP60 as described. Subsequently, all mice were immunized with HSP60 and two weeks thereafter, splenocytes of these mice were re-stimulated in vitro with HSP60. The proliferation was measured as described above.

FLOW CYTOMETRIC ANALYSIS

For the detection of $CD4^+CD25^+Foxp3^+$ T cells, a three color flow cytometry was performed. 4 and 14 days after oral treatment with HSP60, spleen, mesenteric lymph nodes, Peyer's patches, and blood were isolated from HSP60-treated and untreated mice (n=5). Mononuclear cells were isolated using Lympholyte (Cedarlane, Ontario, Canada) conform the manufacturers protocol. Cells were subsequently stained with FITC-conjugated anti-CD4 (0.125 µg/sample) and APC-conjugated anti-CD25 (0.06 µg/sample) mAb (eBioscience, Belgium) for 30 min. Cells were then fixed and permeabilized for 16 hrs with Fixation/Permeabilization solution according to the suggested protocol (eBioscience, Belgium). Subsequently, the cells were stained with PE-conjugated anti-Foxp3 (0.2 µg/sample) (eBioscience, Belgium) for 30 min. Cells were analyzed immediately by flow cytometry on a FACSCalibur. All data were analyzed with CELLQuest software (BD Biosciences, The Netherlands).

CYTOKINE ASSAYS

Mesenteric lymph nodes were isolated from untreated and HSP60-treated mice (n=5) 14 days after oral treatment with HSP60. The lymph nodes were squeezed through a cell strainer and the cells were cultured at $1 \cdot 10^6$ cells per well of a 24-wells plate in the presence or absence of 20 µg/ml HSP60. Culture supernatants were harvested after 48 hours of incubation. IL-10, IFN-γ (both from eBioscience, Belgium) and TGF-β (Bender MedSystems, Austria) concentrations were determined by enzyme-linked immunosorbent assays (ELISA) according to the manufacturers suggestions.

REAL-TIME PCR ASSAYS

Carotid arteries from control and HSP60-treated mice were isolated and mRNA was extracted using the guanidium isothiocyanate (GTC) method and reverse transcribed (RevertAid M-MuLV reverse transcriptase). Quantitative gene expression analysis was performed on an ABI PRISM 7700 sequence detector (Applied Biosystems, CA) using SYBR green technology. Primer pairs as described in table 8.1 were used to quantify Foxp3, CD25, CTLA4 gene expression. Acidic ribosomal phosphoprotein PO (36B4) was used as the endogenous reference gene.

TABLE 7.1: SEQUENCE OF PRIMERS. Primer sequences for the genes analyzed with qPCR.

GENE	FORWARD PRIMER	REVERSE PRIMER
FOXP3	5′-GGAGCCGCAAGCTAAAAGC-3′	5′-TGCCTTCGTGCCCACTGT-3′
CD25	5′-CTTATATTGCAAATGTGGCACAATC-3′	5′-ATCAATCATCAGTGGGACAATCTG-3′
CTLA4	5′-CGAGGTCCTGCACCAACTG-3′	5′-TCCATCACCATCGGTTTATGC-3′
36B4	5′-GGACCCGAGAAGACCTCCTT-3′	5′-GCACATCACTCAGAATTTCAATGG-3′

DETECTION OF ANTI-HSP60 ANTIBODIES

HSP60 (10 µg/ml) dissolved in a $NaHCO_3/Na_2CO_3$ buffer (pH 9.0) was coated. Measurement of IgG1, IgG2a and IgM levels in serum was performed using an ELISA Ig detection kit (Zymed Laboratories, CA) conform the manufacturer's protocol and appropriate controls were performed.

STATISTICAL ANALYSIS

All data are expressed as mean ± SEM. The Mann-Whitney test was used to compare the data. When required an ANOVA test (Kruskal-Wallis test with post-test) was used. *P*-values less than 0.05 are considered to be statistically significant.

RESULTS

T CELLS SPECIFIC FOR HSP60 AND HSP60(253-268) EPITOPES ARE PRESENT IN LDLR$^{-/-}$ MICE

Because of the important role of HSP60-specific T cells in atherosclerosis, we first investigated the presence of T cells specific for HSP60, HSP60(253-268) or HSP70(111-125) epitopes in the LDLr$^{-/-}$ mice. HSP60 is homologue in bacteria, mice, rats and human and therefore mycobacterial HSP60 was used in this study. The peptide (HSP60(253-268)) was used because this peptide is present in all species. Splenocytes were isolated out of naive LDLr$^{-/-}$ mice and were incubated with several concentrations of the HSP epitopes. Incubation with 5 µg/ml HSP60 or HSP60(253-268) had no effect on naive splenocytes while incubation with 20 µg/ml HSP60 or HSP60(253-268) resulted in a 2.70±0.42 and 2.04±0.35 fold increase in proliferation, respectively (Figure 7.1A and B; $P<0.05$). HSP70(111-125) did not stimulate proliferation of the splenocytes (data not shown). In all experiments 2 µg/ml ConA, a general pan T cell mitogen, was used as a positive control, which resulted in a more than 50-fold increase in proliferation (data not shown).

To determine whether the T cell response to HSP-epitopes can be induced *in vivo* we immunized LDLr$^{-/-}$ mice by an intraperitoneal injection of 100 µg of HSP60, HSP60(253-268) or HSP70(111-125) using DDA as adjuvant. After two weeks mice were killed, and isolated splenocytes from HSP60-immunized mice incubated with 5 and 20 µg/ml of HSP60 showed a 7.40±1.29 (P<0.05) and 12.71±2.30 (P<0.05) fold increase in proliferation, respectively (Figure 7.1C). Incubation of splenocytes from HSP60(253-268)-immunized mice with 5 and 20 µg/ml HSP60(253-268) resulted in a 7.29±2.32 (P<0.05) and 9.26±2.58 (P<0.05) fold increase, respectively (Figure 7.1D). Incubation of splenocytes from HSP70(111-125)-immunized mice with HSP70(111-125) did not result in a significant effect on proliferation (Figure 7.1E).

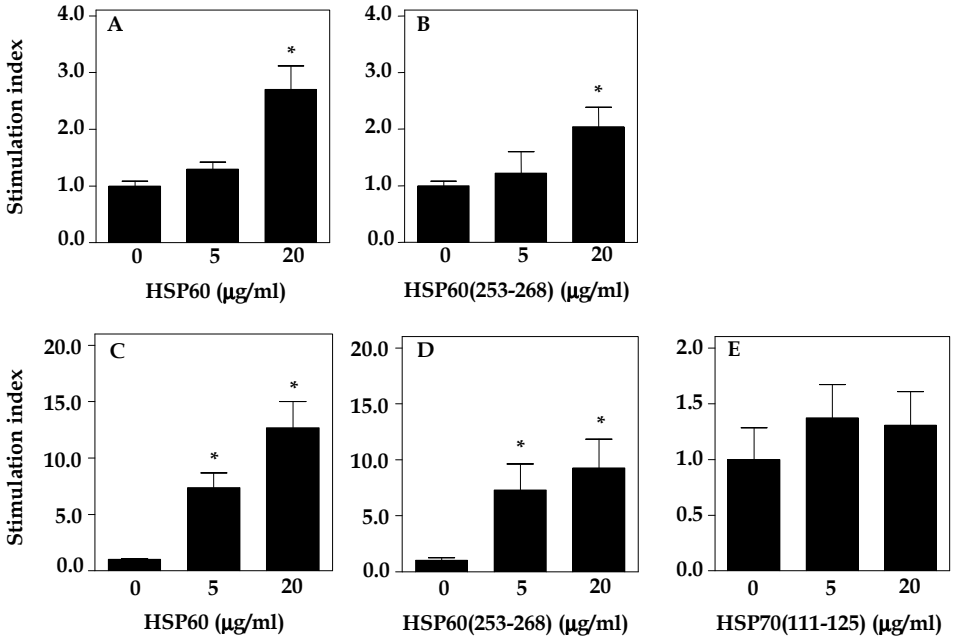

FIGURE 7.1: SPLEEN CELL PROLIFERATION IN RESPONSE TO HSP60 AND HSP60(253-268).
Splenocytes were isolated from naïve LDLr-/- mice (A and B) and mice immunized via one i.p. injection with 100 µg of HSP60, HSP60(253-268) or HSP70(111-125) (C,D and E). The naive and primed splenocytes were re-stimulated in vitro with HSP60 (A and C), HSP60(253-268) (B and D) or HSP70(111-125) (E) for 48 hours. Proliferation was measured by incorporation of 3H-thymidine. Data are shown as the stimulation index (S.I.) ± SEM. The S.I. is the ratio of the mean cpm of cultures with antigen to the mean cpm of cultures without antigen. *P<0.05.

EFFECT OF ORAL TOLERANCE INDUCTION TO HSP60, HSP60(253-268) AND HSP70(111-125) ON ATHEROSCLEROSIS

Next we investigated the immunomodulatory effect of oral tolerance induction to these compounds on atherosclerosis. LDLr$^{-/-}$ mice were put on a Western-type diet for one week prior to oral administration of PBS (n=7) or 30 µg of HSP60 (n=7), HSP60(253-268) (n=6) or HSP70(111-125) (n=6). The oral treatment was given 4 times in total, every other day. Subsequently, mice were equipped with collars around both common carotid arteries and fed a Western-type diet. Six weeks thereafter, atherosclerotic plaque formation was analyzed. Representative hematoxylin-eosin stained cryosections of the carotid arteries of PBS, HSP70(111-125), HSP60, and HSP60(253-268)-treated mice are shown in figure 7.2A-D.

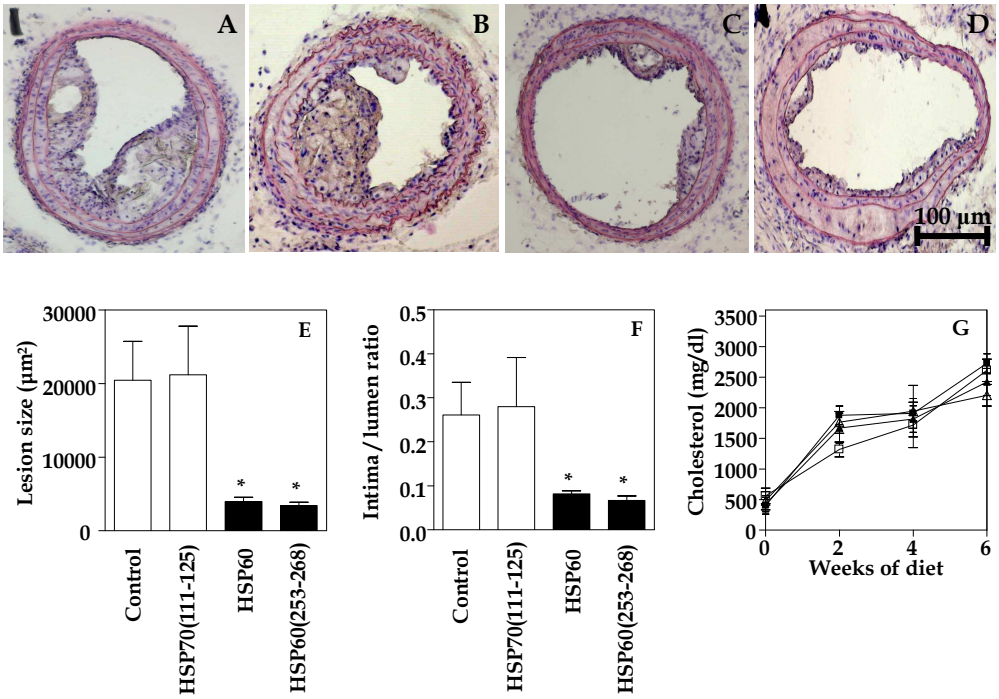

FIGURE 7.2: ORAL TOLERANCE INDUCTION TO HSP60 AND HSP60(253-268) ATTENUATES PLAQUE FORMATION IN COLLAR INDUCED ATHEROSCLEROSIS IN LDLR-/- MICE.
LDLr-/- mice were treated intragastrically with PBS, HSP70(111-125), HSP60 or HSP60(253-268) four times before induction of atherosclerosis and six weeks thereafter mice were sacrificed and the carotid arteries of PBS-treated (A), HSP70(111-125)-treated (B), HSP60-treated (C) and HSP60(253-268)-treated (D) mice were sectioned and stained with hematoxylin-eosin. Lesions were quantified by computer-assisted morphometric analysis and plaque size (E) and intima/lumen ratio (F) were determined. During the experiment plasma cholesterol levels of PBS-treated (closed squares), HSP70(111-125)-treated (closed triangles), HSP60-treated (open squares) and HSP60(253-268)-treated (open triangles) mice were monitored (G). *P<0.05

No significant difference in plaque size was observed in mice fed HSP70(111-125) (21181±5273 μm²) compared to PBS-treated mice (20471±5273 μm²). Oral administration of HSP60 (3959±582 μm²) resulted in a significant 80.7% (P<0.05) reduction in plaque size when compared to PBS-treated mice. Oral tolerance induction to HSP60(253-268) (3419±460 μm²) resulted in an 83.3% (P<0.05) reduction in plaque size (Figure 7.2E).

Furthermore, the intima/lumen ratio was reduced significantly with 68.8% in the HSP60 treated mice (P<0.05; 0.082±0.007) and with 74.3% in the HSP60(253-268)-treated mice (P<0.05; 0.067±0.010) when compared to the PBS-treated mice (0.261±0.074) (Figure 7.2F). During the experiment no significant differences in total plasma cholesterol levels were detected between the different groups (Figure 7.2G). In addition, a 27.4% reduction in plaque size at the aortic root was observed in HSP60-treated mice (377000±37200 μm²) when compared with PBS-treated mice (Figure 7.3 A-C; 519000±44600 μm²; P<0.05). Immunohistochemical analysis of all plaques showed that oral tolerance induction to HSP60 and HSP60(253-268) had no effect on the relative macrophage and smooth muscle cell content (data not shown).

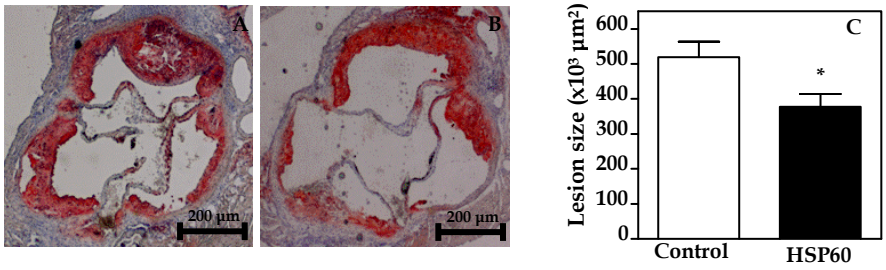

FIGURE 7.3: ORAL TOLERANCE INDUCTION TO HSP60 REDUCES PLAQUE FORMATION AT THE AORTIC ROOT IN LDLR$^{-/-}$ MICE.
LDLr$^{-/-}$ mice were fed a Western-type diet and were treated intragastrically four times with PBS or HSP60 as in figure 7.2. After 8 weeks, sections of the aortic root of PBS-treated (A) and HSP60-treated (B) mice were stained with Oil-red-O and hematoxylin and subsequently lesions were quantified and plaque size was determined (C). Values are mean lesion size ± SEM. *P<0.05

EFFECT OF ORAL TOLERANCE INDUCTION TO HSP60 ON CD4$^+$CD25$^+$FOXP3$^+$ TREGS

To evaluate whether oral tolerance induction to HSP60 was associated with an effect on Tregs, flow cytometry analysis was performed. HSP60-treated LDLr$^{-/-}$ mice were sacrificed 4 and 14 days after the last oral treatment. In untreated control mice, CD4$^+$CD25$^+$Foxp3$^+$ T cells are present in low numbers in Peyer's patches (0.79±0.16%), blood (2.21±0.12%), spleen (0.80±0.07%) and mesenteric lymph nodes (3.82±0.25%).

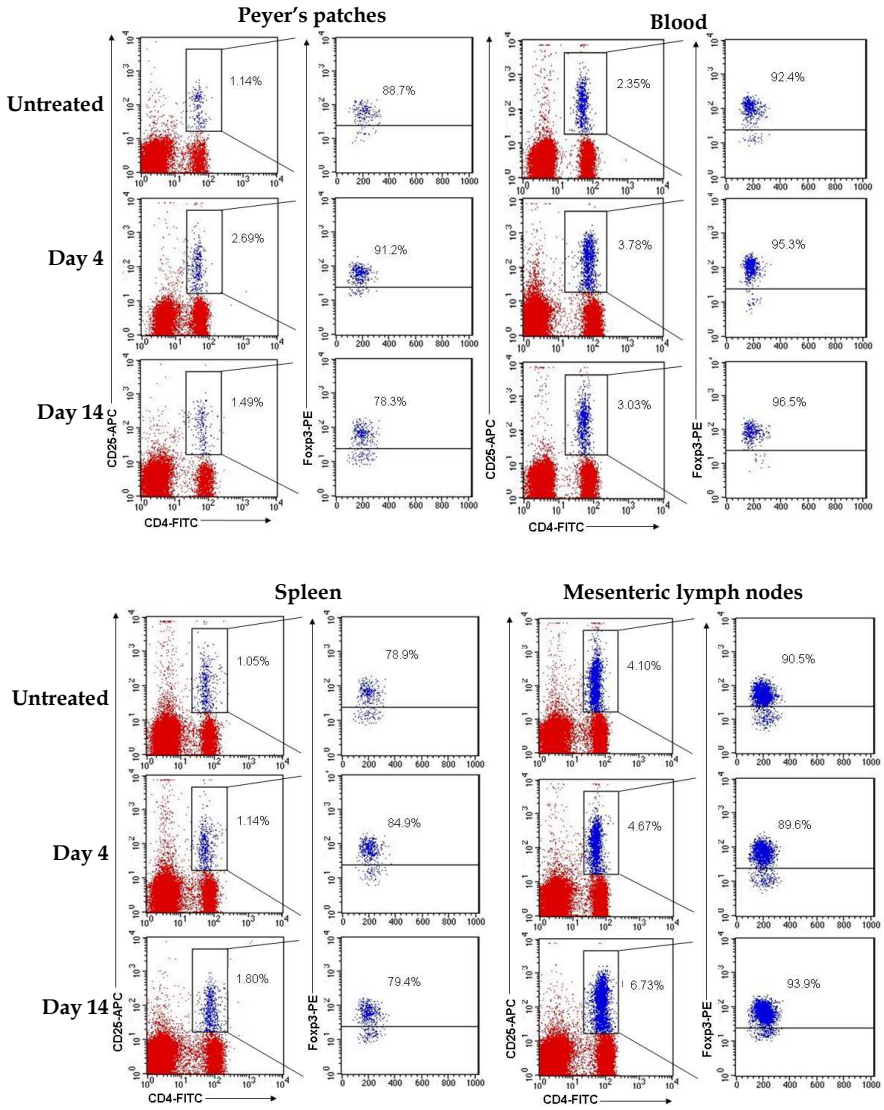

FIGURE 7.4: ORAL TOLERANCE INDUCTION TO HSP60 LEADS TO AN INCREASED AMOUNT OF CD4⁺CD25⁺FOXP3⁺ CELLS.

LDLr-/- mice were fed HSP60 four times and killed 4 and 14 days after oral treatment. As a control, untreated animals were used. The dot plots show representative examples of lymphoid cells isolated from Peyer's patches, blood, spleen and mesenteric lymph nodes stained for CD4 and CD25 (left panel). The right panels show the percentage of Foxp3⁺ cells within the CD4⁺CD25⁺ population.

The dot-plots in figure 7.4 represent examples of FACS analysis on $CD4^+CD25^+$ cells (left panels) and $Foxp3^+$ cells within the $CD4^+CD25^+$ population (right panels) in Peyer's patches, blood, spleen and mesenteric lymph nodes, respectively. 4 days after oral treatment with HSP60, the number of $CD4^+CD25^+Foxp3^+$ T cells in the Peyer's patches and blood was increased significantly to 1.73±0.30% (P<0.05) and 2.86±0.21% (P<0.01), respectively, when compared to untreated mice. No significant change was seen in the spleen (0.85±0.06%) and mesenteric lymph nodes (4.67±0.41%). 14 days after oral treatment, the number of $CD4^+CD25^+Foxp3^+$ T cells decreased again to 1.07±0.08% in the Peyer's patches and was not significantly different from untreated mice whereas the number of $CD4^+CD25^+Foxp3^+$ T cells in blood was still enhanced (2.81±0.20%, P<0.01). In the spleen and mesenteric lymph nodes a significant increase to 1.24±0.11% (P<0.01) and 5.36±0.10% (P<0.01) was observed when compared with the situation without treatment (Figure 7.5).

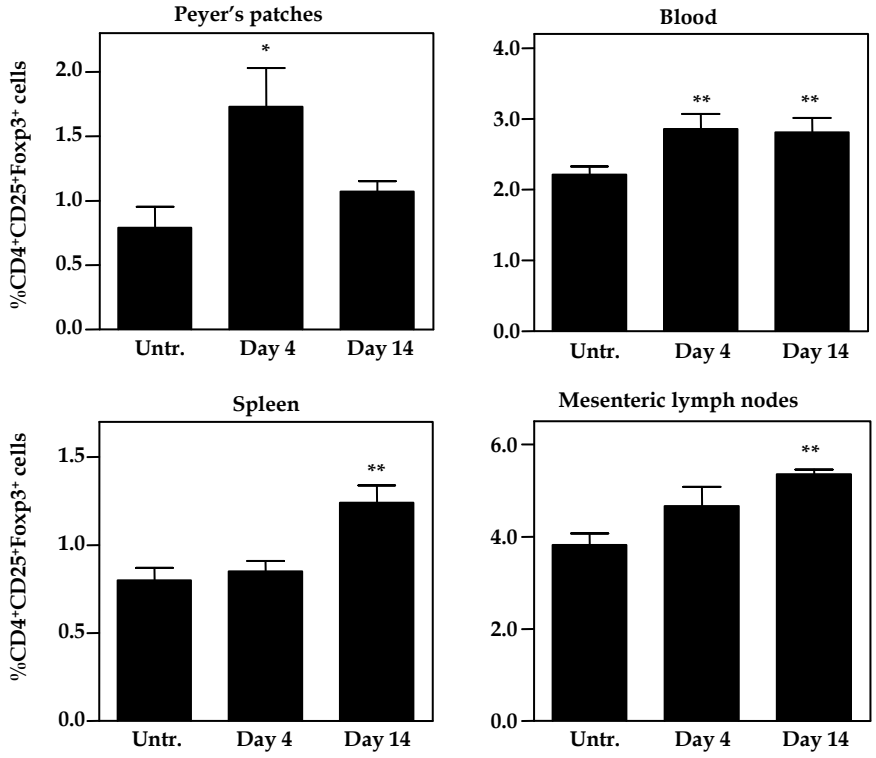

FIGURE 7.5: ORAL TOLERANCE INDUCTION TO HSP60 LEADS TO AN INCREASED AMOUNT OF $CD4^+CD25^+FOXP3^+$ CELLS.

$LDLr^{-/-}$ mice were treated intragastrically four times with HSP60 and killed 4 and 14 days after the last oral treatment. As a control, untreated animals were used. The graphs represent the amount of $CD4^+CD25^+Foxp3^+$ cells in the different organs measured via FACS analysis (mean±SEM). *P<0.05, **P<0.01

REGULATORY T CELL MARKERS IN ATHEROSCLEROTIC PLAQUES

After tolerance induction to HSP60 and the induction of atherosclerosis, carotid arteries were dissected and mRNA was isolated. Subsequently, the expression of different Treg markers (CD25, CTLA-4 and Foxp3) in the atherosclerotic plaques in the carotid arteries was determined. After oral treatment with HSP60 (n=5) and 8 weeks of Western-type diet feeding, the mRNA expression of CD25, CTLA-4 and Foxp3 was significantly upregulated in the atherosclerotic plaque when compared with control mice (n=9). CD25 showed a 4.9-fold increase (P<0.05), CTLA-4 a 4.1-fold increase (P=0.068) and Foxp3 a 6.4-fold increase (P<0.05) (Figure 7.6).

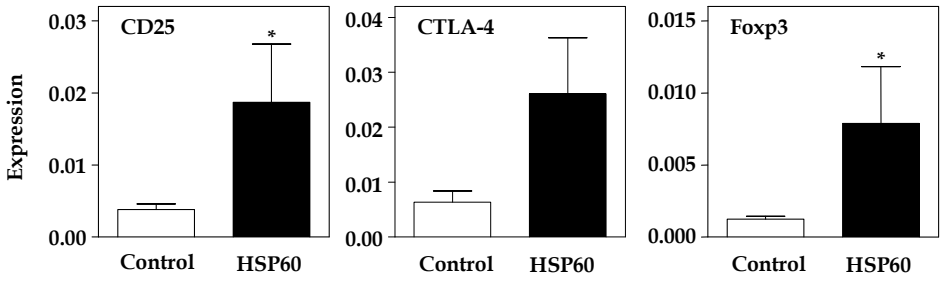

FIGURE 7.6: INCREASED EXPRESSION OF TREG MARKERS IS OBSERVED WITHIN LESIONS OF HSP60-TREATED LDLR$^{-/-}$ MICE.
To investigate the presence of Tregs within atherosclerotic lesions, mRNA was isolated from carotid arteries of PBS (n=9) and HSP60-treated (n=5) mice and the mRNA expression of CD25, CTLA-4 and Foxp3 was quantitatively determined and expressed relative to 36B4. *P<0.05

EFFECT OF TOLERANCE INDUCTION ON CYTOKINE PRODUCTION

Furthermore, we investigated whether the increased number of $CD4^+CD25^+Foxp3^+$ T cells also demonstrated a change in the production of cytokines in response to stimulation with HSP60. Mesenteric lymph node cells, isolated 14 days after the oral treatment with HSP60, were stimulated *in vitro* in presence or absence of 20 µg/ml of HSP60. Incubation with HSP60 resulted in a significant larger production of TGF-β (1.86±0.22 versus 0.93±0.15 ng/ml; P<0.05) and IL-10 (19.52±5.51 versus 6.41±1.72 pg/ml; P<0.05) when compared with mesenteric lymph node cells cultured without HSP60 (Figure 7.7A-B). Furthermore, HSP60-stimulated mesenteric lymph node cells isolated from HSP60-treated mice (14 days after treatment) produced significantly more TGF-β than HSP60-stimulated mesenteric lymph node cells isolated from untreated mice (1.86±0.22 ng/ml versus 0.96±0.22 ng/ml; P<0.05; data not shown). In all cases IFN-γ levels were below the detection threshold.

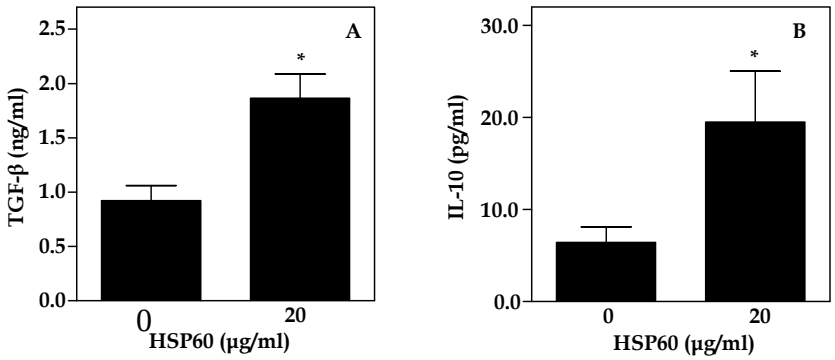

FIGURE 7.7: ORAL TOLERANCE INDUCTION TO HSP60 INDUCES ANTI-ATHEROGENIC CYTOKINE PRODUCTION BY MESENTERIC LYMPH NODE CELLS.
LDLr$^{-/-}$ mice were treated intragastrically four times with HSP60. 14 days after the treatment, mesenteric lymph nodes were isolated from HSP60-treated mice and the lymphocytes were cultured in vitro with or without HSP60 for 48 hours. The production of TGF-β (A) and IL-10 (B) was monitored using ELISA. Data are mean±SEM. *P<0.05.

ORAL TOLERANCE INDUCTION TO HSP60 REDUCES THE PROLIFERATIVE RESPONSE OF SPLENOCYTES TO HSP60

To determine the effect of tolerance induction to HSP60 on the HSP60-specific proliferation, LDLr$^{-/-}$ mice were treated orally with PBS or HSP60. After oral treatment all mice were immunized with HSP60 and two weeks later splenocytes were isolated and cultured with or without 5 and 20 µg/ml of HSP60. Splenocytes from PBS-treated mice respond to HSP60 with an increased proliferation; a stimulation index of 4.4±0.7 and 10.4±2.5 when incubated with 5 and 20 µg/ml of HSP60, respectively. Mice orally treated with HSP60 showed a 56.8% and 68.2% reduction in the proliferative response to 5 and 20 µg/ml of HSP60, respectively (Figure 7.8; 1.9±0.2 and 3.3±0.4; *P*<0.05).

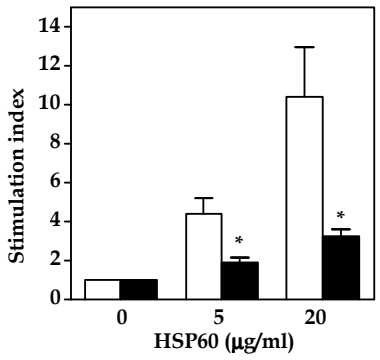

FIGURE 7.8: ORAL TOLERANCE INDUCTION TO HSP60 REDUCES THE PROLIFERATIVE RESPONSE OF SPLENOCYTES TO HSP60.
LDLr$^{-/-}$ mice were treated 4 times intragastrically with PBS or HSP60. Subsequently, all mice were immunized with HSP60 and after two weeks the mice were sacrificed and splenocytes were isolated. Splenocytes of PBS-treated mice (white bars) and HSP60-treated mice (black bars) were cultured with or without 5 and 20 µg/ml of HSP60. The extent of proliferation is shown as stimulation index after incorporation of 3H-thymidine. Values are mean±SEM. *P<0.05

EFFECT OF ORAL TOLERANCE TO HSP60 ON HSP60-SPECIFIC ANTIBODIES

After oral treatment with HSP60 and the induction of atherosclerosis, HSP60-specific IgG1, IgG2a and IgM levels in serum were determined. No detectable differences in HSP60-specific IgG1, IgG2a and IgM levels were observed (Figure 7.9)

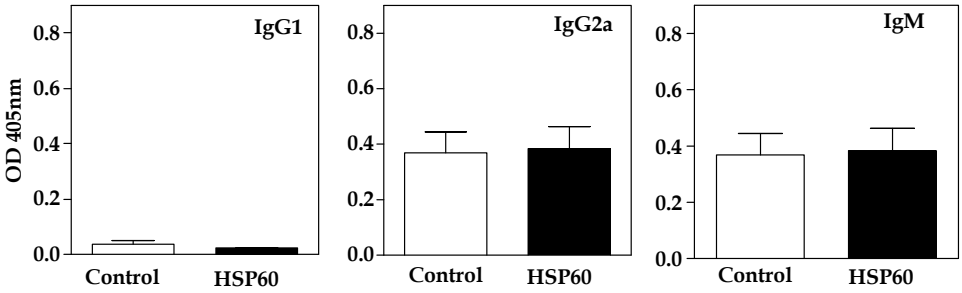

FIGURE 7.9: EFFECT OF TOLERANCE INDUCTION TO HSP60 ON HSP60-SPECIFIC ANTIBODY-LEVELS.
LDLr$^{-/-}$ mice in which atherosclerosis was induced by a combination of Western-type diet feeding and collar placement around both carotid arteries were treated 4 times intragastrically with PBS or HSP60 and serum levels of HSP60-specific IgG1, IgG2a and IgM were measured using a capture enzyme-linked immunosorbent assay. Values are mean OD(405) values ± SEM.

DISCUSSION

Previous studies have demonstrated the importance of HSPs in the pathology of atherosclerosis. HSP70 is expressed in atherosclerotic lesions[34] and oxLDL enhances the expression of HSP70.[35, 36] Autoantibodies to HSP60 causing endothelial damage[12] and macrophage lysis[37], are associated with an increase in susceptibility in atherosclerosis. T cells reactive to HSP60 are found to correlate with early atherosclerotic events[38] and are found in atherosclerotic plaques in rabbits[39] and humans.[40] Furthermore, *Chlamydia pneumoniae*, can induce an immune response because of its HSP60 expression, which is highly homologous to human and mouse HSP60.

We now show that LDLr$^{-/-}$ mice contain T cells specific for HSP60 and for the small peptide HSP60(253-268), but no T cells with any reactivity against the HSP70(111-125) peptide. HSP60 was derived from *Mycobacterium bovis* bacillus, but due to the high degree of amino acid sequence homology between different species, T cells specific for this HSP60 were found to be cross reactive against self-HSP60[41]. A spleen cell proliferation assay demonstrated a 2-3-fold increase in T cell proliferation in response to HSP60 or HSP60(253-268). Immunization of LDLr$^{-/-}$ mice with HSP60 or HSP60(253-268) and a subsequent proliferation assay with HSP60 or HSP60(253-268) resulted in a 13- and 9-fold increase in proliferation,

respectively. These data confirm that HSP60 but also the small HSP60-peptide can induce a T cell response in LDLr$^{-/-}$ mice, while the small HSP70-peptide was not effective.

Intervention in the anti-HSP60 autoimmune response could be beneficial for atherosclerosis. Many strategies are used to interrupt autoimmune responses directed towards autoantigens and one of these strategies is mucosal tolerance induction. Mucosal tolerance induction, can lead to a deletion of Th1 and Th2 cells or to an activation of Tregs depending on the administered dose of the antigen. Tregs, induced by low doses of the antigen, are known for the production of TGF-β and IL-10, which both have anti-atherogenic properties. Recently, Mallat et al. hypothesized that in atherosclerosis an imbalance exists between pathogenic T cells (Th1 and/or Th2) and Tregs specific for 'altered' self and non-self antigens.[21] Tregs play an important role in controlling the development of atherosclerosis in mice[20] and a transfer of HSP60-specific Tregs to RAG1$^{-/-}$LDLr$^{-/-}$ mice reduced the development of atherosclerotic lesions.[42] Consequently, mucosal tolerance induction and the subsequent activation of Tregs may be a useful strategy to ameliorate atherosclerosis. It was already shown that oral tolerance induction to β2-glycoprotein I,[25] HSP65[26, 27] and oxLDL[28] reduced early atherosclerosis. However, the studies on β2-glycoprotein I and HSP65 do not give a clear explanation for the observed reduction in atherosclerosis and they do not show whether Tregs are involved in this process. Oral administration of HSP60 suppresses adjuvant arthritis due to an expansion of T cells specific for HSP60 producing IL-10[43]. We showed that an induction of Tregs after oral administration of oxLDL was observed and these oxLDL specific Tregs were found to be responsible for the reduction in atherosclerotic plaque formation.[28]

We now show that oral tolerance induction to HSP60 and HSP60(253-268) attenuates atherosclerosis. A relatively low dose of HSP60 significantly reduced early atherosclerotic lesion formation by 80.7%. We now propose that an immunogenic peptide present in HSP60 (aa 253-268) can also induce regulatory T cells and reduces plaque size by 83.3%. The specificity of the response is reflected by the finding that HSP70(111-125), a peptide based on a conserved sequence found in the HSP70 protein of men, rats, and mice, was not effective in reducing atherosclerosis. The experimental setup of our current study is comparable with two previous studies on oral tolerance induction to HSP65/HSP60.[26, 27] Both Harats et al.[26] and Maron et al.[27] show a decreased proliferation of splenocytes after oral treatment but no effects on Tregs were described. Maron et al.[27] observed a decreased IFN-γ and an increased IL-10 production by lymphocytes after oral treatment with HSP65. This could indicate an activation of Tr1 cells, a subset of adaptive Tregs, particularly producing IL-10.[28] In our current study low doses of HSP60 and HSP60(253-268) were administered and therefore we investigated the

possible activation of Tregs. Four days after the oral HSP60-treatment, the number of $CD4^+CD25^+Foxp3^+$ Tregs was significantly increased in Peyer's patches and blood. After two weeks, the number of $CD4^+CD25^+Foxp3^+$ T cells was significantly increased in blood, mesenteric lymph nodes and spleen. In the Peyer's patches, the first site of activation, the number of Tregs decreased after two weeks, which may be attributed to the migration of the activated $CD4^+CD25^+Foxp3^+$ T cells to peripheral lymphoid organs and the site of inflammation (atherosclerotic lesions) where they may recognize self-HSP60. We also investigated the mRNA-expression of markers for Tregs within the lesions and we observed an increased expression of CD25, CTLA-4 and Foxp3. In addition, oral treatment with HSP60 reduced the proliferative response of splenocytes to HSP60 which is in line with the studies by Maron et al. and Harats et al.[26, 27] Furthermore, mesenteric lymph node cells of HSP60-treated mice also produced increased levels of TGF-β and IL-10 after *in vitro* re-stimulation with HSP60. All these data suggest that oral administration of HSP60 induced Tregs, which were may be able to dampen the immune response to HSP60 in atherosclerosis prone mice.

Natural Tregs, which are $CD4^+CD25^+Foxp3^+$ T cells can display their specific immunosuppressive effects via cell contact. TGF-β on their surface may bind to TβRII expressed on T cells specific for the same antigen. This TGF-β-TβRII interaction leads to the activation of a Smad-dependent pathway, resulting in a blockade of IL-2 production and a reduced proliferation of antigen-specific T cells. CTLA-4 is also important in the cell-cell interaction between Tregs and other T cells. It is however more likely that adaptive Tregs (Th3 and Tr1 cells) are involved in oral tolerance induction[31]. Th3 cells are known for the production of anti-inflammatory TGF-β and upon activation they may express Foxp3.[44, 45] Tr1 cells, which can also be activated in the periphery, produce particularly anti-inflammatory IL-10 but whether these Tregs express Foxp3 is still not clear. Therefore we assume that oral tolerance induction led to an increase in Foxp3-expressing natural Tregs or Th3 cells, producing excessive amounts of TGF-β but also IL-10. IL-10 may also be produced by activated Tr1 cells, which however do not contribute to the increase in $Foxp3^+$ Tregs. To definitively prove that the protection against atherosclerosis after oral administration of bacterial HSP60 is due to the induction of Tregs specific for murine HSP60 additional experiments are required, for example by deletion of Tregs after tolerance induction or transfer of the Tregs.

In conclusion, we describe that $LDLr^{-/-}$ mice can be tolerized to HSP60 and a HSP60 peptide (HSP60(253-268)) which results in an attenuation of early atherosclerotic lesions. The mechanism underlying this effect can be attributed to the induction of $CD4^+CD25^+Foxp3^+$ Tregs, which may produce TGF-β and IL-10 in atherosclerotic lesions. In this way they may down-regulate the inflammatory

response locally. Altogether, these beneficial results of oral tolerance induction to HSP60 and HSP60(253-268) on atherosclerosis may provide new therapeutic approaches for the treatment of atherosclerosis.

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Chapter 8

Vaccination against Foxp3$^+$ regulatory T cells aggravates atherosclerosis

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ABSTRACT

Regulatory T cells are crucial for immune homeostasis and an impaired regulatory T cell function results in many pathological conditions, such as atherosclerosis. Regulatory T cells can be divided in adaptive regulatory T cells and natural regulatory T cells, the latter expressing the transcription factor Foxp3. Regulatory T cells have already been described to be protective in atherosclerosis. However the exact contribution of Foxp3 expressing regulatory T cells in atherosclerosis has not been elucidated yet. In this study we vaccinated LDL receptor deficient mice with dendritic cells which are transfected with Foxp3 encoding mRNA and studied the effect on initial and advanced atherosclerosis.

Vaccination against Foxp3 resulted in a reduction of Foxp3 expressing cells and an increase in both initial and advanced atherosclerotic lesion formation. Furthermore we observed an increase in plaque cellularity and increased T cell proliferation in the Foxp3 vaccinated mice.

In conclusion, in this study we further establish the protective role of Tregs in atherosclerosis. The results illustrate the important role for Foxp3 expressing regulatory T cells in atherosclerosis, thereby providing a potential opportunity for therapeutic intervention against this disease.

INTRODUCTION

Atherosclerosis is an autoimmune like disease, in which both innate and adaptive immune responses are involved.[1] T helper (Th) cells are crucial for an adequate immune response and can be divided in Th1 (cell mediated immunity) and Th2 (humoral immunity) cells. Several studies show that inflammatory processes in atherosclerosis are associated with a Th1-driven immune response (IFN-γ, IL-12), while the Th2 cells (IL-5 and IL-13) exert an anti-atherogenic role.[2, 3] It was postulated that an imbalance between Th1 and Th2 cells was, at least partially, responsible for the development of atherosclerotic lesions. However, more recently, IL-4, a Th2-cytokine, was found to be pro-atherogenic in early lesion formation.[4, 5] This finding, together with studies establishing an anti-atherogenic role for regulatory T cells (Tregs), suggested another mechanism of immune regulation in atherosclerosis, where T cells (both Th1 and Th2) are suppressed by regulatory T cells (Tregs).

Tregs are characterized by the expression of both CD4 and CD25 and are subdivided in adaptive Tregs and natural Tregs. Adaptive Tregs develop form naive T cells in the periphery and can produce IL-10 (Tr1 cells) and TGF-β (Th3 cells). Natural Tregs originate from the thymus as $CD4^+CD25^+$ cells and exert their suppressive function especially via cell-cell contact and membrane bound TGF-β and CTLA-4. Forkhead box protein P3 (Foxp3) is characteristically expressed in this subclass of Tregs and this transcription factor is necessary for the development of Tregs. Deficiency in Foxp3 leads to a lack of Tregs and severe auto-immune disorders.[6-9]

Recently, we showed that oral administration of atherosclerosis-related antigens (HSP60 and oxLDL) increases the number of Foxp3-expressing Tregs in several organs, which leads to a decrease in development of atherosclerotic lesions in $LDLr^{-/-}$ mice.[10, 11] These results are in line with studies on the role of Tregs in atherosclerosis after oral and nasal tolerance induction[12,13, 14] but our studies specifically demonstrate the contribution of $Foxp3^+$ Tregs. Furthermore, a study showed that a transfer of Tregs selected on basis of CD4 and CD25 expression reduced lesion formation in $ApoE^{-/-}$ mice[15], while another study showed that treatment of $ApoE^{-/-}$ mice with a CD25-specific antibody (PC61) which depletes CD25 positive T cells, results in an increase in lesion size. Additionally, bone marrow transplantation of $CD80^{-/-}CD86^{-/-}$ bone marrow into $LDLr^{-/-}$ mice results in a decrease in the number of Tregs and an increase in lesion size again indicating an inverse relationship between the presence of Tregs and atherosclerotic lesion development.[15]

However, these studies do not directly demonstrate the role of Foxp3 expressing cells in atherosclerosis. This can be achieved via vaccination against

Foxp3 using DCs expressing Foxp3 as described by Nair *et al.* Vaccination with these DCs results in a decline in the number of intratumoral Tregs due to an induction of a cytotoxic T lymphocyte response against Foxp3 and subsequently in an enhanced antitumor immunity.[16]

To specifically establish the role of Foxp3-expressing Tregs, we targeted Foxp3 expressing cells using DCs electroporated with mRNA encoding for Foxp3. Vaccination against Foxp3 results in a reduction of $Foxp3^+$ Tregs and a subsequent increase in both initial and advanced atherosclerotic lesion formation, thereby establishing a prominent role for $Foxp3^+$ Tregs in these processes.

MATERIAL AND METHODS

ANIMALS

All animal work was approved by the regulatory authority of Leiden University and carried out in compliance with the Dutch government guidelines. Female $LDLr^{-/-}$ mice were obtained from the Jacksons Laboratory. Male C57BL/6J mice were from Charles river Laboratories. All mice were kept under standard laboratory conditions and were fed a normal chow diet or a 'Western-type' diet containing 0.25% cholesterol and 15% cocoa butter (Special Diet Services, Witham, Essex, UK). All mice used were 10-12 weeks of age. Diet and water were administered *ad libitum*.

SYNTHESIS OF FOXP3 AND GFP MRNA

The pSP73-Spf/Foxp3/A64 construct was kindly provided by E. Gilbao (Duke University Medical Center, Durham, NC, USA)[16]. As a control we created a pSP73-Spf/eGFP/A64 construct. The pSP73-Spf/Foxp3/A64 and pSP73-Spf/eGFP/A64 constructs were used as a DNA template in a T7 mMessage mMachine® (Ambion, Austin, TX) reaction to produce large amounts of capped Foxp3 and GFP in vitro transcribed mRNA. To eliminate excessive DNA the reaction, a TurboDNase® (Ambion, Austin, TX) treatment was performed. The Megaclear Kit® (Ambion, Austin, TX) was used for purifying mRNA from the in vitro transcription reactions. All reactions were performed according to the manufacturer's protocol.

GENERATION AND ASSESSMENT OF THE DC BASED VACCINE

Bone marrow cells were isolated from the *tibia* and *femora* of C57BL/6J mice. Cells were pooled and cultured for 10 days in IMDM supplemented with 8% fetal calf serum (FCS, PAA), 100 U/ml streptomycin/penicillin (PAA), 2 mM glutamax

(Invitrogen, The Netherlands) and 20 µM β-mercaptoethanol, in the presence of granulocyte-macrophage colony-stimulating factor (GM-CSF). Hereafter, the DCs were harvested, washed, and resuspended in Opti-MEM (GIBCO, Grand Island, NY). The used DC culture media were saved as conditioned media for later use. 5 x 10^6 DCs in 200 µL Opti-MEM were electroporated with either GFP or Foxp3 mRNA as described by Nair *etal*.[17] After electroporation the DCs were transferred to culture petridishes containing GM-CSF and a 1:1 combination of conditioned DC growth media and fresh media. Transfected DCs were incubated at 37 °C, 5% CO_2 overnight, washed 2 times in PBS, for vaccination. To assess the maturation profile the electroporated DCs were stained against surface markers: CD80-FITC, CD86-PE and CD40-PE. To detect surface bound and intracellular Foxp3 expression in the electroporated DCs, the cells were stained with Foxp3-APC. For intracellular staining of Foxp3, DCs were fixated and permeabilized overnight and subsequent stained against Foxp3. All FACS antibodies were purchased from eBioscience (Belgium) and used according manufacturer's protocol. DCs were analyzed by flow cytometry on a FACSCalibur (Becton Dickinson, Mountain View, CA). Data were analyzed using Cell Quest software (BD Biosciences, The Netherlands). Total RNA was isolated from the electroporated DCs using the GTC method.[18] cDNA synthesis was performed using Revert Aid™ M-MuZV Reverse Transcriptase (Fermentas Life Science). Quantitative gene expression analysis was performed on a 7500 fast Real-Time PCR System (Applied Biosystem) using SYBR Green technology. Acidic ribosomal phosphoprotein PO (36B4) and hypoxanthine phosphoribosyl transferase (HPRT) were used as reference genes. Primer pairs as described in table 8.1 were used to quantify Foxp3 gene expression.

TABLE 8.1: SEQUENCE OF PRIMERS. Primer sequences for the genes analyzed with qPCR.

GENE	FORWARD PRIMER	REVERSE PRIMER
FOXP3	5′-GGAGCCGCAAGCTAAAAGC-3′	5′- TGCCTTCGTGCCCACTGT-3′
36B4	5′-GGACCCGAGAAGACCTCCTT-3′	5′-GCACATCACTCAGAATTTCAATGG-3′
HPRT	5′-TTGCTCGAGATGTCATGAAGGA-3′	5′-AGCAGGTCAGCAAAGAACTTATAG-3′

VACCINATION AND THE INDUCTION OF ATHEROSCLEROSIS

Mice (GFP N=15, Foxp3 N=16) were vaccinated with $5x10^5$ DCs in 100 µl per mouse subcutaneously at the base of the ear pinna at day 0. Mice of the control group (N=15) were injected with 100 µl PBS. After vaccination, the mice were directly fed a Western-type diet (0.25% cholesterol and 15% cocoa butter) to induce hypercholesterolemia and atherosclerosis. For the advanced atherosclerosis study, mice were fed a Western-type diet for 10 weeks and were subsequently vaccinated against GFP (N=15) Foxp3 (N=16) or sham vaccinated with PBS (N=15). After vaccination the mice were fed a Western-type diet for 12 more weeks before they were sacrificed. During both experiment blood samples were obtained by tail vein bleeding. The concentrations of serum cholesterol were determined using an enzymatic colorimetric procedure (Roche/Hitachi). Precipath (Roche/Hitachi, Mannheim, Germany) was used as an internal standard. Blood samples of week 5 were also used to determine the percentage Tregs. 8 weeks after vaccination, the mice were sacrificed and tissues were harvested after in situ perfusion using PBS and subsequent perfusion with Zinc Formal-Fixx (Shandon Inc. Pittsburg, USA). Tissues were snap-frozen in nitrogen and stored at –80 °C until further use.

FLOW CYTOMETRY

Peripheral Blood Mononuclear Cells (PBMC) were isolated via orbital bleeding and erythrocytes were removed by incubating the cells with erythrocyte lysis buffer (0.15 M NH_4Cl, 10 mN $NaHCO_3$, 0.1 mM EDTA, pH 7.3; N=5 each group). Spleens, heart lymph nodes (HLN) and mesenteric lymph nodes (MLN) were dissected from the mice and single cell suspension was obtained by passing the organs through a 70 µm cell strainer (Falcon, The Netherlands; N=5 each group). Cells were stained with surface markers (0.20 µg antibody/300.000 cells) and subsequently analyzed by flow cytometric analysis. For the detection of $CD4^+CD25^+Foxp3^+$ T cells, the spleen, blood, MLN and HLN were stained with CD4-FITC and CD25-PE and subsequently with an intracellular Foxp3 staining as described above. All antibodies were purchased from eBioscience (Immunosource, Belgium). All data were acquired on a FACScalibur (Becton Dickinson, Mountain View, CA) and analyzed with CELLQuest software (BD Biosciences, The Netherlands).

SPLEEN CELL PROLIFERATION

The splenocytes (N=5 per group) were cultured for 48 hours in triplicate in a 96-wells round-bottom plate (2×10^5 cells/well) in RPMI 1640 supplemented with L-

Glutamine, 10% FCS and 100 U/ml streptomycin/penicillin. As a positive control cells were stimulated with Concanavalin A (2 µg/ml, Con A, Sigma Diagnostics, MO). Proliferation was measured by addition of ^{3}H-thymidine (0.5 µCi/well, Amersham Biosciences, The Netherlands) after 16 hours. The amount of ^{3}H-thymidine incorporation was measured using a liquid scintillation analyzer (Tri-Carb 2900R). The proliferation is expressed in disintegration per minute (dpm).

HISTOLOGICAL ANALYSIS

The heart was embedded in OCT compound (TissueTek; Sakura Finetek, The Netherlands) and cryosections (10 µm) of the aortic root containing the three aortic valves were made. Cryosections were routinely stained with Oil-Red-O and hematoxylin (Sigma Diagnostics, MO). Corresponding sections on separate slides were also stained for collagen using Masson trichrome staining according to manufacturers protocol (Sigma Diagnostics). For the cellularity assessment a hematoxylin staining was performed. The different histological stainings were quantified using a Leica DM-RE microscope and Leica Qwin Imaging software (Leica Ltd., Germany).

STATISTICAL ANALYSIS

All data are expressed as mean ± SEM. The two-tailed student's t-test was used to compare individual groups of mice or cells. When indicated, a Mann-Whitney test was used to analyze not normally distributed data. The frequency of thickened cap structure was analyzed by a Fisher's exact test. *P* values of <0.05 were considered significant.

RESULTS

ELECTROPORATION OF DCS WITH MRNA RESULTS IN AN ACTIVATED PHENOTYPE

To test whether a reduction in Foxp3-expressing Tregs affects atherosclerosis, LDLr$^{-/-}$ mice were vaccinated with DCs transfected with mRNA encoding for Foxp3. A significant increase in the surface expression of the co-stimulatory molecules, CD80, CD86 and CD40 was observed in DCs that were electroporated with mRNA encoding Foxp3 or GFP, when compared with electroporation without mRNA (Control) (Figure 8.1).

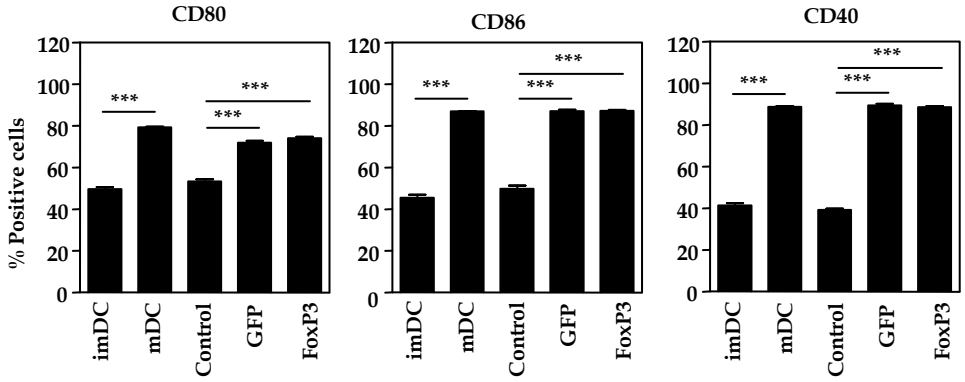


FIGURE 8.1: EXPRESSION OF SURFACE MARKERS ON ELECTROPORATED DCS. After electroporation with either PBS (control, N=3), GFP mRNA (N=3) or Foxp3 mRNA (N=3) the DCs were cultured o/n. DCs were also stimulated with LPS as a positive control for maturation (mDCs). Unstimulated DCs (imDCs) were used as a negative control for maturation. DCs were subsequently stained for CD80, CD86 and CD40 and analyzed by FACS. ***P<0.001

The increase upon transformation with mRNA is comparable to the LPS induced TLR4 dependent maturation of DCs (mDCs). Interestingly, no upregulation was observed in DCs electroporated without mRNA. These DCs had the same expression level of the activation markers as immature DCs (imDCs), indicating that exposure of DCs to electroporation conditions alone did not result in the maturation of DCs (Figure 8.1A).

ELECTROPORATION WITH MRNA ENCODING FOR FOXP3 RESULTS IN THE EXPRESSION OF FOXP3

After electroporation with mRNA encoding for Foxp3 a strong increase in Foxp3 expression was observed on mRNA level, which indicated a successful transfection of the DCs (Figure 8.2A). To determine the intracellular protein expression of Foxp3, an intracellular Foxp3 FACS staining was performed (Figure 8.2B and C). Foxp3 transfected DCs expressed 6.3 fold more intracellular Foxp3 compared to GFP transfected DC, which expressed no Foxp3.

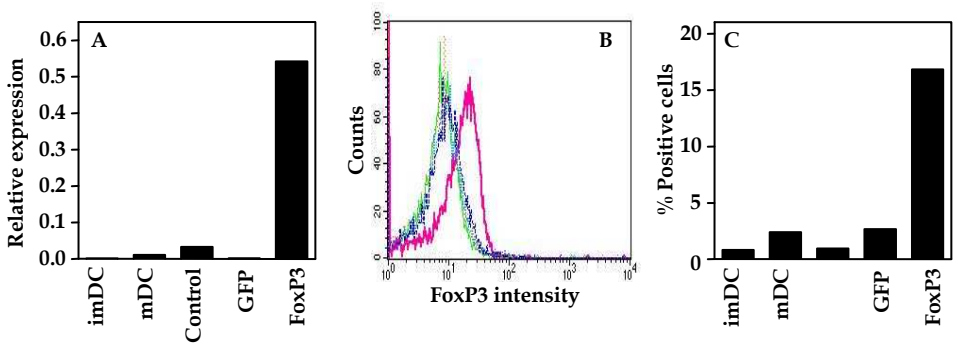


FIGURE 8.2: DETECTION OF FOXP3 SPECIFIC MRNA WITH QPCR AND SUBSEQUENT EXPRESSION BY FACS ANALYSIS.
After electroporation total RNA was isolated and cDNA was synthesized. Subsequently the amount of mRNA was detected with Foxp3 specific qPCR primers (A). The expression is relative to HPRT and 36B4. Next we determined, with FACS analysis, the intracellular expression of Foxp3 in PBS (green), GFP (blue) and Foxp3 (pink) electroporated DCs. DCs were cultured o/n after electroporation and stained intracellularily for Foxp3 and analyzed by FACS. A representative histogram (B) and percentage of intracellular Foxp3 expression is depicted (C).

EFFECT OF VACCINATION AGAINST FOXP3 ON FOXP3 EXPRESSING T CELLS

To asses the efficacy of the Foxp3 vaccine, LDLr$^{-/-}$ mice were vaccinated with Foxp3 electroporated DCs. As a control, mice were vaccinated with DCs electroporated with mRNA encoding for GFP. Five weeks after vaccination, the number of Foxp3^{+} Tregs in blood was analyzed. A 34% reduction in the number of Foxp3^{+} Tregs in Foxp3 vaccinated mice was observed, compared to GFP vaccinated mice (Figure 8.3A; 0.773±0.032% versus 1.175±0.065%; P<0.001). Eight weeks after vaccination, mice were sacrificed and blood, spleen, mesenteric lymph nodes (MLN) and mediastinal lymph nodes draining from the aorta (HLN) were isolated to determine the percentage of Tregs. Vaccination with Foxp3 transfected DCs resulted in a 27-30% decrease in Foxp3^{+} Tregs in blood (0.67±0.045% versus 0.92±0.096%, P<0.05), spleen (2.88±0.24% versus 3.94±0.34%, P<0.05), MLN (3.59±0.10% versus 5.01±0.45%, P<0.05) and HLN (3.68±0.08% versus 5.50±0.46%, P<0.01), when compared to mice vaccinated with DCs electroporated with mRNA encoding for GFP (Figure 8.3B). As a control for the DC vaccination, one group of mice was treated with PBS. We observed no differences in the numbers of Foxp3^{+} Tregs in lymphoid organs and blood between mice treated with PBS alone and mice vaccinated with DCs electroporated with mRNA encoding for GFP (data not shown).

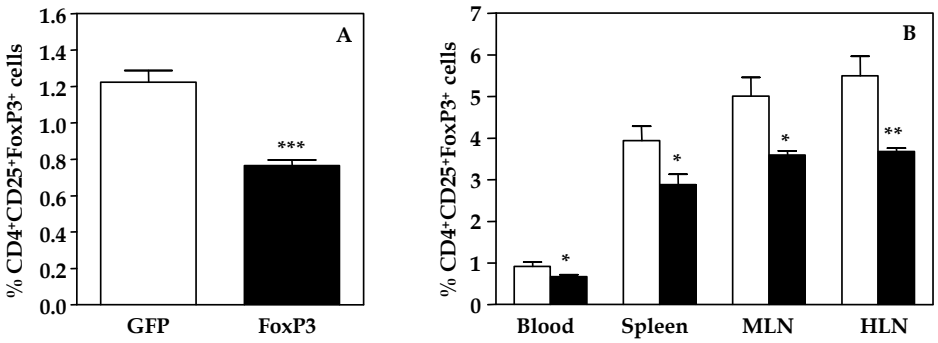


FIGURE 8.3: EFFECT OF FOXP3 VACCINATION ON THE PERCENTAGE OF TREGS IN THE LYMPHOID ORGANS. 5 weeks after vaccination blood was taken from the mice vaccinated with either GFP (N=5, white bar) or Foxp3 (A, N=5, black bar) and we stained for CD4+CD25+Foxp3+ cells and subsequently performed a FACS analysis. 8 weeks after vaccination the mice vaccinated with either GFP (B, N=5, white bar) or Foxp3 (B, N=5, black bar) were sacrificed. Blood, spleen, MLN and HLN, were isolated and stained for CD4+CD25+Foxp3+ cells and analyzed. *P<0.05, **P<0.01, ***P<0.001

VACCINATION AGAINST FOXP3 INCREASES PLAQUE FORMATION IN LDLR$^{-/-}$ MICE IN BOTH INITIAL AND ADVANCED ATHEROSCLEROSIS

Eight weeks after vaccination and Western-type diet feeding the plaque size at the aortic root was analyzed. Representative slides of the aortic root of GFP and Foxp3 vaccinated mice are shown in Figure 8.4A and B. Mice vaccinated against Foxp3 showed a significant 34% increase in plaque size compared to the GFP vaccinated mice (Figure 8.4C; 538.932±46.043 μm^2 versus 382.865±29.044 μm^2, P<0.01). During the experiment, all mice developed hypercholesterolemia, however no significant differences in serum cholesterol levels (Figure 8.4D) and body weight (data not shown) were observed between the different groups of mice. Again, no differences in plaque size were observed between PBS treated mice and mice vaccinated against GFP (data not shown).

In an advanced atherosclerosis study, LDLr$^{-/-}$ mice were vaccinated against GFP and Foxp3 while they were on a Western-type diet for already 10 weeks. 12 weeks after vaccination and continuous Western-type diet feeding, mice were sacrificed and lesions at the aortic root were investigated. Representative slides of the aortic root of GFP and Foxp3 vaccinated mice are shown in Figure 8.5A and B. A 14% increase in lesion size, although not significant, was observed between Foxp3 vaccinated mice and GFP vaccinated mice (Figure 8.5C; 993.620±44.946 μm^2 versus 869.165±46.884 μm^2; P=0.07). However, during analysis we observed an increased diameter of the hearts and therefore we calculated the ratio between the intima and the luminal space. We observed an increase of 28% in intima/lumen ratio, indicating aggravated atherosclerosis (Figure 8.5D; 0.37±0.015 versus 0.473556±0.028, P<0.01). These effects are unrelated to serum cholesterol levels,

since there were no differences observed in cholesterol levels between the two groups (data not shown). Again, no difference in lesion size was observed between PBS treated mice and GFP vaccinated mice (data not shown).

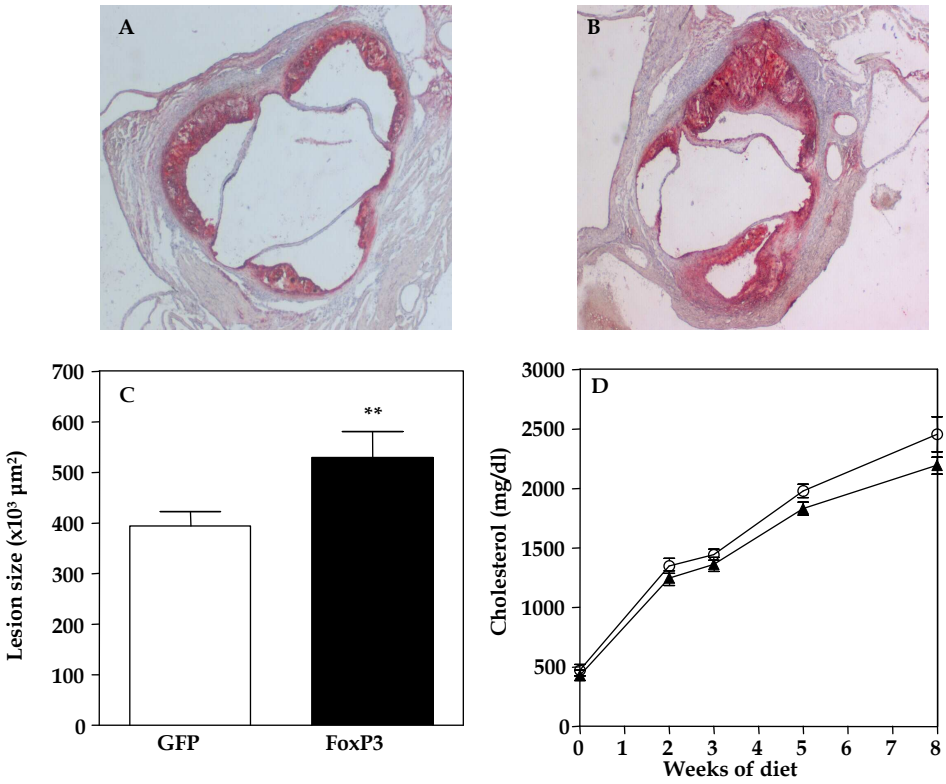


FIGURE 8.4: EFFECT OF FOXP3 VACCINATION ON INITIAL LESION SIZE. After 8 weeks of vaccination and Western type diet feeding the LDLr$^{-/-}$ mice were sacrificed and the heart of GFP treated (A) and Foxp3 treated (B) mice were sectioned and stained with Oil-red-O and hematoxylin. The lesions were quantified and the plaque size was determined (C). During the experiment, blood was taken by tail vein bleeding and total cholesterol concentration was determined within the serum (D). **P<0.01

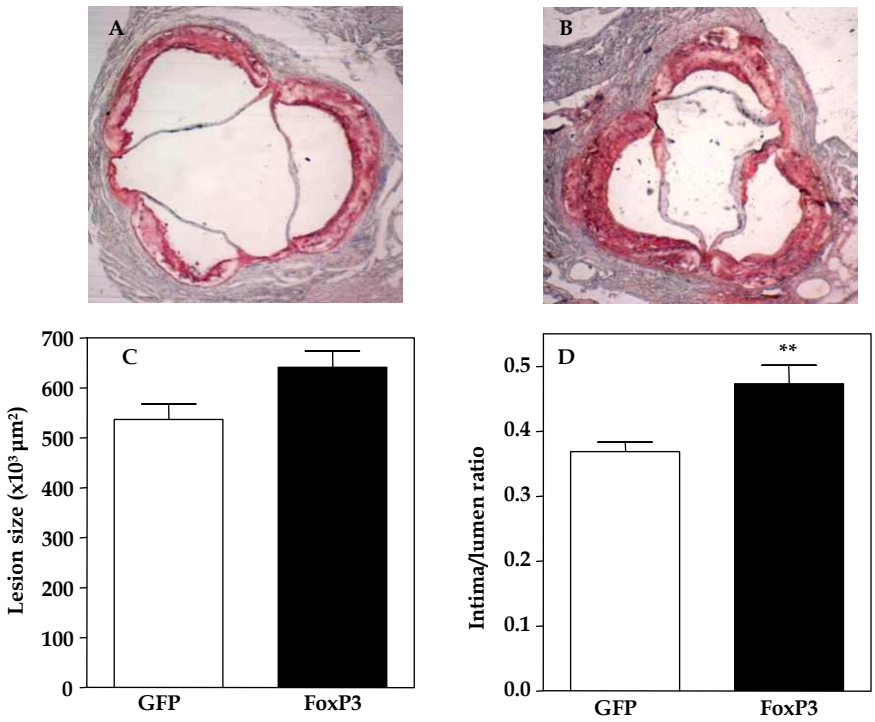


FIGURE 8.5: THE EFFECT OF FOXP3 VACCINATION ON ADVANCED LESION SIZE. After 22 weeks on Western type diet feeding and 12 weeks after vaccination the LDLr$^{-/-}$ mice were sacrificed and the heart of GFP treated (A) and Foxp3 treated (B) mice were sectioned and stained with Oil-red-O and hematoxylin. The lesions were quantified and the plaque size was determined (C). Additionally, the ratio between intima and luminal space was calculated (D). **P<0.01

VACCINATION AGAINST FOXP3 RESULTS IN INCREASED PLAQUE CELLULARITY

To investigate whether there is a change in plaque composition in the enlarged initial plaques of the Foxp3 vaccinated mice we quantified the number of cells within the lesions. The plaques of Foxp3 vaccinated mice showed a 27% increase in cellularity compared to GFP vaccinated mice (Figure 8.6; $3.84x10^{-3}\pm0.19x10^{-3}$ cells/µm² versus $2.81x10^{-3}\pm0.79x10^{-3}$ cells/µm²; $P<0.01$). Increased cellularity may indicate an increased level of inflammation within the plaque. Therefore we determined the plaque stability by measuring both the amount of collagen within the plaque and the fibrous cap thickness. The collagen content of the plaque was not significantly changed between the Foxp3 and GFP vaccinated mice (Figure 8.7, 9.0±1.1% versus 6.5±1.9%). Additionally, we noticed that the lesions in 9 of the 13 Foxp3-vaccinated mice displayed significant characteristic changes in morphology. These lesions are characterized by a thickened structure in the fibrous cap (Figure 8.8, arrowhead). When compared with GFP vaccinated mice in which 2 out of 14

mice showed these thick structures, a significant difference is observed when a Fisher's exact test is performed (Figure 8.8, $P<0.01$). Overall there was no significant difference between the cap thickness in the different groups (data not shown).

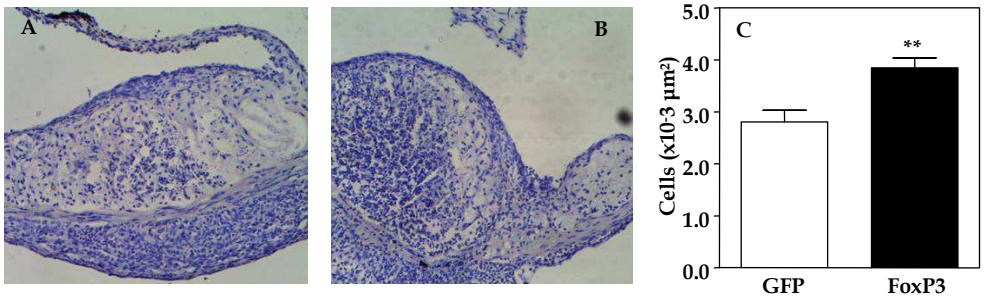


FIGURE 8.6: EFFECT OF FOXP3 VACCINATION ON PLAQUE CELLULARITY. After 8 weeks of vaccination and Western type diet feeding the LDLr$^{-/-}$ mice were sacrificed and the heart of GFP treated (A) and Foxp3 treated (B) mice were sectioned and stained with hematoxylin to stain the nucleus of the cells within the lesion. The number of nuclei was quantified (C). $^{**}P<0.01$

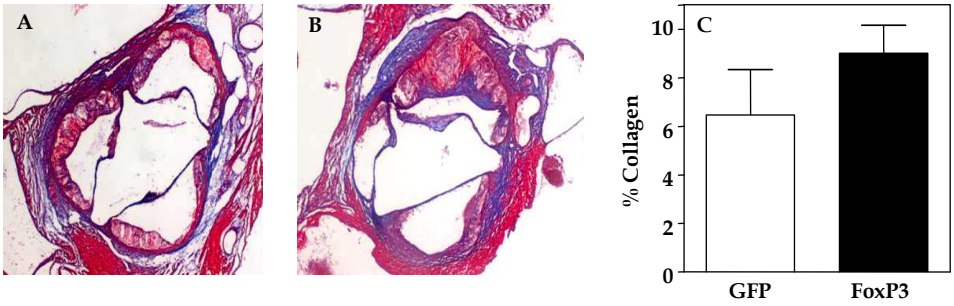
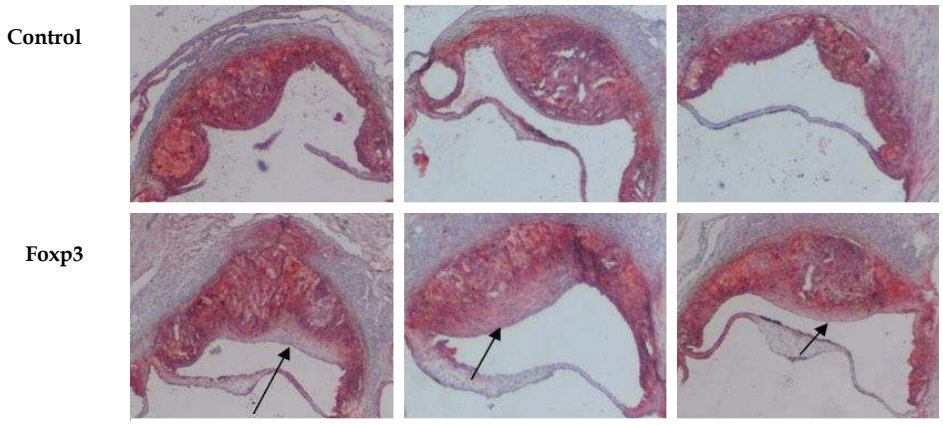


FIGURE 8.7: EFFECT OF FOXP3 VACCINATION ON COLLAGEN CONTENT WITHIN THE LESION AT THE AORTIC ROOT. After 8 weeks of vaccination and Western-type diet feeding the LDLr$^{-/-}$ mice were sacrificed and the heart of GFP treated (A) and Foxp3 treated (B) mice were sectioned and stained with Masson's Trichrome staining which stains collagen blue. The percentage of collagen relative to the lesion size was determined (C).

INCREASED SPLEEN CELL PROLIFERATION IN FOXP3 VACCINATED MICE

The regulatory function of Tregs is partly mediated by cell-cell contact and surface-bound TGF-β and CTLA-4.[19] Since we observed an increased cellularity within the lesions of Foxp3 vaccinated mice, we hypothesized that vaccination against Foxp3 results in less suppression of T cell proliferation. Therefore we studied the proliferation of the spleen cells from the vaccinated mice. We observed a significant 1.9-fold increase compared to the GFP vaccinated mice (Figure 8.9: 4,148.5±941.4 dpm versus 8,230.5±1 542.5 dpm $P<0.05$).



	No thickened cap	Thickened cap
Control	**12**	**2**
Vaccinated	**4**	**9**

FIGURE 8.8: THICKENED CAP STRUCTURE IN FOXP3 VACCINATED MICE. After 8 weeks of vaccination and Western type diet feeding the LDLr$^{-/-}$ mice were sacrificed. Within the Foxp3 vaccinated group we observed more thickened structures in the fibrous cap (arrow), compared to the control. Frequency of thickened structure between control and vaccinated mice is depicted in the table. A Fisher's exact test was used (P<0.01).

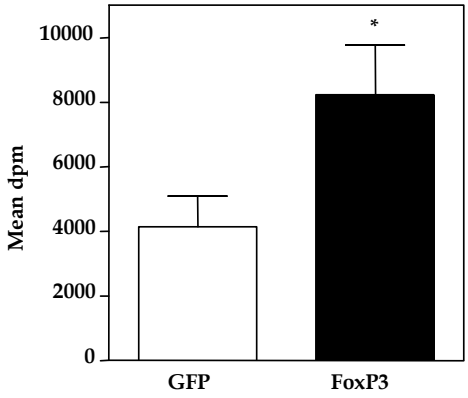


FIGURE 8.9: EX VIVO SPLEEN CELL PROLIFERATION IN VACCINATED MICE. 8 weeks after vaccination, the mice vaccinated with either GFP (N=5, white bar) or Foxp3 (N=5, black bar) were sacrificed. The spleen was dissected and cultured *ex vivo*. Proliferation was assessed by the amount of ^{3}H incorporation in dividing cells. The amount of ^{3}H-thymidine incorporation was measured using a liquid scintillation analyzer (Tri-Carb 2900R). The proliferation is expressed in disintegrations per minute (dpm). *P<0.05

DISCUSSION

In this study we demonstrate that vaccination against Foxp3+ Tregs aggravates atherosclerotic lesion formation, thereby directly establishing the protective role of Tregs in atherosclerosis. The beneficial role of Tregs in atherosclerosis is already indicated in a number of publications.[15, 20] We showed before that induction of Foxp3 positive Tregs via oral tolerance induction against HSP60 and oxLDL reduces atherosclerotic lesion formation.[10, 11] Furthermore, it had been demonstrated that a deficiency in co-stimulatory molecules promotes atherosclerosis because of a decrease in the number of Tregs[15] and Ait-Oufella and colleagues showed that a depletion of CD25+ cells using anti-CD25 antibodies increased lesion formation.[15] However, the final proof for the involvement of Foxp3 expressing T cells in atherosclerosis has not been provided since CD25 is not exclusive for Tregs. CD25 is for example also expressed on other cell types such as activated T cells[21], NK cells[22] and myeloid DCs.[23]

To specifically study the role of Foxp3 positive Tregs in atherosclerosis we vaccinated atherosclerosis prone mice against Foxp3 using a DC vaccination strategy. DCs are electroporated with mRNA encoding for Foxp3 and this approach, described by Nair *et al*, results in a cytotoxic T lymphocyte (CTL) response against Foxp3 and a subsequent depletion of Foxp3+ Tregs.[16] As a control, mRNA encoding for GFP was used to exclude that mRNA electroporation into DCs activates the DCs leading to immune modulatory effects. In all the experiments, the treatment with GFP electroporated DCs was compared with PBS treatment alone and no differences were observed between both treatments. After electroporation of DCs with mRNA, either with GFP or with Foxp3, an activated phenotype of DCs was observed. This was not observed when DCs were electroporated without mRNA. It is known that single stranded RNA (ssRNA), such as mRNA is a natural ligand for Toll like receptors 7 and 8,[24,25] which may explain the activated phenotype of the DCs after electroporation with mRNA.

We successfully demonstrated that the DCs were transfected with the mRNA coding for Foxp3 via qPCR and a FACS analysis. Foxp3 is a nuclear product and is not expressed on the cell surface. However, we use a truncated Foxp3 in which the nuclear localization sequence is removed. This results in relatively high concentration of cytosolic Foxp3 protein and therefore may results in the cross-presentation of Foxp3-peptides on MHC class I. Furthermore, mRNA may act as a natural agonist of TLR7/8 and thereby even further enhance cross-presentation, which is already described by other groups.[24-27] This may lead to the triggering of CD8+ T cells, which subsequently target Foxp3 expressing cells.

Our present data show a significant reduction in Foxp3+ Tregs in blood five weeks after vaccination. This may be an indication for the induction of Foxp3-

specific cytotoxic CD8$^+$ T cells. A similar reduction in Foxp3$^+$ Tregs was observed within the blood, HLN, MLN and spleen, 8 weeks after vaccination, indicating a systemic reduction in Foxp3$^+$ Tregs and a persistent effect of the vaccination. The reduction in Foxp3$^+$ Tregs resulted in a 34% increase in initial lesion size and additionally 14% increase in advanced lesion size. The increase in atherosclerosis is not related to a change in total cholesterol serum levels since there is no significant difference in cholesterol levels between the different groups.

Besides an increase in lesion size, vaccination against Foxp3 also induced a 30% increase in cellularity of the initial lesions. The increased cellularity may indicate an increase in inflammation within the lesion, which may be caused by an increase in proliferation of inflammatory cells or by an increase in influx of inflammatory cells. Which cells are responsible for this increase needs to be determined, but we suggest that at least a part of these cells are T cells, since a reduction in Foxp3$^+$ Tregs may result in more proliferation of effector T cells (Th1 and Th2).[28] This is in line with the observed increase in proliferation of spleen cells isolated from Foxp3 vaccinated mice as compared with both control groups. This may illustrate that a lowered number of Foxp3$^+$ Tregs led to a less suppression of Th1 and Th2 cells and thereby led to an increased proliferation of effector T cells. In addition to plaque cellularity, we checked plaque stability but the collagen content was not changed. In spite of the fact that the fibrous cap thickness was not changed between vaccinated and control mice, we observed significant more thickened cap structures in the fibrous cap of the Foxp3 vaccinated mice. The composition of this thickening and whether this thickened structure results in a more stable plaque phenotype remains to be determined.

The results in this study are in line with the observations that the induction of Tregs exert an atheroprotective effect. The increase in lesion size is comparable with the decrease of initial lesion size upon the induction of Tregs via oral administration of oxLDL (30.0%) or HSP60 (27.5%).[10, 11] Oral tolerance induction against oxLDL also resulted in a decrease in advanced lesion size, which is comparable with the increase in advanced lesion size in this study.[10]

In conclusion, we confirmed the protective role of Foxp3$^+$ Tregs in atherosclerosis by vaccinating LDLr$^{-/-}$ mice using a DC based Foxp3 vaccination strategy. The results illustrate an important role for Foxp3$^+$ Tregs in atherosclerosis, thereby providing a potential opportunity for therapeutic intervention against atherosclerosis.

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Chapter 9

A vaccine against atherosclerosis: myth or reality?

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ABSTRACT

Atherosclerosis is a chronic inflammatory disease that develops in the context of enhanced serum lipid levels. Nowadays, many studies focus on the modulation of inflammatory responses to reduce atherosclerosis. The most powerful strategy to achieve this is vaccination. In several immune diseases vaccination is shown to be very effective, resulting in a drastic decline in the incidence of the disease. But is vaccination also realistic in atherosclerosis?

In this article, several approaches to vaccinate against atherosclerosis are described. Vaccination (based on protein or DNA) against bioactive molecules and disease-related proteins successfully reduces experimental atherosclerosis. In addition, passive immunization with antibodies against atherosclerosis-specific antigens and tolerance induction, in which antigen-specific regulatory T cells are elicited, are described.

In the near future, we expect an increased interest in vaccination against atherosclerosis and, maybe, the myth may become reality when the first clinical trials are performed.

INTRODUCTION

Atherosclerosis, the main underlying pathology of cardiovascular disease, is a multifactorial, chronic, autoimmune-like disease initiated by both lipid accumulation and inflammatory processes.[1–3] Both the innate immune response, represented by monocytes and macrophages, and adaptive immune responses, represented by T and B cells, are important in the onset and progression of atherosclerosis.

The modification of lipoproteins and the subsequent activation of endothelial cells results in an increased expression of chemokines and adhesion molecules, leading to the attraction of monocytes and subsequent diapedesis into the vessel wall. The monocytes ingest the modified lipoproteins and differentiate into foam cells. These foam cells produce chemokines and cytokines that enhance the recruitment of more monocytes and also T cells, thereby aggravating atherosclerosis.

Dendritic cells are pivotal in bridging the innate and the adaptive immune responses, and the switch towards an adaptive immune response is considered to be of utmost importance in the development of autoimmune diseases. Locally (within the lesion), but also systemically (periphery), dendritic cells ingest autoantigens such as oxidatively modified-LDL (ox-LDL) and heat-shock proteins (HSPs), and migrate to secondary lymphoid organs – the spleen and draining lymph nodes – where they will activate both naive and antigen-specific T cells. Subsequently, the T cells migrate towards the atherosclerotic lesion.

These effector T cells mainly produce T helper (Th)1-associated cytokines such as IL-12, IFN-γ, IL-6 and IL-1β within the lesion, which are pro-atherogenic and lead to enhanced activation and recruitment of T cells, macrophages and dentric cells in the plaque. A disturbed balance between Th-1 and -2 cytokines has long been suggested as the underlying cause of the auto-inflammatory pathology in atherosclerosis. Th2 cytokines such as IL-5 and -10 are anti-atherogenic and may counteract the Th1 cytokines.[1–3] During recent years, this theory has been challenged by a new hypothesis in which regulatory T cells (Tregs) play a pivotal role, since they can dampen inflammatory responses in several autoimmune diseases. Therefore, Tregs may hold promise in the future treatment of atherosclerosis.[3,4]

CURRENT TREATMENT OF ATHEROSCLEROSIS

Currently, the treatment of atherosclerosis is mainly based on reducing risk factors such as high circulating cholesterol levels and hypertension. Statins are currently the most prescribed drug and exert their effect largely by lowering plasma

cholesterol levels. In addition, statins may improve endothelial function, modulate inflammatory responses, maintain plaque stability and prevent thrombus formation. Other less frequently prescribed medications to reduce cardiovascular diseases are anti-platelet therapies and anti-inflammatory aspirin. Although statins produced a decline in the incidence of atherosclerosis, cardiovascular diseases are still the leading cause of death in the Western world.

The role of inflammation in atherosclerosis has been demonstrated extensively in experimental studies, but no treatment that directly modulates the inflammatory response has reached the clinic as yet. At the moment, a number of attractive approaches are available to interfere in the autoimmune reactions. Suppressing the function or expression of pro-atherogenic cytokines and/or stimulating the function and expression of anti-atherogenic cytokines are the most described techniques in atherosclerosis research. However, intervention in cytokine pathways may have a disadvantage, since some pro-inflammatory cytokines, such as IL-12 and IFN-γ, are crucial for an efficient immune response against pathogens.

VACCINATION

For many diseases, vaccination is used as a preventive therapy. Traditional vaccination protocols use weakened or dead pathogens or purified components from these pathogens, for example, proteins, to induce immunity. It is an effective, relatively easy way to protect against diseases caused by viruses or bacteria such as smallpox, polio, measles and rubella.

Nowadays, vaccination against nonpathogenic particles such as cytokines and immunogenic antigens is also a commonly used strategy to prevent autoimmune diseases in animal models. In our opinion, the terminology of vaccination and immunization is somewhat unclear. In this perspective review we use the term vaccination as a treatment that results in immunization. Immunization is the induction of immune resistance to a specific disease in humans (or other mammals) by exposing the individual to a disease-related antigen in order to raise antibodies and/or other immune reactions towards that antigen (e.g., tolerance induction). Although passive immunization is not strictly believed to be a vaccination strategy, we include this technique as an immunomodulating tool for treatment of autoimmune diseases, such as atherosclerosis, in the scope of this review.

In different autoimmune diseases, vaccination has already proved to be an effective strategy to attenuate disease pathology.[5–7] However, the complication in atherosclerosis is that it is a multifactorial disease. Many studies suggest that several pathogens influence the inflammatory responses towards a more pro-atherosclerotic condition, probably by cross-reacting with homologous 'self' proteins in a form of 'molecular mimicry'. Pathogens such as Chlamydia pneumoniae and cytomegalovirus are related to atherosclerosis, but their role is still unclear.[8–10] As in other autoimmune diseases, autoantigens play an important role in the initiation and aggravation of the disease.

In patients with manifestations of atherosclerosis, several autoantigens can be identified, such as modified lipoproteins (ox-LDL, malondialdehyde [MDA]-modified-LDL), HSPs (HSP60 and HSP70), ApoB100 peptides and β2 glycoprotein I. The immunogenecity of these autoantigens is reflected in the levels of autoantibody levels against epitopes of ox-LDL and HSPs, since they are significantly increased in patients with atherosclerosis. In addition, T cells specifically responding to these auto antigens have been detected within atherosclerotic plaques.[11,12] Considering atherosclerosis as an autoimmune disease, vaccination can provide an alternative treatment for atherosclerosis.

Many of the vaccination strategies mentioned in this article are successfully performed in other autoimmune diseases, such as collagen-induced arthritis and experimental autoimmune encephalitis (EAE),[13,14] but it should be taken into account that atherosclerosis is, in some aspects, different from other autoimmune diseases. Atherosclerosis is a disease that slowly progresses with age, and the onset of lesion formation begins at a very young age (<20 years). Furthermore, atherosclerosis is a multifactorial disease in which inflammation and an altered lipid metabolism play essential roles, resulting in a more complex pathogenesis. Removal or blockade of proatherogenic factors in childhood could be very effective in the prevention of lesion formation, and the interesting question is whether vaccination is a realistic goal in the treatment of atherosclerosis? We will further discuss different ways of vaccination that could become potential treatments for atherosclerosis.

PROTEIN VACCINATION: ELIMINATION/BLOCKADE OF BIOACTIVE MOLECULES

Most traditional vaccination strategies use adjuvants to enhance and modulate the immune response. Commonly used adjuvants are Freund's adjuvant and aluminium salts adjuvant. The choice of the adjuvant together with the target determines the outcome of the vaccination. This approach has been used in other

autoimmune diseases, such as vaccination against IL-17A in EAE[15] and vaccination against TNF-α in experimental arthritis.[6]

In a previous study we performed a protein vaccination against IL-12 with MPL/QS21 adjuvant. To break tolerance against self-antigens, we coupled IL-12 to an immunodominant Th epitope, termed PADRE. Functional antibodies were detected and IL-12 was successfully neutralized. In addition, the downstream pathway of IL-12 was blocked as was seen in reduced IFN-γ levels. With this approach, a 60% smaller sized and more stable plaque was formed.[16] Another possible target for vaccinating against atherosclerosis is cholesteryl ester transfer protein (CETP). CETP transfers cholesteryl esters from anti-atherogenic HDLs to pro-atherogenic ApoB-containing lipoproteins, including VLDLs and LDLs. Studies show that anti-CETP antibodies can inhibit CETP activity, increase plasma HDL-C and attenuate atherosclerosis.[17,18] As a next step, a vaccine was developed to induce antibodies that continuously inhibit CETP activity for a long time in vivo. A chimeric protein containing CETP and a helper T-cell epitope consisting of residues of tetanus toxin was constructed. The T-cell epitope from tetanus toxin was selected on the basis of its ability to bind many MHC haplotypes. New Zealand white rabbits were vaccinated with the chimeric enzyme and CETP-neutralizing antibodies were formed. There was an increase in plasma HDL-C, a decrease in plasma LDL-C and an attenuation in the development of atherosclerosis in vivo.[18]

Both vaccinations result in the formation of specific antibodies and attenuation of atherosclerosis. Despite the successful results in animals, protein vaccination has some drawbacks. Large quantities of protein (antigen) have to be synthesized or isolated, and its storage and production are rather expensive processes. Furthermore, immunomodulatory components need to be added, such as adjuvants and immune dominant T-cell epitopes, in order to break tolerance and to get an appropriate immune response. The addition of these immunomodulatory components might induce a risk for side effects. In our opinion, methods without adjuvant addition are, therefore, more favorable in the clinic as very few traditional adjuvants are approved by the US FDA for human use.[19,20]

Besides these considerations, there is also the risk of side effects related to blocking the bioactive molecules. For example, blocking CETP may increase HDL levels and a clinical trial demonstrates that this may cause serious problems, resulting in high blood pressure and increased deaths and cardiovascular events.[21] Even more riskful is the neutralization of immunoregulatory molecules, such as IL-12. Blocking IL-12 will impair a proper host defense against several pathogens not related to atherosclerosis.

PROTEIN VACCINATION: ELIMINATION/BLOCKADE OF DISEASE-RELATED PROTEINS

The most preferred vaccination strategy is to develop a vaccine that induces a protective immune response against disease-specific autoantigens. Vaccination against a glycosylated collagen type II peptide reduces active arthritis,[22] and epicutaneous immunization with type II collagen inhibits collagen-induced arthritis.[23] Furthermore, vaccination against an Alzheimer-specific peptide (Aβ1–11) protects against Alzheimers disease[24] and epicutaneous immunization with myelin basic protein protects against EAE.[25]

In atherosclerosis, many different antigens and epitopes that induce the immune response are known. Interesting candidates are indicated by the presence of naturally occurring antibodies against ox-LDL or HSPs in patients with cardiovascular diseases or suffering a carotid artery occlusion. [26,27] Vaccination against these more disease-specific molecules is often called immunization (active immunization).

Immunization against HSP65 resulted in an enhanced fatty-streak formation in C57BL/6 mice and it promoted early atherosclerosis in $LDLr^{-/-}$ mice. In both cases, an increase in anti- HSP65 antibodies was found and it was, therefore, suggested that these antibodies are pro-atherogenic.[28,29] By contrast, immunization against modified LDL induced a protective antibody response (both IgM and IgG). IgM antibodies are shown to be protective by blocking the uptake of ox-LDL via scavenger receptor CD36, thereby reducing foam-cell formation. The role of specific IgG antibodies against ox-LDL is, however, still not understood.[30] Lopes-Virella et al. showed a correlation between increased ox-LDL-specific IgG levels and the development of coronary artery disease.[31] Fredrikson et al., however, showed that activation of Th2-specific antibodies (IgG1) as a result of immunization with MDA-modified ApoB100 peptide sequences protects against atherosclerosis.[32,33] Binder et al. showed that active immunization of atherosclerosis-prone mice against Streptococcus pneumoniae led to a high level of IgM antibodies against phosphorylcholine (PC), owing to molecular mimicry and a reduction in the degree of atherosclerosis.[34] Subsequently, atherosclerosis-prone mice immunized with PC displayed smaller atherosclerotic lesions and an increase in serum titers of anti-PC IgG and IgM. In this study, it is again suggested that anti-PC antibodies, at least partially, blocked ox-LDL uptake by macrophages. So the decline in foam-cell formation may contribute to the reduction in atherosclerotic lesion development.[35] More research must be performed to determine the exact mechanism of action of these autoantibodies.

Although very effective, immunization against disease-specific antigens has a disadvantage owing to the use of adjuvants. Therefore, as stated above, strategies without adjuvant addition are more favorable. It would be an

improvement when the immunization results in long-term protection. Then you would only need one injection, taken once within a certain period, for example 10 years, as is the case with several general vaccination protocols. This will further reduce the incidence of the negative side effects.

DNA VACCINATION

To overcome the obstacles with protein vaccination, a new approach has been used, DNA vaccination. This method uses a eukaryotic expression vector that encodes the antigen of interest. In accordance with protein vaccination, an immunomodulatory component must be added to enhance the immunogenecity. Therefore, the DNA of the antigen is altered or coupled to bacterial motifs, coding regions for interleukins, or costimulatory molecules. Furthermore, the plasmid can be transfected to an attenuated pathogen and subsequently be administered to the patient. This approach induces an enhanced immune response and, thus, functions both as an adjuvant and a carrier at the same time.

The DNA-vaccination technique is already applied in mice models for other autoimmune diseases. Vaccination with DNA constructs encoding MOG and PLP resulted in a reduction of EAE in mice,[36] while vaccination with plasmids containing human TNF-α DNA elicits the production of protective antibodies against TNF-α and prevents collagen-induced arthritis in mice.[37] We recently used a DNA-vaccination strategy in which an attenuated Salmonella typhimurium containing a VEGF-receptor (VEGFR) 2-encoding plasmid is administered orally to induce a cytotoxic CD8+ T-cell response against this molecule. The specific CD8+ T cells target activated endothelial cells within the damaged endothelial lining, which results in the subsequent removal of these cells and in a reduction in atherosclerotic lesion formation.[38] DNA-vaccination techniques are very promising, but again, many of the side effects against self antigens seen with protein vaccination occurs with DNA vaccination. For example, blocking VEGFRs might impair angiogenesis and vasculogenesis, which causes bleedings and abnormalities to the vasculature.

It should be taken into account that with DNA vaccination, some posttranslational modifications might be different when compared with the native protein expression. In addition, the epitopes may be altered and the specific recognition may be ameliorated. However, the main advantage of DNA vaccination is that it is relatively fast and easy to develop the vaccine as compared with the labor-intensive isolation of proteins and antibodies.

PASSIVE IMMUNIZATION

Another vaccination strategy is passive immunization, in which specific antibodies are injected. This approach has already been applied in the clinic as treatment for rheumatoid arthritis.[39] Treatment with anti-PC IgM antibodies inhibited accelerated atherosclerosis in a vein graft in atherosclerosis-prone ApoE-/- mice.[40] A human IgG1 antibody against a MDA-modified ApoB100 peptide is protective against atherosclerosis an ApoE-/- mice. Low numbers of the MDA-ApoB100 peptide were found in plaques of the treated mice and it is suggested that the injected IgG antibodies inhibited the uptake of ox-LDL in the lesion and/or facilitated the removal of ox-LDL from the circulation or plaques.[41]

Passive immunization may be a safer and more controllable treatment compared with active immunization (DNA and protein). Furthermore immunocompromised patients may benefit from passive immunization. A disadvantage is that the treatment is quite expensive and labor-intensive. Humanized antibodies are needed before patients can be treated with these antibodies.

TOLERANCE INDUCTION

The last method of vaccination discussed here is tolerance induction. Tolerance induction is a relatively new vaccination strategy in which the vaccine is administered orally or nasally without the addition of adjuvants or other immunomodulatory components. Tolerance can be induced against several autoantigens, such as type II collagen peptide 250–270, which protects against collagen-induced arthritis via the induction of specific cellular and humoral immune responses.[42] Low-dose oral tolerance can also be induced against a MOG peptide, resulting in decreased EAE in mice.[43] Several years ago, oral and nasal tolerance against HSP60/65 was shown to successfully suppress early atherosclerosis.[44,45] Some years later, oral-tolerance induction against β2-glycoprotein I also proved to be protective against atherosclerosis.[46] In all these studies, an increased production of anti-atherogenic cytokines was observed, but the underlying mechanism remained to be clarified.

Recently, we performed two studies in which we demonstrated that oral administration of HSP60, a small HSP60 peptide (253–268) and ox-LDL resulted in a reduction in atherosclerosis.[47,48] These are the first studies on oral vaccination that demonstrate the suppression of atherosclerosis is due to the induction of Tregs. In both cases, we found an initial increase in numbers of Tregs in the Peyer's patches. Subsequently, an increase in Treg numbers is observed in blood, mesenteric lymph nodes and spleen 2 weeks after oral vaccination. We now suggest that the Tregs migrate from the Peyer's patches via blood to the secondary lymphoid organs. In

addition, an increase in Tregs is observed within the atherosclerotic lesion, and mesenteric lymph node cells produced more IL-10 and TGF-β upon restimulation with the antigen.

Oral, but also nasal vaccination in the context of tolerance induction may be very effective in reducing atherosclerosis. Recently, it was shown that injections with anti-CD25 antibodies, thereby depleting Tregs, resulted in an increased lesion size in ApoE-/- mice.[49] Depletion of costimulatory molecules such as CD-28, -80 and -86, also resulted in a reduction in the number and function of Tregs and subsequently in higher atherosclerosis susceptibility.[49]. On the other hand, treatment of LDLr-/- mice with anti-CD3 antibodies, stimulating the activation and proliferation of Tregs, reduced the development and progression of atherosclerotic lesions.[50] Furthermore, a transfer of antigen-specific Tr1 cells, a subset of Tregs, elevated IL-10 levels in ApoE-/- mice and reduced lesion formation.[51] In addition, injection of in vitro-generated HSP60-specific Tregs prevented atherosclerotic lesion development.[52]

However, some important questions remain unanswered. What is the exact mechanism involved in Treg suppression of pro-atherogenic immune responses? Do Tregs act via cell–cell contact, do they act cytokine-dependently or a combination of these two pathways? Do these Tregs suppress the immune responses within the lymphoid organs such as lymph nodes and spleen or do they act within the lesion? Finally, the opportunity to manipulate Treg responses as a treatment for atherosclerosis requires a better characterization of the antigens that pro-atherogenic T cells recognize. In addition, the antigens that cause activation and proliferation of Tregs that migrate into atherosclerotic lesions or draining lymphoid tissues need to be characterized.

CONCLUSION

In summary, a number of different vaccination strategies have been applied in atherosclerosis research during the last 10 years (Table 1). One of the first strategies was the targeting of pro-atherogenic interleukins that play an important role in a normal host defense, such as IL-12. This strategy can be subdivided into protein and DNA vaccinations, and it has been demonstrated to be successful in experimental autoimmune encephalomyelitis and may, therefore, also be successful in atherosclerosis.[5]

TABLE 9.1: AN OVERVIEW OF THE DIFFERENT VACCINATION STRATEGIES THAT HAVE BEEN APPLIED IN ATHEROSCLEROSIS RESEARCH DURING THE LAST 10 YEARS.

IMMUNOMODULATION METHOD	TARGETS	ADJUVANS	PHARMA-CEUTICAL APPLICABILITY	PATIENTS DISCOMFORT	EXPECTED SIDE EFFECTS	REF.
Protein vaccination						
Bioactive molecules	**IL-12**	**+**	**-**	**+**	**+**	**16**
	CETP	**+**	**-**	**+**	**+**	**18**
Disease related proteins	**Ox-LDL**	**+**	**-**	**+**	**±**	**30**
	MDA-modified $ApoB_{100}$	**+**	**-**	**+**	**±**	**32,33**
	Streptococcus pneumoniae	**+**	**-**	**+**	**±**	**34**
DNA vaccination	**VEGF-receptor 2**	**-**	**±**	**+**	**+**	**38**
Passive immunization	**PC**	**-**	**-**	**±**	**-**	**40**
	MDA-modified $ApoB_{100}$	**-**	**-**	**±**	**-**	**41**
Tolerance induction	**HSP60/65**	**-**	**+**	**-**	**-**	**44,45,47**
	β2-glycoprotein I	**-**	**+**	**-**	**-**	**46**
	HSP60 peptide	**-**	**+**	**-**	**-**	**47**
	Ox-LDL	**-**	**+**	**-**	**-**	**48**

Passive immunization uses (humanized) antibodies to block the pro-inflammatory action of specific cytokines or antigens. However, selection and production of these specific humanized antibodies or the production of proteins for protein vaccination is very labor intensive and expensive as compared with DNA vaccination. It is challenging for the industry to synthesize humanized antibodies that are appropriate for administration to patients in, for example, 4-week intervals that can modulate the function of the pro-inflammatory cytokines or antigens.

We conclude that the optimal approach to introduce a vaccine against atherosclerosis is to focus on atherosclerosis-specific components. This can be achieved by either immunization, resulting in the production of protective antigenspecific antibodies, or by mucosal tolerance induction, resulting in the induction of antigenspecific Tregs. Atherosclerosis is a complex disease in which several antigens – many of which are not yet characterized – play an important role. A vaccination that results in the generation of antibodies that are limited in time in their functioning and in which a cocktail of the different antigens is administered may be an attractive approach. This cocktail can either be injected

intravenously or intraperitoneally to immunize against the antigens, or it can be administered orally or nasally to induce protective Tregs against these antigens.

A major consideration when developing a new therapeutic treatment is the inconvenience for the patient. Repeated injections and invasive treatment and, thus, repeated visits to the clinic are not favorable, but may not form a major problem. A better, more promising approach may be to supplement the vaccine to the individual's diet. This is convenient for the patient and easy to apply every day or once a week. The best vaccination strategy will be one that results in plaque stabilization in addition to reducing plaque development. Since atherosclerosis prevails at a very young age but only manifests itself later in life, the majority of patients will not benefit from strategies that focus on early plaque development, but will benefit from a vaccination strategy early in life that prevents the development of lesions.

FUTURE PERSPECTIVE

A number of different strategies can be developed to protect people against atherosclerosis or to treat patients with clinical symptoms thereof. In our opinion, DNA and protein vaccination in experimental models for atherosclerosis will form the initial strategy to investigate the possibility of whether vaccination against the target protects against atherosclerosis. The studies described in this article investigated the effect of vaccination and immunomodulation on the development of atherosclerosis. It would be favorable for the clinical application that a vaccination against a specific molecule increases the plaque stability, and we foresee that this can be achieved by short-term vaccination with proteins or DNA or a short-term treatment with humanized antibodies. In the next 5–10 years, we expect to see an increase in studies using protein and DNA vaccination as a strategy to investigate the blockade of the molecule of interest in experimental models for atherosclerosis, with specific interest in pro-atherosclerotic cytokines.

Treatment with protective antibodies is a very powerful strategy and a may form a good achievable alternative for protein or DNA vaccination. This technique is nonpermanent, controllable and side effects may be limited. Because the antibodies have a defined half-life once injected, one can determine the exact administration protocol and the optimal timing for repetitive injections. This strategy is, however, very expensive given that it may also be needed to treat the majority of the population given the widespread occurrence of cardiovascular disease, although this approach is attractive for pharmaceutical companies.

In our opinion, the strategy with the best future perspective is oral-tolerance induction. This vaccine is relatively cheap, easy to administer and no adjuvants or immunomodulatory components are necessary. The risk for side

effects appears minimal and the patient can easily be 'vaccinated' by processing the tolerant agent in food or drinks. Nowadays, a few antigens – ox-LDL, HSP60 and β2 glycoprotein I – have been used to induce oral tolerance in atherosclerosis. We expect that in the near future a great deal more possible antigens will be tested in oral tolerance. The best strategy would be to use a cocktail of different antigens as a powerful vaccine. However, a lot of research is still necessary before this strategy will be applicable in the clinic, and several questions remain unanswered. How to obtain the adequate antigens for tolerance induction? Should we use, for example, the ox-LDL from the patients themselves? Which concentration is optimal in humans? Although these results look very promising, it should be mentioned that all these experiments are performed in mice models for atherosclerosis. In order to extrapolate these data, they need to be verified in the human situation.

Therefore, further research is required and we expect that in the next couple of years the first clinical trials will be performed using both antigen-based vaccines (oral tolerance induction) and recombinant IgGs targeting epitopes in ox-LDL. For a lot of people, vaccination against atherosclerosis is still a myth, but it may become reality when one of the above described strategies turns out to be successful in reducing atherosclerosis without severe side effects.

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Chapter 10

Summary and perspectives

Thomas van Es

SUMMARY AND PERSPECTIVES

Atherosclerosis, the predominantly underlying pathology of cardiovascular events, is the consequence of lipid deposition in the arterial wall, mostly as consequence of high levels of serum cholesterol. Treatment of atherosclerosis is mainly focused at the reduction of cholesterol levels by lipid lowering medication, such as statins.

Despite the use of statins and prophylactic treatments, such as a reduction in blood pressure and a reduction in risk factors to prevent atherosclerosis, cardiovascular disease is still the major cause of death in the Western world.[1-3] As the struggle against atherosclerosis continues and its prevalence is increasing in the world, it is pivotal to find new targets for implementing new strategies against atherosclerosis.

Inflammation is considered to be a major component in the process of atherosclerosis and is involved in initiation, progression and destabilization of the atherosclerotic lesion. Although the beneficial effects of statins on atherosclerosis may partly be ascribed to their anti-inflammatory properties, relatively little is known about the exact mechanism and contribution of different inflammatory cells and products in atherosclerosis. The aim of this thesis was to further elucidate the contribution of various components of the inflammatory response in atherosclerosis and thereby finding new intervention points to reduce the incidence and the consequences of atherosclerosis.

INTERLEUKIN 15: BRIDGING THE INNATE AND ADAPTIVE IMMUNE SYSTEM

Macrophages are key inflammatory cells in the process of atherosclerosis and play an important role in the development of the atherosclerotic lesions, by taking up modified LDL via pattern recognition receptors (PRRs). This results in activation of macrophages and subsequently in production of inflammatory mediators, such as cytokines.

One of the cytokines produced by macrophages is IL-15. Expression of IL-15 is detected in human and murine atherosclerotic lesions.[4, 5] IL-15 enhances T cell recruitment and activation of peripheral blood T cells within the plaque.[6] In chapter 2 the role of IL-15 in atherosclerosis is demonstrated by targeting IL-15 overexpressing cells, using DNA vaccination, in LDL receptor deficient mice. The increase in number of IL-15 expressing cells in the circulation and spleen upon induction of hypercholesterolemia suggests that IL-15 is involved in atherosclerotic plaque formation. To further elucidate the role of IL-15 in atherosclerosis, LDLr$^{-/-}$ mice were vaccinated against IL-15 by using an attenuated *Salmonella typhimurium*

carrying an IL-15 encoding plasmid. In mice vaccinated against IL-15 a 75% reduction of the atherosclerotic plaque is observed in the carotid artery.

Interestingly, we observed not only 75 % decrease in plaque size, but also an increase in relative macrophage content within the atherosclerotic plaque of mice vaccinated against IL-15, indicative for a less advanced state of the lesions in the vaccinated mice. IL-15 is, in association with IL-15Rα, also expressed in its biologically active form on the surface of monocytes and activated macrophages. This surface expressed IL-15 is approximately five times more effective than soluble IL-15 in the induction of T cell proliferation.[7-9]We speculate that the macrophages in the lesions of IL-15 vaccinated mice do not, or to a lesser extent, express IL-15. This is supported by the observation that the number of IL-15 expressing cells in the circulation was declined to the same level as before the induction of hypercholesterolemia. Blocking the IL-15 pathway may result in an impairment of the innate switch towards an adaptive immune response thereby attenuating atherosclerosis.

The vaccination strategy used in this study and described in chapter 2, successfully evoked a cytotoxic response targeting IL-15 expressing cells. This resulted in a vast reduction in atherosclerosis, thereby providing new insights in the process of atherosclerosis and the contribution of IL-15 in this process. These new insights may contribute to the future immunomodulating treatment of patients with cardiovascular disease.

A NEW PLAYER IN THE FIELD, THE TH17 CELL

Macrophages exert an important bridge function between the innate and adaptive immune response. Activated macrophages are also able to present antigens in association with MHC class-II molecules to T cells. Depending on the microenvironment, T cells can develop in different subsets and T cells are able to either aggravate or attenuate atherosclerosis. Already in 1986 Mosmann *et al.* described two distinct Th subsets, Th1 and Th2 cells, with their own specific production of cytokines.[10] An imbalance between these cells is often related to autoimmune diseases, such as atherosclerosis. An exaggerated pro-inflammatory response due to an overexpression of Th1 associated cytokines, like IL-12 and IFN-γ, enhances atherosclerosis.[11] It was speculated that the restoration of the Th1/Th2 balance may attenuate atherosclerosis. However, a deficiency in IL-4, a typical Th2 interleukin, reduced atherosclerosis.[12] Moreover, IL-4 is associated with increasing MMP-1 production by macrophages and may therefore have negative effects on plaque stability.[13] These data indicate that the Th1/Th2 balance does not appropriately describe the immune responses in atherosclerosis. Therefore researchers looked for different inflammatory and regulatory pathways.

One of these pathways is related to the recently identified T cell subset, the Th17 cell. These cells provide more complexity and may provide a better understanding in the process of atherosclerosis. Th17 mainly produce IL-17, although IL-22 and IL-21 are also produced in large quantities.[14,15] Recently, newly obtained data and insights resulted in a more complex model where TGF-β and IL-6 initiate Th17 development.[16] This is interesting as TGF-β is also required for Treg cell differentiation and partly mediates the suppressive function of Treg cells.[17,18] Furthermore, Tregs and TGF-β are associated with reduced atherosclerosis.[19-22]

The role of Th17 cells and its hallmark, IL-17, is not known in the context of atherosclerosis. Therefore we studied the role of IL-17 in atherosclerosis as described in chapter 3. In this study we observed that IL-17 expression within the spleen of LDLr$^{-/-}$ mice does increase under hypercholesterolemic conditions. This indicates that IL-17 may be involved in the initiation of atherosclerosis. To unravel the role of IL-17 in atherosclerosis we used DNA vaccination to block IL-17. The DNA vaccine used, encodes the IL-17 coding sequence, preceded by a specific part of the HEL sequence, which binds with high affinity to MHC class II and is able to activate T cells, thereby breaking tolerance.[23,24] Vaccination against IL-17 results in a 90.2% decrease in atherosclerotic lesion formation in the carotid artery, indicates that IL-17 has profound pro-atherosclerotic properties.

Since IL-17 has also pleiotropic effects on several cell types within the atherosclerotic lesion, IL-17 may stimulate a pro-inflammatory environment resulting in aggravation of atherosclerosis.[25,26] An important matrix metalloproteinase (MMP) in atherosclerosis is MMP-9,[27] which is upregulated in macrophages upon IL-17 stimulation. However, the plaques of the HEL-IL-17 vaccinated mice were too small to accurately determine plaque stability and MMP-9 expression. Therefore more research has to be performed to study the effect of IL-17 on more advanced atherosclerotic lesions.

We set out to investigate the role of the IL-17 receptor in atherosclerosis, by performing a bone marrow transplantation (BMT) with IL-17 receptor deficient (IL-17R$^{-/-}$) bone marrow (BM) in LDLr$^{-/-}$ recipient mice. In this study (described in chapter 4) we further establish the pro-atherosclerotic role of IL-17, since IL-17R$^{-/-}$ BM recipients demonstrated a 46% reduction in plaque size. The reduced lesion size relates to a reduced number of IL-17R expressing cells in blood after IL-17R$^{-/-}$ BMT. Furthermore, spleen cells from IL-17R$^{-/-}$ BM recipient mice demonstrate a decreased IL-6 and an increased IL-10 production. This indicates a shift towards a more anti-inflammatory response, which favors a beneficial outcome for atherosclerosis. Interestingly, we detected a decrease in IgG auto antibodies against modified LDL. This is likely due to an impaired B cell regulation in the germ line centre (GC) as a consequence of the disturbed IL-17 pathway.[28]

Based on the obtained results described in chapter 3 and 4, we continued to study whether Th17 cells, the prominent IL-17 producing T cell, are involved in atherosclerosis. An important interleukin in the development of Th17 cells is IL-23. IL-23 is a heterodimeric interleukin consisting of a p40 and a p19 subunit and is closely related to its family member IL-12 by both sharing the p40 subunit (Figure 10.1).[29] In our laboratory, we already demonstrated that vaccination against p40 (IL-12/IL-23) results in a reduction of almost 70% in atherosclerotic lesion formation.[30] However, p40 is shared by both IL-12 and IL-23 and consequently it does not elucidate the individual contribution of IL-12 and IL-23 on atherosclerosis (Figure 10.1).

To unravel the role of IL-23, we performed a BMT using $p19^{-/-}$ mice, which do not express functional IL-23 (chapter 5). Although the BMT was successful, there was no change in lesion size detected between $p19^{-/-}$ BM recipients and mice receiving control BM. Furthermore, BMT with $p19^{-/-}$ BM does not result in a decrease of Th17 cells. These results may partially be explained by data form other research groups, which state that IL-23 acts more as a stabilizing factor for Th17 cells in stead of initiating Th17 cells.[14] Another possibility is the observation that $CD3^+$ T cells are able to express the p19 subunit (Lubberts *et al.*, personal communication). Whether p40 is also expressed by these T cells to form functional IL-23, remains to be elucidated.

Interestingly, unpublished reports of S. Pflanz & R.A. Kastelein mention a heterodimeric interleukin consisting of p19 and EBI3 *in vitro*.[31] Whether these heterodimers may be formed *in vivo* and what function this new interleukin exerts, remains to be elucidated (Figure 10.1).

Another question which remains is, whether the $p19^{-/-}$ BMT results in an increased formation of p40 homodimers (12p80). 12p80 can act as an IL-12 and an IL-23 antagonist by binding to the IL-12Rβ1 but it can not mediate a biological response.[32-34] However, other reports illustrate an agonistic function of 12p80 by stimulating the differentiation of $CD8^+$ cytotoxic cells and $CD4^+$ IFN-γ producing cells.[35] The connection of these new insights with our data has to be elucidated in future research.

NEW T CELL SUBSETS, NEW BALANCES

IL-27 is a recently discovered interleukin that structurally resembles IL-12 and IL-23. IL-27 is a heterodimeric cytokine composed of Epstein-Barr virus induced gene 3 (EBI3) and p28 and it is important in the regulation of T cell function and cytokine production (Figure 10.1).[36] Interestingly, IL-27 was firstly described as a pro-inflammatory interleukin by Goldberg *et al.* in EAE and RA, both autoimmune

diseases.[37, 38] More recently, IL-27 has been associated with suppression of Th17 cell and may thereby attenuate diseases such as EAE.[39]

Little is known about IL-27 and to our knowledge the role of IL-27 in atherosclerosis is not studied yet. Therefore, we studied the role of IL-27 in relation to atherosclerosis, which is described in chapter 6. The expression of both IL-27 subunits (EBI3 and p28) was upregulated in the atherosclerotic plaques of LDLr$^{-/-}$ mice. The fact that both subunits are upregulated suggests the production of functional IL-27, since p28 alone cannot be excreted without EBI3.[40] After vaccination against p28, the lesion size in the vaccinated mice increased, which indicates that IL-27 has a protective role in atherogenesis.

Additionally, we demonstrate that blocking IL-27 was associated with a reduced number of Treg cells and with an augmented number of Th17 cells within the spleen. These observations are of great interest as they demonstrate a shift in the balance between the pro-inflammatory Th17 cells and the anti-inflammatory Treg cells, thereby aggravating atherosclerosis. Furthermore, is has been shown by other groups that there is a balance between Th17 and Treg cells, thereby adding another layer of regulation in the inflammatory response.[16, 41-44]

The results described in chapter 6 demonstrate that blocking IL-27 results in aggravated atherosclerosis. Therefore is would be interesting to over-express IL-27 in LDLr$^{-/-}$ mice to study whether this reduces Th17 cells and induces Treg cells, resulting in attenuated atherosclerosis.

ROLE OF TREG CELLS IN ATHEROSCLEROSIS

Treg cells are important in maintaining immune homeostasis and preventing autoimmunity.[45] Based on the observations that a disturbed balance between Th17 and Treg cells aggravates atherosclerosis, further research was performed to establish the role of Treg cells in atherosclerosis. Mallat *et al.* hypothesized that there is a balance between pathogenic T cells and Treg cells. Furthermore, Puijvelde *et al.* demonstrated that oral administration of an atherosclerosis associated antigen, oxLDL, leads to the induction of Treg cells and subsequently ameliorates atherosclerosis.[21] Along this line we studied another atherosclerotic associated antigen, HSP60, to induce tolerance, which is described in chapter 7.

Tolerance against HSP60 and a small conserved sequence of HSP60(253-268) was induced by oral administration with a low dose of the antigen. The induction of tolerance upon oral administration of either HSP60 or a HSP60 derived peptide results in a significant reduction of 80.7 -83.3% in lesion size. Furthermore, tolerance induction results in an increased Foxp3^{+} Treg cell population, indicating a systemic effect of the tolerance induction. Upon stimulation of spleen cells with HSP60, the production of IL-10 and TGF-β

increased in HSP60-treated mice. Moreover, we observed a decreased proliferation when spleen cells were stimulated with HSP60. These results indicate that tolerance against HSP60 evoked an immune suppression mediated by Treg cells.

Of great interest is the increased expression of CTLA-4, Foxp3 and CD25 within the atherosclerotic lesion, which indicates that Treg cells migrated from the regional lymph nodes towards the local inflamed tissue and exert their immunosuppressive effect, explaining the reduced atherosclerotic lesion size in HSP60 treated mice.[46]

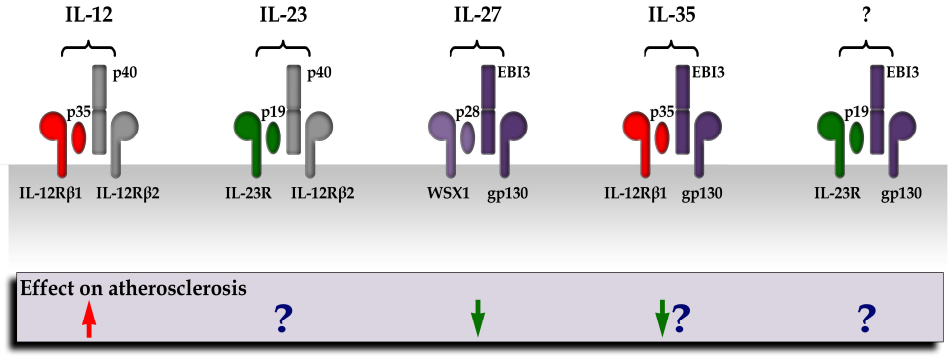

FIGURE 10.1 A SCHEMATIC OVERVIEW OF THE DIFFERENT INTERLEUKINS AND RECEPTORS DESCRIBED IN THIS THESIS. IL-12, consisting of p35 and p40, is already established as a pro-atherosclerotic interleukin. Its close family member IL-23, which consists of p19 and p40, is less defined in atherosclerosis. Although an aggravating role is suspected, based on the role of IL-23 in other autoimmune disease, more research on atherosclerosis in relation to IL-23 has to be performed. Although IL-27, consisting of p28 and EBI3, is already described in 2002, its pleiotropic effects are not completely understood yet. In this thesis we observed a protective role for IL-27 in initial atherosclerosis, however more information is needed to elucidate its exact role in atherosclerosis. Recently, a novel interleukin is described consisting of p35 (shared with IL-12) and EBI-3 (shared with IL-27). This interleukin is called IL-35 and has been associated with immune suppression. Interestingly, IL-35 is probably an exclusive product of Treg cells whereas IL-27 and IL-12 are predominantly expressed by APCs. Finally, unpublished results of S. Pflanz & R.A. Kastelein describe a novel heterodimeric interleukin, consisting of p19 (shared with IL-23) and EBI3 (shared with IL-27). Thus far, this heterodimer has been detected *in vitro*. Whether this interleukin exists *in vivo* and if it has a biological function, has yet to be determined (More details and references see text).

Treg cells which originate from the thymus constitutively express Foxp3 and Tregs may also acquire foxp3 after oral tolerance. Treg cells play an essential role in immune homeostasis.[21,22,47-50]. To study the effect of the elimination of Foxp3$^+$ Tregs in atherosclerosis, we used a novel vaccination strategy directed against Foxp3 expressing cells. The effect of Foxp3 elimination on the initial atherosclerosis and on advanced atherosclerosis is described in chapter 8. A dendritic cell (DC) based vaccine was used to provoke a cytotoxic T cell response against Foxp3 expressing cells.

During the time course of the experiment we observed significant less Foxp3$^+$ Treg cells in the circulation of Foxp3 vaccinated mice. Also within the lymphoid tissues the Foxp3$^+$ cells are significantly reduced. Furthermore, vaccination against Foxp3 results in an increase of atherosclerotic plaque formation in both initial and advanced atherosclerosis and the lesions have an increased cellularity. This may suggest that an impaired inhibition of pathogenic T cells within the plaque upon depletion of Treg cells. However, more research has to be performed to investigate which cells are responsible (e.g. Th1, Th2 or Th17 cells) for the increased cellularity and plaque size. The results from this study further established the role of Foxp3$^+$ Treg cells in atherosclerosis and are in line with the results of Ait-Oufella *et al*. [51]

TH17/TREG BALANCE, A NEW INTERVENTION POINT

In this thesis, we demonstrate in chapter 6 that blocking of IL-27 results in an increased population of Th17 cells and in a decreased number of Treg cells. The shift in Treg/Th17 balance favoring Th17 cells, results in an increased lesion size. This observation indicates that manipulating the balance between these cells may provide a potential therapeutic intervention point. This is further supported by the observation that vaccination against Foxp3 aggravates atherosclerosis, which is described in chapter 8. On the other hand, when Treg cells are induced via oral administration of atherosclerotic specific antigens, this results in ameliorated atherosclerosis as is described in chapter 7.

Therefore, therapeutic approaches must be aimed at inducing Treg cell specific for atherosclerosis specific antigens. This possibility is discussed in more detail in chapter 9, were vaccination is evaluated as a potential therapy against atherosclerosis. Furthermore, based on the results in chapter 6, overexpressing IL-27 or administration of recombinant IL-27 may also prove to be a new approach to shift the balance towards Treg cells favoring a beneficial outcome for atherosclerosis.

VACCINATION AGAINST INFLAMMATORY MEDIATORS

Besides interfering in the T cell balance, vaccination against interleukins involved in atherosclerosis may also provide an interesting strategy for therapeutic intervention. IL-17, described in chapter 4, and IL-15, described in chapter 2, are potential candidates that can be blocked via vaccination. In chapter 9 DNA vaccination as a therapeutic approach is discussed. Although vaccination against self-antigens may provide a potential therapy to redirect the inflammatory response, additional studies have to be performed to establish the long-term effect

of these vaccination strategies. Independent of the therapeutical implications, DNA vaccination strategy provides an excellent research tool to block endogenous proteins with relatively low costs. This can be used to study, for example the role of interleukins in different stages of atherosclerotic disease.

CONSIDERATIONS

Most studies on atherosclerosis in mice are performed on the initial phase of atherosclerosis and are thus not necessarily relevant to advanced and complicated lesions. Most patients with clinical symptoms do however already posses large and complicated atherosclerotic lesions. Therefore treatment should also be focused at stabilizing existing plaques, reducing progression of plaques or inducing plaque regression. Intervention at Treg cell level, by inducing Treg cells, may fulfill this promise. Puijvelde *et al.* demonstrated that mice, which are tolerized against oxLDL display attenuated lesion progression.[21] The role of Treg cell depletion in advanced atherosclerosis was further demonstrated in chapter 8 of this thesis.

These data suggests that regulation of the Treg cells may be an attractive clinically relevant target to further develop an immunotherapy to treat atherosclerosis in the near future.

To add one final remark, recently a new interleukin is discovered, which consists of two subunits and both subunits are, direct or indirect, studied in this thesis. This novel interleukin is named IL-35 and consist of p35 (subunit of IL-12) and EBI3 (subunit of IL-27) and is thereby another member of the IL-12 family (Figure 10.1). In contrast to other known IL-12 family members, IL-35 has anti-inflammatory properties instead of immunostimulatory or proinflammatory features.[52-54] IL-35 is produced by Treg cells and contributes to the suppressive activity of these cells.[52-54] Therefore IL-35 may represents a novel potential future target for the therapeutic intervention in atherosclerosis.

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Nederlandse samenvatting

Thomas van Es

ADERVERKALKING

Hart- en vaatziekten zijn de voornaamste doodsoorzaak in de Westerse wereld. Het overtreft de aan kanker gerelateerde doodsoorzaken. Patiënten met hart- en vaatziekten ervaren pas op latere leeftijd de klinische symptomen zoals angina pectoris (drukkend/pijnlijk gevoel op de borst) of een infarct (hartaanval, beroerte). Deze symptomen zijn gerelateerd aan een vernauwd of geblokkeerd bloedvat, waardoor de bloedtoevoer naar distaal gelegen weefsel geblokkeerd of gelimiteerd wordt. De vernauwingen zijn het gevolg van een chronische ontsteking in het bloedvat wat resulteert in het ontstaan van een laesie. Deze laesie bestaat uit ontstekingscellen en cholesterol deposito's. Dit proces staat bekend als aderverkalking, ook wel atherosclerose genoemd.

Atherosclerose begint al vroeg in het leven doordat er cholesterol wordt afgezet in de vaatwand en dit vormt de zogenoemde "fatty streak". Dit leidt echter niet tot klinische symptomen, omdat deze fatty streaks nog niet tot een obstructie van de doorbloeding leidt. Gedurende het leven worden deze laesies groter en dit kan uiteindelijk leiden tot klinische symptomen. De leeftijd waarop de symptomen optreden is in grote mate afhankelijk van de blootstelling aan risicofactoren. Bekende risicofactoren zijn overgewicht, een hoog bloed cholesterolgehalte, roken, hoge bloeddruk en te weinig lichaamsbeweging. Daarnaast dragen ziektes zoals diabetes en reuma ook bij aan een verhoogd risico voor hart- en vaatziekten. Het is dan ook niet verwonderlijk dat hart- en vaatziekten voornamelijk in de "Westerse wereld" voorkomen.

Op dit moment is behandeling van atherosclerose voornamelijk gericht op het verminderen van risicofactoren. Een veel voorgeschreven behandeling is de toediening van statines. Statines zijn in staat om het cholesterol gehalte te doen verlagen en in combinatie met aanbevolen lichaamsbeweging resulteert dit in een verbeterde klinische uitkomst. Ondanks deze behandeling blijft het sterftecijfer, dat gerelateerd is aan hart- en vaatziekten, erg hoog. Daarom is er ook een grote noodzaak om nieuwe behandelmethodes te ontwikkelen.

ONTSTEKING EN ADERVERKALKING

Recentelijk is geconstateerd dat het immuunsysteem (de cellen en organen die betrokken zijn bij een ontstekingsreactie) ook een grote bijdrage levert aan atherosclerose. Aangezien statines naast een cholesterol verlagende werking ook een ontstekingsremmend effect hebben, zou dit dus deels hun gunstige effect op atherosclerose verklaren.

De ontstekingsreactie tijdens atherosclerose begint wanneer de vaatwand beschadigd raakt. Beschadiging gebeurt voornamelijk op plaatsen in het bloedvat

waar een turbulente bloedstroom is, bijvoorbeeld bij een aftakking van een bloedvat. Door deze beschadiging raakt de vaatwand geactiveerd en wordt deze meer doorlaatbaar voor cholesterol vervoerende componenten, ook wel lipoproteïnes genoemd. De lipoproteïnes worden in de vaatwand gemodificeerd waardoor deze immunogeen worden en dus in staat zijn het immuunsysteem te activeren. Vervolgens worden macrofagen aangetrokken door de geactiveerde vaatwandcellen (endotheelcellen) en de gemodificeerde lipoproteïnes. Macrofagen zijn ontstekingscellen die in staat zijn om ongewenste deeltjes zoals de gemodificeerde lipoproteïnes op te nemen, waarna deze geactiveerd raken en allerlei signaaleiwitten produceren (cytokines). De cytokines op hun beurt rekruteren andere immuuncellen en activeren deze cellen, zodat zij hulp kunnen bieden om de ongewenste deeltjes te verwijderen. In de vaatwand nemen de macrofagen de lipoproteïnes op en hierdoor veranderen de macrofagen langzaam in opgezwollen cellen die gevuld zijn met cholesterol, de “schuimcellen”. Deze schuimcellen produceren veel ontstekingsstimulerende cytokines.

Een belangrijk celtype dat gerekruteerd wordt is de T-cel. Na activatie, kunnen T-cellen zich ontwikkelen in verschillende subklassen T-cellen. De verschillende typen T-cellen kunnen het immuunsysteem verder activeren of juist remmen. Een belangrijke functie van macrofagen is het “presenteren” van de opgenomen deeltje (antigenen) aan T-cellen. T-cellen die het antigeen herkennen worden geactiveerd en maken vervolgens allerlei cytokines om het ontstekingsproces in goede banen te leiden. In het geval van atherosclerose zijn er te veel ontstekingstimulerende T-cellen, waardoor er een buitenproportionele ontstekingsreactie plaatsvindt. Dit leidt tot het aantrekken van een nieuwe golf ontstekingscellen. De laesie zal hierdoor groeien en uiteindelijk kunnen “scheuren”. Hierdoor komen de verschillende componenten van de laesie in contact met het bloed, waardoor er een bloedstolsel ontstaat. Dit stolsel kan terplekke het bloedvat afsluiten, maar als het stolsel los raakt, kan het ook worden meegevoerd door de bloedstroom en kan het stolsel verder gelegen bloedvaten afsluiten. Door de afsluiting van een bloedvat krijgt het onderliggende weefsel te weinig voeding en zuurstof, wat leidt tot het afsterven en functieverlies van het weefsel/orgaan. Dit leidt tot de klinische symptomen zoals een hartaanval of een beroerte.

In dit proefschrift is onderzocht hoe verschillende ontstekingscellen betrokken zijn bij atherosclerose. Verder is er bestudeerd welke cytokines deze cellen maken en welke functie deze cytokines hebben in relatie tot atherosclerose. In hoofdstuk 1 is gedetailleerd weergegeven wat er tot nu toe bekend is over de ontstekingscellen die zijn betrokken bij atherosclerose. Verder is daar ook ingegaan op verschillende belangrijke cytokines. Om ontstekingscellen en cytokines in atherosclerose te kunnen bestuderen hebben we gebruik gemaakt van proefdieren.

In de studies die zijn beschreven in dit proefschrift wordt gebruik gemaakt van muizen die genetisch zijn veranderd zodat ze atherosclerose ontwikkelen. Als deze muizen worden gevoed met een vetrijk dieet (Western-type dieet), krijgen ze een verhoogde concentratie cholesterol in het bloed, waardoor atherosclerose ontstaat.

"NIEUWE" CELLEN EN CYTOKINES IN ATHEROSCLEROSE

INTERLEUKINE 15

In hoofdstuk 2 is een belangrijk ontstekingstimulerend cytokine bestudeerd, interleukine-15 (IL-15). IL-15 is een cytokine dat voornamelijk gemaakt wordt door macrofagen en vooral effect heeft op T-cellen. Aangezien zowel macrofagen als T-cellen belangrijk zijn in atherosclerose is het interessant om de rol van IL-15 in atherosclerose te bepalen. In dit hoofdstuk demonstreren wij dat het aantal cellen dat IL-15 tot expressie brengt sterk toeneemt door de muizen te voeden met een vetrijk dieet. Dit kan betekenen dat IL-15 is betrokken bij het ontstaan van atherosclerose.

Om de functie van IL-15 te bestuderen gebruikten we een vaccinatietechniek die gebruik maakt van een levende, maar wel verzwakte, bacterie. In deze bacterie, de *Salmonella typhimurium*, hebben we DNA ingebracht dat codeert voor IL-15. Wanneer we deze bacterie toedienen wordt er een immuunrespons opgewekt die uiteindelijk zorgt voor het doden van cellen die IL-15 hoog tot expressie brengen. We zien in de tegen IL-15 gevaccineerde muizen een sterke afname van atherosclerose. Verder zien we ook dat het aantal cellen dat IL-15 tot expressie brengt wordt verminderd tot hetzelfde niveau voordat atherosclerose werd geïnduceerd. We zien echter wel meer macrofagen in de laesie, maar die brengen waarschijnlijk minder of geen IL-15 tot expressie en daardoor zal de laesie niet toenemen in grootte.

INTERLEUKINE 17 EN DE TH17 CEL

Zoals hierboven vermeld zijn er verschillende soorten T-cellen, waaronder de T-helper 1 (Th1) en T-helper 2 (Th2) cellen. De algemene gedachte is dat tijdens atherosclerose voornamelijk de Th1-cel verantwoordelijk is voor het verergeren van atherosclerose en de Th2-cel juist voor het verminderen van atherosclerose. Een balans tussen deze twee types T-cellen is van cruciaal belang voor een goede uitkomst van de ontsteking. In verschillende auto-immuun ziekten is een verstoorde balans richting Th1 verantwoordelijk voor de verergering van de ziekte. Echter niet alle resultaten kunnen hiermee verklaard worden. Dit leidde tot de

ontdekking van nieuwe T-cellen, de T-helper 17 (Th17) cel en de regulatoire T (Treg)-cel.

De Th17-cel is een T-cel die voornamelijk het ontsteking-stimulerende cytokine IL-17 produceert en zijn rol is geïdentificeerd in verschillende ziekte modellen, maar de rol van deze nieuwe Th17-cel is nog niet beschreven in atherosclerose.

In hoofdstuk 3 bestuderen we dan ook het effect IL-17, het prominent geproduceerde cytokine door Th17-cellen, doormiddel van het blokkeren van de functie van IL-17. Als eerste hebben we bestudeerd of de productie van IL-17 omhoog gaat na het voeden van de muizen met een vetrijk dieet. We hebben geconstateerd dat in de milt (een belangrijk orgaan betrokken bij het immuunsysteem) een verhoogde productie van IL-17 plaatsvindt, wat inhoudt dat IL-17 waarschijnlijk een rol speelt bij atherosclerose. Om dit verder te bevestigen vaccineren we de muizen tegen IL-17 door middel van een DNA vaccin, coderend voor IL-17. Dit resulteerde in een grote vermindering van 90% in atherosclerose in de tegen IL-17 gevaccineerde muizen.

In hoofdstuk 4 bestuderen we het effect van de receptor voor IL-17, door gebruik te maken van een beenmergtransplantatie. We hebben een beenmergtransplantatie uitgevoerd met het beenmerg van muizen waarvan de receptor van IL-17 (IL-17R) is verwijderd. Deze cellen kunnen dus niet meer reageren op IL-17. Op deze manier kunnen we de rol van IL-17 in atherosclerose bestuderen in het muismodel. We zien in muizen die het IL-17R deficiënte beenmerg hebben gekregen een sterke vermindering van atherosclerose. Verder zagen we een afname van IL-6 (dat betrokken is bij een verergering van atherosclerose) en een toename van IL-10 (een interleukine dat atherosclerose vermindert). Een interessante bevinding is de vermindering van antilichamen tegen gemodificeerde lipoproteïnes, welke in sommige gevallen atherosclerose kunnen verergeren en dus ook een verklaring kunnen vormen voor verminderde atherosclerose.

De resultaten uit hoofdstuk 3 en 4 bevestigen een rol voor IL-17 in de ontwikkeling van atherosclerose. Nu we hebben aangetoond dat IL-17 een belangrijke rol speelt in atherosclerose is het waarschijnlijk dat de Th17-cel ook een rol speelt in het ziekteproces.

Onlangs is een nieuw cytokine ontdekt, IL-23, die betrokken is bij het ontstaan van Th17-cellen. IL-23 is een interleukine dat uit twee subunits bestaat, p40 en p19. Het p40 deel is ook een onderdeel van een ander cytokine, het IL-12. IL-12 is een sterk ontstekingstimulerend interleukine en in een eerdere studie is gebleken dat na vaccinatie tegen p40 atherosclerose sterk verminderd wordt. Omdat p40 zowel een deel van IL-23 als IL-12 is, is het achteraf niet mogelijk vast te stellen welk interleukine exact voor de remming van in atherosclerose heeft

gezorgd. Om dit verder uit te zoeken hebben we de functie van IL-23 in atherosclerose bestudeerd door middel van een beenmergtransplantatie met beenmerg waarin het p19 deel van IL-23 is uitgeschakeld. De cellen zonder het p19 gen kunnen geen functioneel IL-23 meer maken en zodoende kan de rol van dit interleukine worden bestudeerd in atherosclerose, zoals is beschreven in hoofdstuk 5.

We vinden echter geen effect van p19 deficiëntie op atherosclerose in het beenmerg transplantatie model. We zien ook geen verschil in de Th17-cellen. Echter recent onderzoek heeft uitgewezen dat IL-23 waarschijnlijk niet betrokken is in de initiatie van de Th17, maar meer betrokken is bij de instandhouding van deze cellen. Mogelijk kan dit een verklaring bieden voor het feit dat we geen verschil in atherosclerose zien. Er dient echter nog meer onderzoek verricht te worden om de rol van IL-23 en p19 te begrijpen.

INTERLEUKINE 27

Een ander recentelijk ontdekt interleukine is IL-27, dat ook uit 2 subunits bestaat, een p28 subunit en een EBI3 deel. Dit interleukine werd in beginsel beschreven als ontstekingsstimulerend en betrokken bij een verergering van auto-immuunziekten. Door de jaren heen is echter meer onderzoek gedaan en is de aan IL-27 toegeschreven ontstekingsstimulerende rol herzien. Zo is er aan het licht gekomen dat IL-27 de potentie heeft om de Th17-cel te remmen en dit zou kunnen resulteren in een verminderd ziektebeeld.

IL-27 is echter nog niet bestudeerd in atherosclerose en gezien de rol van IL-17 en waarschijnlijk ook de Th17 cel in atherosclerose, is IL-27 een interessant interleukine om te bestuderen in de context van atherosclerose. In hoofdstuk 6 wordt een studie beschreven waarin muizen gevaccineerd worden tegen p28. Uit dit onderzoek blijkt dat de 2 delen van IL-27 (p28 en EBI3) sterk tot expressie komen in de atherosclerotische laesie, wat aangeeft dat IL-27 mogelijk betrokken is bij atherosclerose. Vervolgens zijn enkele muizen gevaccineerd met DNA, coderend voor het interleukine p28, en is atherosclerose geïnduceerd.

Uit de resultaten van dit onderzoek blijkt dat atherosclerose was verergerd in de muizen die gevaccineerd waren tegen p28. Dit betekent dat IL-27 beschermend werkt in atherosclerose. De verergering van atherosclerose zou verklaard kunnen worden door de gevonden toename van Th17-cellen. Daarnaast hadden de gevaccineerde muizen ook minder Treg-cellen. Treg-cellen zijn betrokken bij vermindering van atherosclerose. Het lijkt er dus op dat de balans tussen de Th17-cellen en Treg-cellen is omgeslagen naar Th17-cellen in de gevaccineerde muizen tegen p28, met verergerde atherosclerose tot gevolg.

REGULATOIRE T-CELLEN

Treg-cellen verminderen ontsteking en kunnen daardoor beschermend zijn in auto-immuunziekten, wellicht ook in atherosclerose. Een manier om Treg cellen te stimuleren is door een antigeen oraal toe te dienen. Door dit via het spijsverteringskanaal toe te dienen, ontstaat er in de darmen een immuunreactie die ervoor zorgt dat er tolerantie ontstaat tegen dit antigeen. Bij het ontstaan van tolerantie wordt het antigeen herkend en vindt er activering van Treg-cellen plaats. Hierdoor wordt vervolgens de immuunrespons afgeremd. Dit is bijvoorbeeld van belang bij tolerantie opwekking tegen voedingsmiddelen. Deze methode is al eerder uitgeprobeerd tegen gemodificeerde lipoproteïnes, wat resulteerde in meer Treg-cellen en minder atherosclerose.

In hoofdstuk 7 zijn door middel van orale toediening van HSP60 Treg cellen geïnduceerd. HSP60 is een antigeen dat is geïdentificeerd in atherosclerose. In atherosclerose is er een immuunreactie tegen HSP60 die niet wenselijk is. Door Treg-cellen te induceren kunnen we deze immuunreactie remmen om atherosclerose te verminderen. We zien in deze studie dat atherosclerose inderdaad afneemt en dat dit komt door meer Treg cellen.

Vervolgens willen wij ook het effect bestuderen van de blokkering van Treg cellen om de rol van Treg cellen in atherosclerose verder te bevestigen. Dit is beschreven in hoofdstuk 8. In dit hoofdstuk wordt een vaccinatiemethode beschreven waarbij dendritische cellen de “opdracht” hebben gekregen om een immuunrespons op te wekken tegen een antigeen. Dendritische cellen zijn speciale cellen van het immuunsysteem die zeer goed in staat zijn een bepaald antigeen te presenteren aan andere immuuncellen om zo een imuunrespons op te wekken. In deze studie hebben we de dendritische cellen speciaal gericht tegen cellen die Foxp3 tot expressie brengen. Foxp3 is een eiwit dat voornamelijk gemaakt wordt door Treg cellen. Door cellen die Foxp3 tot expressie brengen te vernietigen kan de bijdrage van Treg cellen in atherosclerose bestudeerd worden. We zien in deze studie dat de vaccinatie leidt tot minder Treg cellen in de muis. Dit heeft tot gevolg dat door de Foxp3 vaccinatie, de atherosclerotische laesie toeneemt in grootte. Hetzelfde is ook waargenomen in muizen met een vergevorderde atherosclerotische laesie. Dit geeft aan dat Treg cellen zowel bij beginnende als gevorderde atherosclerose betrokken zijn. Verder zien we dat de laesie van gevaccineerde muizen veel meer cellen bevat. Mogelijk duidt dit op meer ontsteking in de laesie, waardoor deze in grootte toeneemt. Welke cellen dit precies zijn moet nog worden uitgezocht in een toekomstig onderzoek. Kortom, in de hoofdstukken 7 en 8 hebben we aangetoond dat Treg cellen een belangrijk celtype is in de regulatie van atherosclerose en mogelijk kan dit leiden tot een therapeutische toepassing.

CONCLUSIE

Het onderzoek beschreven in dit proefschrift is er op gericht meer inzicht te verkrijgen in de rol van ontstekingscomponenten in atherosclerose. De nieuwe bevindingen kunnen leiden tot nieuwe inzichten en idealiter resulteren in een klinische toepassing zodat patiënten met atherosclerose in de toekomst beter behandeld kunnen worden. In dit proefschrift wordt veel gebruik gemaakt van vaccinatietechnieken om de functie van bepaalde cellen en interleukines te bestuderen. In hoofdstuk 9 staat beschreven hoe deze inzichten, die zijn verkregen door onder andere een bijdrage uit dit proefschrift, kunnen leiden tot therapeutisch haalbare toepassingen.

Vooralsnog is de beste remedie om atherosclerose te verminderen het voorkomen van blootstelling aan risico factoren. Helaas is dit niet altijd mogelijk, door bijvoorbeeld erfelijke factoren, en zal de zoektocht naar een behandelmethode tegen atherosclerose voort blijven gaan. Een veel belovende toepassing kan het induceren van tolerantie tegen atherosclerose gerelateerde eiwitten kunnen zijn. Deze non-invasieve behandeling die tolerantie opwekt tegen ziekte specifieke antigenen kan een uitkomst bieden in veel auto-immuunziekten en dus ook in atherosclerose. Echter, er dient nog veel onderzoek te worden verricht naar de haalbaarheid en effectiviteit in patiënten.

List of abbreviations

Thomas van Es

LIST OF ABBREVIATIONS

-/-	homozygous knock out
36B4	acidic ribosomal phosphoproteinP0
β2GPI	β2-glycoprotein I
Ab	antibody
ACS	acute coronary syndromes
APC	antigen presenting cell
apoE/apoB	apolipoprotein E/apolipoprotein B
BM	Bone marrow
BMT	Bone marrow transplantation
CC/CXC	chemokine
CCL/CXCL	chemokine ligand
CD	cluster of differentiation
CETP	cholesteryl ester transfer protein
CIA	collagen induced arthritis
CMV	Cytomegalovirus
conA	concanavalin A
CTL	cytotoxic T lymphocyte
CTLA	cytotoxic T-lymphocyte antigen
CTX-I	cardiotoxin I
CVD	cardiovascular disease
DC	dendritic cell
EAE	experimental acquired encephalomyelitis
EBI3	Epstein-Barr virus induced gene 3
ECM	Extracellular matrix
ELISA	enzyme-linked immunosorbent assay
FACS	fluorescent-activated cell sorting
Foxp3	forkhead box p3
GC	germinal center
(e)GFP	(enhanced) green-fluorescent protein
GTC	guandium isothiocyanate
HDL	high-density lipoprotein
HEL	hen egg-white lysozyme
HPRT	hypoxanthine phosphoribosyl transferase
HSP	heat shock protein
imDC	immature dendritic cell
i.m.	intra muscular
i.p.	intra peritoneal
iTreg	inducible regulatory T cell

i.v.	intravenous
ICAM-1	intercellular adhesion molecule-1
IFN	interferon
Ig	immunoglobulin
IL	interleukin
IL-.R	interleukin . receptor
imDC	immature dendritic cell
JAM-1	junctional adhesion molecule-1
LDL	low-density lipoprotein
LDLr	low-density lipoprotein receptor
LFA-1	lymphocyte function associated antigen-1
LPS	lipopolysaccharide
MCP-1	monocyte chemotactic protein
M-CSF	macrophage colony stimulating factor
MDA-LDL	malondialdehyde modified LDL
mDC	mature dendritic cell
MHC	major histocompatibility complex
MMP	matrix metalloproteinase
NF-κB	nuclear factor κB
NK cell	natural killer cell
NKT cell	natural killer T cell
oxLDL	oxidized low-density lipoprotein
PAMP	pathogen-associated molecular pattern
PBS	phophate buffered saline
PBMC	peripheral blood mononuclear cell
PCR	polymerase chain reaction
PC	phosphatidyl choline
PE	phosphatidyl ethanolamine
PECAM	platelet endothelial cell adhesion molecule
PGE_2	prostaglandin E_2
PRR	pattern recognition receptor
qPCR	quantative polymerase chain reaction(also RT-PCR)
RA	rheumatoid arthritis
RAG	recombination activating gene
SCID	severe combined immunodeficient
s.i.	Stimulation index
SMC	smooth muscle cell
SNP	single nucleotide polymorphism
SOCS	suppressor of cytokine signaling
ScR	scavenger receptor

STAT	signal transducers and activators of transcription
TβRII	transforming growth factor β receptor II
TCR	T cell receptor
TGF	transforming growth factor
Th1/Th2/Th3/Th17	T helper 1/T helper 2/T helper 3/T helper 17
TLR	Toll-like receptor
TNF	tumor necrosis factor
Tr1	regulatory T cell type 1
Treg	regulatory T cell
VCAM-1	vascular cell adhesion molecule-1
VEGF (R)	vascular endothelial growth factor (receptor)
VLA-4	very late antigen-4
VLDL	very low-density lipoprotein
vSMC	vascular smooth cell

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Thomas van Es

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Curriculum Vitae

Thomas van Es

CURRICULUM VITAE

Thomas van Es werd geboren op 8 april 1979 te Gouda. In juni 1997 werd het H.A.V.O diploma behaald aan de scholengemeenschap "de Goudse waarden" te Gouda. In datzelfde jaar werd begonnen met de studie medische biologie aan de Hogeschool van Utrecht te Utrecht, alwaar het propedeutisch examen werd behaald in juni 1998. Als afstudeerrichting werd moleculaire biotechnologie gekozen. De hoofdstage werd gelopen bij de Erasmus Universiteit Rotterdam, afdeling kindergeneeskunde, onder begeleiding van Dr. J.W.A. Rossen en Dr. A.W.C. Einerhand. Gedurende die stage werd onderzoek gedaan naar de receptor waaraan het Rotavirus kan binden ten einde de gastheer te infecteren. Deze stage werd afgesloten met een verslaglegging getiteld: "Mechanisme van rotavirusinfectie – opzoek naar de receptor met behulp van het Y2H-systeem". Het ingenieursdiploma werd behaald in juni 2001. In datzelfde jaar werd de studie medische biologie aan de Vrije universiteit te Amsterdam aangevangen. Zijn hoofdvakstage werd verricht bij TNO Leiden, preventie en gezondheid, afdeling vaat- en bindweefsel onder begeleiding van Dr. P.H.A. Quax en Dr. M.M.L. Deckers. Hij deed hier onderzoek naar een non-virale methode om MCP-1 tot overexpressie te brengen teneinde arteriogenese te induceren. Deze stage werd afgesloten met een verslaglegging getiteld: "Therapeutische arteriogenese door non-virale gentherapie met MCP-1". Het doctoraal examen werd behaald in augustus 2003 aan de Vrije universiteit Amsterdam. Van januari 2004 tot juni 2008 was hij werkzaam als assistent in opleiding bij de vakgroep Biofarmacie van het Leiden/Amsterdam Center for drug research aan de Universiteit Leiden. Het onderzoek, beschreven in dit proefschrift, werd verricht onder begeleiding van Dr. J. Kuiper en Prof. dr. Th.J.C. van Berkel en werd gefinancierd door de Nederlandse Hartstichting. Sinds september 2008 is hij werkzaam als postdoc bij de afdeling moleculaire celbiologie en immunologie in de groep van Prof. dr. Y van Kooyk aan het VU medisch centrum te Amsterdam.

Dankwoord

Thomas van Es

DANKWOORD

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Thomas van Es