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Begeleidingsformulier aanvraag dierproef DEC- UM

Versie 2006

DECNR: 2011-038

Ontvangen:11-03-2011

Herziene versie

DEC datum goedkeuring#	Type aanvraag [2]	VROM/GGONR[3]	LNV/CB DNR[4]
07-04-2011	Nieuw	Ig08071	

Hoofdproject	CARIM
Deelproject	3.
Financieel beheerder	
Budgetnummer	30983109N

Titel van het onderzoek:

Studying the atheroprotective role of plasmacytoid dendritic cells in atherosclerosis.

startdatum	01-03-11	einddatum [9]	30-12-2012	*Duur van de proef [10]: 16 weeks*

1.Verantwoordelijk onderzoeker (VO)		Art.9
2. Vervanger VO (VVO)		Art.9
3. Verantwoordelijk medewerker (VM) GGO[7]		Art.9
4. overige uitvoerenden		Art.12 Art.9 Art.9
5.		Art.9
6. Principle Investigator (PI)		Art.9

Diergroep	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28
ctrl/exp/sham	exp	exp	exp	exp	exp	exp	exp	exp	exp	exp	exp	exp	exp	exp	exp	exp	exp	ctrl	ctrl	ctrl	ctrl	ctrl	ctrl	ctrl	ctrl	ctrl	exp	ctrl
Diersoort	01	01	01	01	01	01	01	01	01	01	01	01	01	01	01	01	01	01	01	01	01	01	01	01	01	01	01	01
Stam	C57 Bl6	C5 7Bl 6	C5 7Bl 6	C5 7Bl 6	C5 7Bl 6	C5 7Bl 6	C5 7Bl 6	C5 7Bl 6	C5 7Bl 6	C5 7Bl 6	C5 7Bl 6	C5 7Bl 6	C5 7Bl 6	C5 7Bl 6	C5 7Bl 6	C5 7Bl 6	C5 7Bl 6	C5 7Bl 6	C5 7Bl 6	C5 7Bl 6	C5 7Bl 6	C5 7Bl 6	C5 7Bl 6	C5 7Bl 6	C5 7Bl 6	C5 7Bl 6	C5 7Bl 6	C5 7Bl 6
Construct / mutatie ?	LDL r-/-	LDL r-/-	LDL r-/-	LDL r-/-	LDL r-/-	LDL r-/-	LDL r-/-	LDL r-/-	LDL r-/-	LDL r-/-	LDL r-/-	LDL r-/-	LDL r-/-	LDL r-/-	LDL r-/-	LDL r-/-	LDL r-/-	LDL r-/-	LDL r-/-	LDL r-/-	LDL r-/-	LDL r-/-	LDL r-/-	LDL r-/-	LDL r-/-	LDL r-/-	LDL r-/-	LDL r-/-
Herkomst (leverancier) *	01	01	01	01	01	01	01	01	01	01	01	01	01	01	01	01	01	01	05	05	05	05	05	05	05	05	01	01
Aantal	40	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	3	21	21	6	6	6	6	6	6	20	15
Geslacht	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V	M	M	M	M	M	M	M	M	M	V	M/V
Dieren immuuncompetent ?	ja	ja	ja	ja	ja	ja	ja	ja	ja	ja	ja	ja	ja	ja	ja	ja	ja	ja	ja	ja	ja	ja	ja	ja	ja	ja	ja	ja
Leeftijd/gewicht	10-24 wk	10-24 wk	10-24 wk	10-24 wk	10-24 wk	10-24 wk	10-24 wk	10-24 wk	10-24 wk	10-24 wk	10-24 wk	10-24 wk	10-24 wk	10-24 wk	10-24 wk	10-24 wk	10-24 wk	10-24 wk	10-24 wk	10-24 wk	10-24 wk	10-24 wk	10-24 wk	10-24 wk	10-24 wk	10-24 wk	10-24 wk	10-24 wk
Doel van de proef *	31	31	31	31	31	31	31	31	31	31	31	31	31	31	31	31	31	31	31	31	31	31	31	31	31	31	31	31
Belang van de proef *	01	01	01	01	01	01	01	01	01	01	01	01	01	01	01	01	01	01	01	01	01	01	01	01	01	01	01	01
Toxicologisch onderzoek *	01	01	01	01	01	01	01	01	01	01	01	01	01	01	01	01	01	01	01	01	01	01	01	01	01	01	01	01
Bijzondere technieken *	1	1	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	2	2	2	2	2	2	2	2	2	1	2
Anesthesie *	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	1	1	1	1	1	1	1	1	1	4	1
Pijnbestrijding *	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	1	1	1	1	1	1	1	1	1	4	1
Mate ongerief *	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	1	1	1	1	1	1	1	1	1	4	1
Toestand dier einde exp.	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1

3 Verantwoording

Aanvraag dierproef DEC-UM
Titel: Studying the atheroprotective role of plasmacytoid dendritic cells in atherosclerosis

1. Doel van de proef.

Despite the efficacy of drug therapy (e.g. statins and hypotensive drugs) in the treatment and prevention of cardiovascular disease, the later disease continues to be the most prominent cause of death worldwide. Interventions are awaited that interfere in the actual process of vascular stenosis and thrombotic occlusion rather than drugs that alleviate its risk factors. In recent years much progress has been achieved in our understanding of the natural history of disease development (how do vascular lesions (plaques) develop?), and new insights point to acute inflammatory processes that trigger the escalation of the disease. Inflammatory cells are believed to play a crucial role in atherosclerosis.

Plasmacytoid dendritic cells (pDC) are a subset of dendritic cells derived from precursors in the bone marrow. pDC are important players between the innate and adaptive immunity by producing large amounts of type I interferons (IFN-α, IFN-β) upon stimulation. This in turn induces the activation of effector T cells and natural killer (NK) cells. While activated pDC promote immunity, resting or alternatively activated pDC function tolerogenic. The presence of pDC has been shown in human advanced stable and ruptured atherosclerotic lesions. pDC were found to be expressed mainly in regions with inflammation where they are believed to regulate T cell function through the release of type I interferons. Recently, it has been shown that pDC are also present in mouse atherosclerotic lesions. Here, pDC seem to play a protective role since pDC depletion led to exacerbated atherosclerotic lesions. Furthermore pDC seem to act atheroprotective by dampening $CD8^+$ T cell responses. Despite the recent findings in mice, it is still unknown through which mechanisms pDC could act atheroprotective and if they interact with specific cell types, such as natural killer T (NKT) to fulfill their atheroprotective function.

In this study we therefore aim to investigate whether NKT cells interact with pDC and by this function as protective players against atherosclerosis. In addition, we will study through which mechanisms pDC could induce tolerance causing an attenuation of lesion formation. Herefore, we will address the relevance of molecules, such as indoleamine-2,3-dioxygenase (IDO), inducible costimulator ligand (ICOS-L), programmed death ligand 1 (PD-L1), IL-10, SiglecH and the Interferon-γ receptor II (IFNγR II) that are upregulated in tolerogenic pDC and by this regulate immune responses by dampening effector T cell responses. A specific knockdown of theses molecules in combination with a specific pDC depletion using two different pDC depletion models will give us inside whether these molecules are upregulated in pDC present in atherosclerotic mice. Furthermore, we will investigate if pDC are able to mediate oral tolerance by administration of modified lipids such as oxidized LDL in atherosclerotic mice and by this prevent the development of atherosclerosis.

The goal of performing these studies will be to reveal whether pDC are critically involved in the attenuation of lesion formation and by this are potential targets for therapeutical interventions.

2. Maatschappelijke relevantie en/of wetenschappelijk belang

The inflammatory process of atherosclerosis is the underlying cause of cardiovascular disease, and will likely continue to be the main cause of global death in the coming 15 years. Despite the recent advance in the treatment of the disease (e.g. statins), we are far from offering an adequate treatment to affected patients. Progress in the treatment is mainly hampered by the incomplete understanding of the mechanism of the disease process and its major players and new insight and targets are eagerly awaited. Recent studies have shown that pDCs are present in both, in human and mouse atherosclerotic lesions,

suggesting a role of this cell subset in cardiovascular disease. We expect that this study will not only contribute to the understanding of lesion development and progression but also pave the way towards a new therapeutic entry for this disabling disease.

3. Alternatieven

In vitro methods using cell cultures represent important tools in the study of the role of pDCs in cardiovascular disease in our studies. For instance we will assess in vitro aspects such as cytokine expression, proliferation, apoptosis, cholesterol uptake, etc, which provide a better insight into the disease process. However atherosclerosis is a multifactorial disease involving many components and cell types at different stages of disease development. Isolated cells will behave differently than cells in their natural environment. In addition, an adequate multicellular vessel culture system for the induction of plaque formation is unavailable. Collectively, in vitro methods alone will never reveal the whole spectrum of effects of pDC modulation in the complex context of atherosclerosis. Therefore, the animal experiments are an essential part of this investigation to deliver conclusive experimental proof of a role of this inflammatory cell.

4. Ethische afweging

Cardiovascular disease and the underlying cause atherosclerosis, is the main cause of mortality & morbidity in western society, despite recent advance in therapeutic intervention. Cardiovascular disease thus not only puts a sizable burden to health care expenditure but also due to its disabling nature, is accompanied by great losses for economy, and personal well-being. This justifies further efforts on the development of the disease onset and on the identification of new targets for therapy or prevention that add to the currently available modalities.

In this study we will study the contribution of pDC to the atherosclerotic disease progression and their relevance as therapeutic target. Given the complex, multifactorial character of the disease we consider it imperative that studies of the role of pDCs will be executed in the complex context of atherosclerosis using a mouse animal model that is very representative for human disease.

Wetenschap

5. Wetenschappelijke onderbouwing

Atherosclerosis is viewed as a chronic inflammatory disease typified by the accumulation of lipid material and inflammatory cells in the subendothelial space of large and middle-sized arteries. Atherosclerosis becomes clinically manifest only at a late stage, when initial plaques have developed into complex fibroatheromatous lesions with a thin fibrous cap and a large lipid core that is vulnerable to stress induced rupture and thrombosis. Both the initiation and progression of the atherosclerotic plaque towards a rupture-prone, unstable plaque is driven by the recruitment of specific leukocyte subsets, a process that is mediated by various immunomodulators. pDCs could play a crucial role in the plaque development and progression since it has recently been shown that they are not only present in human, but also in mouse atherosclerotic lesions and that a depletion of pDC in atherosclerotic mice led to an aggravated lesion formation. In this study we are interested to find out if pDCs function atheroprotective and through which specific mechanisms (activation of specific intracellular signalling pathways, expression of specific

surface receptors) they could induce their tolerogenic function. Additionally, we are interested, if pDC interact with specific cell subtypes, especially with NKT cells, to fulfill their tolerogenic function. NKT cells are innate-like T lymphocytes that can influence a wide array of immune responses. It has been shown that NKT cells have a dual role in the regulation of immune responses. Although they promote T cell responses against pathogens, they usually dampen T cell responses against self-antigens. More recently, it has been reported that NKT cells play a crucial role in the inflammatory process of atherosclerosis. Here activated NKT cells functioned in a protective way by reducing plaque formation. Both, pDC and NKT cells, are relevant mediators in the induction of tolerance and could therefore be important therapeutic targets dampening immune responses in atherosclerosis. In a further study our goal is to see whether pDC can mediate oral tolerance in atherosclerotic mice and by this attenuate lesion development. Oral tolerance is the physiologic mechanism by which the mucosal immune system prevents T cell mediated reactions to modified self-antigens and exogenous antigens. Previous animal studies have shown that antigen feeding prior to systemic immunization with the same antigen prevented T cell mediated immunopathologies. Here, orally administered antigens were presented to T cells by antigen-presenting cells in the intestinal lamina propria and the mesenteric lymph nodes. This induced dampening of $CD4^+$ and $CD8^+$ T cell responses to these antigens and promoted induction of regulatory T cells. These studies have shown that pDC were involved in maintaining the immune tolerance.

To study a possible interaction of NKT cells with pDC in the development of atherosclerosis (focus on early lesions), we will study two different models. In the first model we will activate NKT cells via a specific NKT cell agonist, α- galactoside ceramide (α-GalCer) in the presence or absence of pDC (pDC depletion by the pDC specific depletion antibody 120G8). In a second model we will deplete NKT cells and pDC using a specific NKT cell/ pDC depletion antibody (anti-mouse NK1.1/Gr-1). Both depletion antibodies (120G8 and ant-mouse NK1.1/Gr-1) are provided by

These two studies will give us insight whether pDC and NKT cells cooperate with each other in an atheroprotective way abating lesion formation in early atherosclerotic plaques.

In a further in vivo study we will investigate the tolerogenic function of immature and mature pDC in atherosclerosis (focus on advanced lesions) by using the BDCA2-DTR model to study the function of immature pDC and the CD11c-DTR model to study the function of mature pDC (DTR= diphtheria toxin receptor). The diphtheria toxin receptor models (BDCA2-DTR and CD11c-DTR) will allow us to deplete pDC specifically based on the generation of transgenic mice that selectively express the diphtheria toxin receptor (DTR) under the control of the BDCA2 or CD11c promoter. pDC will be eliminated selectively by the administration of diphtheria toxin (DT), a compound that is normally not harmful to mice. Both , the BDCA2-DTR and the CD11c-DTR model, are extremely new models and could be used for the specific depletion of either immature or mature pDC in vivo what is at the moment not possible by using a depletion antibody. The BDCA2-DTR model will

. The model will be provided by our collaborator . To study through which mechanisms pDC could induce tolerance, we will produce double chimerism mice. Here $LDLR^{-/-}$ mice will get a mixed bone marrow transplantation (BMT), receiving BDCA2-DTR or CD11c-DTR bone marrow to deplete either immature or mature pDC and bone marrow from $IDO^{-/-}$, ICOS-$L^{-/-}$, PD-$L1^{-/-}$, IL-$10^{-/-}$, $SiglecH^{-/-}$ or IFNyR $II^{-/-}$ knockout mice. These molecules are upregulated in tolerogenic pDC promoting the generation of IL10-producing regulatory T cells and the attenuation of effector $CD8^+$ T cells. $IDO^{-/-}$, ICOS-$L^{-/-}$, PD-$L1^{-/-}$, IL-$10^{-/-}$ and IFNyII $R^{-/-}$ knockout mice will be

To determine whether pDC can mediate oral tolerance in atherosclerotic $LDLR^{-/-}$mice, we will induce intragastric administration of modified lipids (oxidized LDL) over a period of 14 days before mice are put on a western type diet for further six weeks. To investigate whether pDC are involved in the mediation of

oral tolerance and could attenuate lesion size and immunological processes in the lesion we will use the collar model to induce plaque formation in the mouse carotids. Furthermore we will perform the study in the presence or absence (pDC depletion by the specific depletion antibody 120G8) of pDC.

6. Wetenschappelijke beoordeling

The principle investigator (PI) of the ( ) group, has evaluated and approved the scientific content of this DEC proposal.

Proefdier

7. Proefdier keuze

7a. Soort, stam / herkomst / eindbestemming

$LDLR^{-/-}$ mice will be used, because after cholesterol rich diet they develop atherosclerotic lesions in a manner similar to those present in humans, especially those in familial hypercholesterolemia. The animals will be provided by the pathology breeding at the department of the CPV or they will be ordered at Jackson's Laboratories, depending on the availability of mice in Maastricht-CPV at the time we need them.

will be used to deplete specifically immature pDC. These animals will be provided by from the
CD11c-DTR transgenic mice will be used to deplete specifically mature pDC. These animals will be provided by
$IDO^{-/-}$, $ICOS\text{-}L^{-/-}$, $PD\text{-}L1^{-/-}$, $IL\text{-}10^{-/-}$, $IFN\gamma II\ R^{-/-}$ and $SiglecH^{-/-}$ knockout mice will be used to study mechanisms through which immature and mature pDC could fulfill their tolerogenic function. These animals will be provided by and
from the

7b. Sexe

In order to keep the experimental number of mice as low as possible, using one sex is preferred. Atherosclerotic lesions vary between male and female $LDLr^{-/-}$ mice [1]. This will induce a larger number of mice if an equal partitioning of male and female is used. Because atherosclerotic lesions seem less variable in females, we prefer to use female $LDLr^{-/-}$ mice for all in vivo experiments. For the bone marrow transplantation experiments male mice will be used as donors in order to be able to monitor the hematopoietic chimerism after transplantation. For in vitro experiments, both male and female mice will be used as there are no indications of gender differences.

7.c. Aantallen

In vivo intervention studies:

We will perform 4 major in vivo intervention studies: 1) studying the effects of activated NKT cells in the presence or absence of pDC in atherosclerosis, 2) studying the effects of NKT cell and pDC depletion in atherosclerosis, 3) studying the depletion and dysfunction of pDC in atherosclerosis and 4) studying oral tolerance by administration of modified lipids.

1. **NKT cell activation ± pDC depletion study (diergroup1)**

For the NKT cell activation ± pDC depletion study, 40 female LDLR$^{-/-}$ mice are required. These 40 mice will be divided over 4 groups: in the four groups we will study the effects of NKT cell activation, using α-GalCer as a stimulator of NKT cells or β-GalCer as control analogue, in the presence (administration of theIgG2a isotype control) or absence of pDC (pDC depletion by the depleting antibody 120G8) in atherosclerotic mice (n=10 mice/ group).

groups	treatment/ plaque development	animal number
A	α-GalCer + IgG2a isotype control	10
B	α-GalCer +120G8 antibody	10
C	β-GalCer (control) + IgG2a isotype control	10
D	β-GalCer (control) +120G8 antibody	10
	Total	40

2. **NKT cell depletion/ pDC depletion study (diergroup 2)**

For the NKT cell and pDC depletion study, 20 female LDLR$^{-/-}$ mice are required. These 20 mice will be divided over 2 groups: in the two groups we will study the effects of NKT cell and pDC depletion in the development of atherosclerotic lesions, using an anti-mouse NK1.1/ Gr-1 depletion antibody for the depletion of both cell subsets. An IgG2 isotype control will be administered for the control group (n=10 mice/group).

groups	treatment	animal number
A	anti-mouse NK1.1/ Gr-1 antibody	10
B	IgG2 isotype control	10
	Total	20

3. **pDC depletion and dysfunction study (diergroup 3-17)**

In the pDC depletion and dysfunction study, we will study if immature and mature pDC play a protective role in mouse atherosclerotic lesions and through which mechanisms they fulfill their protective function. For this study 300 female recipient LDLR$^{-/-}$ mice will be required in total. To induce deficiency of immature pDC, LDLR$^{-/-}$ mice will receive BDCA2-DTR bone marrow (n=20). To induce deficiency of mature pDC, LDLR$^{-/-}$ mice will receive CD11c-DTR bone marrow (n=20).A control group of 20 LDLR$^{-/-}$ mice receives WT bone marrow. To study effects of pDC dysfunction (immature or mature pDC) on atherosclerosis, we will establish double chimerism mice by performing mixed BMT (1:10 ratio). Herefore, LDLR$^{-/-}$ mice that receive either BDCA2-DTR or CD11c-DTR bone marrow will also receive IDO$^{-/-}$ bone marrow (n=20), ICOS-L$^{-/-}$ bone marrow (n=20), PD-L1$^{-/-}$ bone marrow (n=20), IL-10$^{-/-}$ bone marrow (n=20), SiglecH$^{-/-}$ bone marrow (n=20) or IFNγR II$^{-/-}$ bone marrow (n=20). For these twelve groups we will use the same control group as for the mice that receive only BDCA2-DTR or CD11c-DTR

bone marrow to reduce the number of mice.

	LDLR$^{-/-}$
Adoptive transfer with WT bone marrow	**20**
Adoptive transfer with BDCA2-DTR bone marrow	**20**
Adoptive transfer with CD11c-DTR bone marrow	**20**
Adoptive transfer with BDCA2-DTR + IDO$^{-/-}$ bone marrow	**20**
Adoptive transfer with BDCA2-DTR + ICOS-L$^{-/-}$ bone marrow	**20**
Adoptive transfer with BDCA2-DTR + PD-L1$^{-/-}$ bone marrow	**20**
Adoptive transfer with BDCA2-DTR + IL-10$^{-/-}$ bone marrow	**20**
Adoptive transfer with BDCA2-DTR + IFNyR II$^{-/-}$ bone marrow	**20**
Adoptive transfer with BDCA2-DTR + SiglecH$^{-/-}$ bone marrow	**20**
Adoptive transfer with CD11c-DTR + IDO$^{-/-}$ bone marrow	**20**
Adoptive transfer with CD11c-DTR + ICOS-L$^{-/-}$ bone marrow	**20**
Adoptive transfer with CD11c-DTR + PD-L1$^{-/-}$ bone marrow	**20**
Adoptive transfer with CD11c-DTR + IL-10$^{-/-}$ bone marrow	**20**
Adoptive transfer with CD11c-DTR + IFNyR II$^{-/-}$ bone marrow	**20**
Adoptive transfer with CD11c-DTR + SiglecH $^{-/-}$ bone marrow	**20**
Total	**300**

Donor mice:
3 male donor mice will be needed for 20 female LDLR$^{-/-}$ recipient mice.

WT donor:	3 for WT bone marrow adoptive transfer
BDCA2-DTR donor:	21 for adoptive bone marrow cell transfer
CD11c-DTR donor:	21 for adoptive bone marrow cell transfer
IDO$^{-/-}$ donor:	6 for adoptive bone marrow cell transfer
ICOS-L$^{-/-}$ donor:	6 for adoptive bone marrow cell transfer
PD-L1$^{-/-}$ donor:	6 for adoptive bone marrow cell transfer
IL10$^{-/-}$ donor:	6 for adoptive bone marrow cell transfer
SiglecH$^{-/-}$ donor:	6 for adoptive bone marrow cell transfer
IFNyRII$^{-/-}$ donor:	6 for adoptive bone marrow cell transfer

4. Oral tolerance study (diergroup 27)

In the oral tolerance study we will investigate whether pDC mediate oral tolerance by administration of modified lipids such as oxidized LDL in atherosclerotic mice and by this prevent the development of atherosclerosis. For this study 20 female LDLR$^{-/-}$ are required. These 20 mice will be divided over 2 groups. To study the impact of pDC in oral tolerance induction, modified lipids will be administered intragastrically in the presence (IgG2a isotype control) or absence (pDC depletion by the pDC specific

depletion antibody 120G8) of pDC (n=10 mice/ group).

groups	treatment	animal number
A	oral oxLDL administration + IgG2a isotype control	**10**
B	oral oxLDL administration + 120G8 antibody (pDC depletion)	**10**
	Total	**20**

In vitro studies (diergroup 28):
15 $LDLr^{-/-}$ mice will be needed to be able to isolate sufficient pDCs out of spleen, blood and lymph nodes for the envisioned in vitro experiments.

The number of animals requested is 380 for the in vivo studies ($LDLr^{-/-}$ recipients), 3 for the WT donors, 21 for the BDCA2-DTR donors, 21 for the CD11c-DTR donors, 6 for the $IDO^{-/-}$ donors, 6 for the $ICOS\text{-}L^{-/-}$ donors, 6 for the $PD\text{-}L1^{-/-}$ donors, 6 for the $IL\text{-}10^{-/-}$ donors, 6 for the $IFNyRII^{-/-}$ donors, 6 for the $SiglecH^{-/-}$ donors and 15 for the in vitro experiments which add to a total number of 476 mice.

Powerberekening:

1. NKT cell activation ± pDC depletion study (diergroup 1)
From previous depletion studies in our group, the average is σ= 31.5 and δ is 42 (plaque content as parameter). According to the formula of Sachs ($n = 2(z_{\alpha/2} - z_{\pi})^2 * (\sigma/\delta)^2$ with (80% confidence): F 0.8= 15.7. $n = 15.7 * (\sigma/\delta)^2$) we will need $n = 15.7\ (31.5.5/42)^2$ mice, which corresponds to a total number of animals (n) of 8.8. Taking into account 10% withdrawals due to surgical interventions e.g. collar placement, we can calculate that n= 10 (more precise 9.8) mice per treatment group should be enough for the planned NKT cell activation ± pDC depletion study. In total we will need 10 animals/groups * 4 groups = 40 mice.

2. NKT cell depletion/ pDC depletion study (diergroup 2)
From previous depletion studies in our group, the average is σ= 31.5 and δ is 42 (plaque content as parameter). According to the formula of Sachs ($n = 2(z_{\alpha/2} - z_{\pi})^2 * (\sigma/\delta)^2$ with (80% confidence): F 0.8= 15.7. $n = 15.7 * (\sigma/\delta)^2$) we will need $n = 15.7\ (31.5.5/42)^2$ mice, which corresponds to a total number of animals (n) of 8.8. Taking into account 10% withdrawals due to surgical interventions e.g. collar placement, we can calculate that n= 10 (more precise 9.8) mice per treatment group should be enough for the planned NKT cell/ pDC depletion study. In total we will need 10 animals/groups * 2 groups = 20 mice.

3. pDC depletion and dysfunction study (diergroup 3-17)
From earlier bone marrow transplantation experiments in our group, the average is σ= 47.5 and δ is 45 (plaque area as parameter). According to the formula of Sachs ($n = 2(z_{\alpha/2} - z_{\pi})^2 * (\sigma/\delta)^2$ with (80% confidence): F 0.8= 15.7. $n = 15.7 * (\sigma/\delta)^2$) we will need $n = 15.7\ (47.5/45)^2$ mice, which corresponds to a total number of animals (n) of 17.5. Taking into account 10% withdrawals due to surgical interventions e.g. collar placement, we can calculate that n= 20 (more precise 19.4) mice per treatment group should be enough for the planned bone marrow transplantation studies. In total we will need 20 animals/group * 15 groups = 300 recipient mice. With our previous experience in transplantation of bone marrow cells, we have determined that 3 donors will be enough for 20 recipients.

4. Oral tolerance study (diergroup 27)

From previous studies in our group, the average is σ= 31.5 and δ is 42 (plaque content as parameter). According to the formula of Sachs ($n = 2(z_{\alpha/2} - z_{\pi})^2 * (\sigma/\delta)^2$ with (80% confidence): F 0.8= 15.7. n =15.7 * $(\sigma/\delta)^2$) we will need n = 15.7 $(31.5.5/42)^2$ mice, which corresponds to a total number of animals (n) of 8.8. Taking into account 10% withdrawals due to surgical interventions e.g. collar placement, we can calculate that n= 10 (more precise 9.8) mice per treatment group should be enough for the planned NKT cell/ pDC depletion study. In total we will need 10 animals/groups * 2 groups = 20 mice.

In vitro experiments (diergroup 28):

Taking into account the amount of cells obtained per animal (dendritic cells), the amount of experimental procedures (migration, proliferation, cytokine production, T cell activation) and repetition of experiments (previous experiments have shown that the variance is on average 35% and that in cytokine production is approximately 50%), it can be estimated that the experiment needs to be performed at n>10. An estimation of 15 $LDLr^{-/-}$ animals will be sufficient to obtain the required amount of plasmacytoid dendritic cells to perform in vitro experiments.

Overview of animals:

BMT=bone marrow transplantation, pDC=plasmacytoid dendritic cell, DTR=diphtheria toxin receptor, Ab=antibody

Experiment	**Intervention**	**Genotype**	**N**	**Origin**
NKT cell activation **(diergroep 1)**	+ αGal-Cer + 120G8 antibody	LDL-$r^{-/-}$	10	PATH breeding (CPV)/ Jackson
	+ αGal-Cer + isotype IgG2a control	$LDLr^{-/-}$	10	PATH breeding (CPV)/ Jackson
	+ βGal-Cer + 120G8 antibody	$LDLR^{-/-}$	10	PATH breeding (CPV)/ Jackson
	+ βGal-Cer + isotype IgG2a control	$LDLR^{-/-}$	10	PATH breeding (CPV)/ Jackson
NKT cell depletion **(diergroep 2)**	+ anti-mouse NK1.1/Gr-1 Ab	LDL-$r^{-/-}$	10	PATH breeding (CPV)/ Jackson
	+ IgG2 isotype control	$LDLr^{-/-}$	10	PATH breeding (CPV)/ Jackson
BMT **(diergroep 3)**	WT bone marrow	$LDLR^{-/-}$	20	PATH breeding (CPV)/ Jackson
BMT **(diergroep 4)**	BDCA2-DTR bone marrow	$LDLR^{-/-}$	20	PATH breeding (CPV)/ Jackson
BMT **(diergroep 5)**	CD11c-DTR bone marrow	$LDLR^{-/-}$	20	PATH breeding (CPV)/ Jackson
BMT **(diergroep 6)**	BDCA2-DTR + $IDO^{-/-}$ bone marrow	$LDLR^{-/-}$	20	PATH breeding (CPV)/ Jackson

BMT **(diergroep 7)**	BDCA2-DTR + ICOS-L $^{-/-}$ bone marrow	LDLR$^{-/-}$	20	PATH breeding (CPV)/ Jackson
BMT **(diergroep 8)**	BDCA2-DTR + PD-L1$^{-/-}$ bone marrow	LDLR$^{-/-}$	20	PATH breeding (CPV)/ Jackson
BMT **(diergroep 9)**	BDCA2-DTR + IL10$^{-/-}$ bone marrow	LDLR$^{-/-}$	20	PATH breeding (CPV)/ Jackson
BMT **(diergroep 10)**	BDCA2-DTR + SiglecH$^{-/-}$ bone marrow	LDLR$^{-/-}$	20	PATH breeding (CPV)/ Jackson
BMT **(diergroep 11)**	BDCA2-DTR + IFNγRII$^{-/-}$ bone marrow	LDLR$^{-/-}$	20	PATH breeding (CPV)/ Jackson
BMT **(diergroep 12)**	CD11c-DTR + IDO$^{-/-}$ bone marrow	LDLR$^{-/-}$	20	PATH breeding (CPV)/ Jackson
BMT **(diergroep 13)**	CD11c-DTR + ICOS-L $^{-/-}$ bone marrow	LDLR$^{-/-}$	20	PATH breeding (CPV)/ Jackson
BMT **(diergroep 14)**	CD11c-DTR + PD-L1$^{-/-}$ bone marrow	LDLR$^{-/-}$	20	PATH breeding (CPV)/ Jackson
BMT **(diergroep 15)**	CD11c-DTR + IL10$^{-/-}$ bone marrow	LDLR$^{-/-}$	20	PATH breeding (CPV)/ Jackson
BMT **(diergroep 16)**	CD11c-DTR + SiglecH$^{-/-}$ bone marrow	LDLR$^{-/-}$	20	PATH breeding (CPV)/ Jackson
BMT **(diergroep 17)**	CD11c-DTR + IFNγRII$^{-/-}$ bone marrow	LDLR$^{-/-}$	20	PATH breeding (CPV)/ Jackson
WT donor **(diergroep 18)**	BMT	WT	3	PATH breeding (CPV)/ Jackson
BDCA2-DTR **(diergroup 19)**	BMT	BDCA2-DTR	21	
CD11c-DTR **(diergroup 20)**	BMT	CD11c-DTR	21	
IDO$^{-/-}$ donor **(diergroup 21)**	BMT	IDO$^{-/-}$	6	
ICOS-L$^{-/-}$ donor **(diergroup 22)**	BMT	ICOS-L$^{-/-}$	6	

PD-L1$^{-/-}$ donor **(diergroup 23)**	BMT	PD-L1$^{-/-}$	6	
IL10$^{-/-}$ donor **(diergroup 24)**	BMT	IL10$^{-/-}$	6	
SiglecH$^{-/-}$ donor **(diergroup 25)**	BMT	SiglecH$^{-/-}$	6	
IFNyRII$^{-/-}$ donor **(diergroup 26)**	BMT	IFNyRII$^{-/-}$	6	
Oral tolerance **(diergroep 27)**	+ 120G8 antibody	LDLR$^{-/-}$	10	PATH breeding (CPV)/ Jackson
	+ isotype IgG2a control	LDLr$^{-/-}$	10	CPV breeding
In vitro experiment **(diergroep 28)**	pDC isolation	LDLr$^{-/-}$	15	CPV breeding
Total			**476**	

Dierproef

8. Experiment

In vivo experiments:

We have planned to do four in vivo experiments to investigate an atheroprotective role of pDCs in atherosclerotic lesions and as therapeutic target for cardiovascular disease. **For all studies we will use the carotid artery collar model (for a detailed description see Von der Thuesen et a., Circulation, 103: 1164-1170, 2001;). This model will allow us to assess plaque formation and perivascular tissue build up and yields lesions that can be investigated by two photon laser scanning microscopy (local intervention studies: ex vivo imaging).**

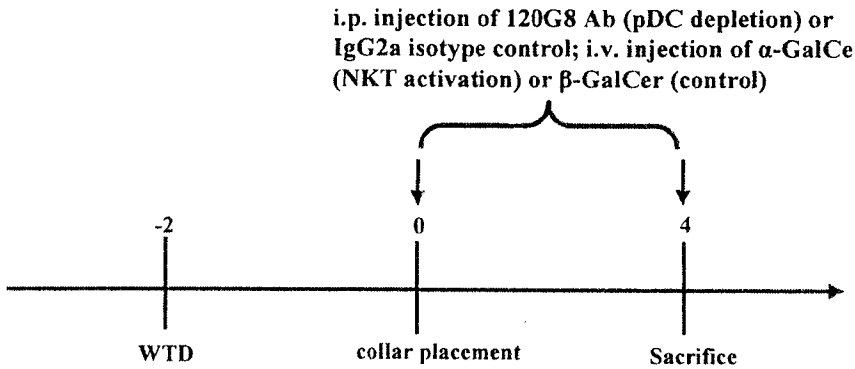
In study 1, 2 and 4 we will investigate plaque development 4 weeks after collar placement (early lesions). In study 3 we will investigate plaque development 8 weeks after collar placement (advanced lesions). In all studies, we will analyze effects on plaque size and morphology (studying mainly T cells, DC, macrophages, cytokine expression) as well as morphology on spleen, lymph nodes, thymus, liver and intestines. Furthermore we will study by FACS the cellular composition and activation characteristics of splenic, circulating and lymph node cells. All data will be analyzed blindly.

In study 1, 2 and 4 we will inject the depleting or control antibodies (120G8 or IgG2a isotype control; anti-mouse NK1.1/Gr-1 or IgG2 isotype control) intraperitoneally and not via a different route, because previous studies in our own group have shown that an i.p. injection leads to the most effective and fastest distribution of the antibody to all the organs.

1. **NKT cell activation study (α-GalCer) ± pDC depletion (120G8) (diergroup 1):**

To study the interaction between NKT cells and pDC in early atherosclerotic lesions (LDLR$^{-/-}$ mice on a

western type diet), we will specifically activate NKT cells by a synthetic ligand, α-GalCer. Control mice will receive the control analogue β-GalCer. Immediately after **collar placement**, mice will be injected twice a week with 2μg of α-GalCer or β-GalCer intravenously (i.v.) for a period of four weeks. In addition mice will be treated with either a pDC specific depletion antibody (120G8) or an IgG2a isotype control (250μg/mouse). The antibody will be administered for 3 consecutive days the first week and then ones per week for the rest of the experiment by intraperitoneal injection. Since the antibody is not stable at 37°C, it cannot be replaced by an osmotic pump. Synthetic ligands and antibodies are diluted in 0.9% NaCl. Mice will be sacrificed at the end of the experiment. This study will give us inside whether NKT can be protective against atherosclerosis through an interaction with pDC.



Mice : **LDLr$^{-/-}$ mice + αGal-Cer +180G8 antibody** (10 female)
LDLr$^{-/-}$ mice + αGal-Cer + control antibody (10 female)
LDLr$^{-/-}$ mice + βGal-Cer (control) +180G8 antibody (10 female)
LDLr$^{-/-}$ mice + βGal-Cer (control) + control antibody (10 female)

Week -2 : Start of western type diet (0.25% cholesterol) up till the end of the experiment

Week 0: Collar placement (2 weeks after initiation of western type diet) + start of i.v. injection of αGal-Cer or β-GalCer and i.p. injection of 120G8 antibody or IgG2a isotype control till the end of the experiment

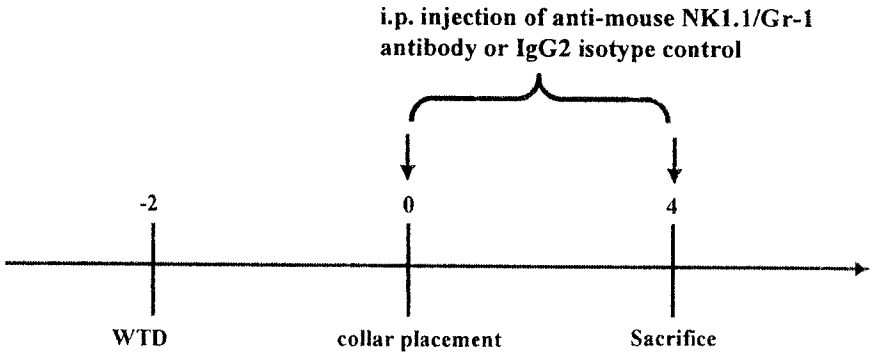
Week 4: Sacrifice of animals

At weeks 0 and at sacrifice, blood will be drawn via the vena saphena (100-200 microliters) to determine lipid levels (indicated by arrow in flow-chart). **At sacrifice, animals will be perfused with PBS-nitroprusside, carotid arteries and aortal tree will be excised and fixed. Excised carotid arteries will partly be used for ex vivo imaging by two photon laser scanning microscopy. Carotids, aortic arch and branch points will be cut and stained to evaluate atherosclerotic lesion area, collagen content, IHC staining of macrophages, T cells, (CD4, CD8, CD25), staining of CTSC, smooth muscle actin, etc.** All analyses will be evaluated blindly. In addition, blood, lymph node and spleen cells will be analyzed for the presence of CD3/CD4/CD8; CD4/CD25/Foxp3 (Tregs); CD3/CD69 Activation antigen (NK cells, T cells); GR-1/Mac1 (granulocyte/macrophages);CD36/MHCII.

2. NKT cell/ pDC depletion (anti-mouse NK1.1/Gr-1) study (diergroup 2):

To study the interaction between NKT cells and pDC in early atherosclerotic lesions (LDLR$^{-/-}$ mice on a western type diet), we will specifically deplete NKT cells and pDC by an anti-mouse NK1.1/Gr-1 monoclonal antibody that is depleting both cell subsets. Control mice will receive an IgG2 isotype

control. Immediately after collar placement, mice will be injected intraperitoneally (i.p.) twice per week with the anti-mouse NK1.1/Gr-1 depletion antibody or the isotype control (4µg/ mouse) till the end of the experiment. Since the antibody is not stable at 37°C, it cannot be replaced by an osmotic pump. Antibodies are diluted in 0.9% NaCl. Mice will be sacrificed at the end of the experiment. This study will give us inside whether NKT can be protective against atherosclerosis through an interaction with pDC.



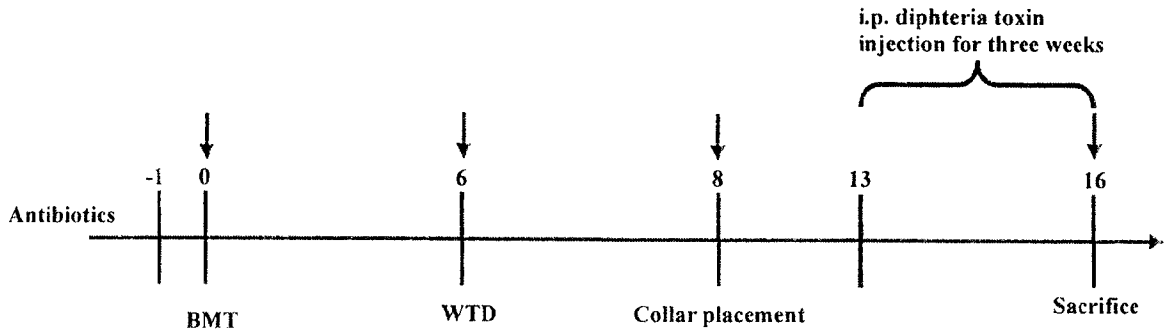
Mice : **LDLr$^{-/-}$ mice + anti-mouse NK1.1/Gr-1** (10 female)
LDLr$^{-/-}$ mice + IgG2 isotype control antibody (10 female)
Week -2 : Start of western type diet (0.25% cholesterol) up till the end of the experiment
Week 0: Collar placement (2 weeks after initiation of western type diet) + start of i.p. injection of anti-mouse NK1.1/Gr-1 antibody or isotype control
Week 4: Sacrifice of animals

At weeks 0 and at sacrifice, blood will be drawn via the vena saphena (100-200 microliters) to determine lipid levels (indicated by arrow in flow-chart). **At sacrifice, animals will be perfused with PBS-nitroprusside, carotid arteries and aortal tree will be excised and fixed. Excised carotid arteries will partly be used for ex vivo imaging by two photon laser scanning microscopy. Carotids, aortic arch and branch points will be cut and stained to evaluate atherosclerotic lesion area, collagen content, IHC staining of macrophages, T cells, (CD4, CD8, CD25), staining of CTSC, smooth muscle actin, etc.** All analyses will be evaluated blindly. In addition, blood, lymph node and spleen cells will be analyzed for the presence of CD3/CD4/CD8; CD4/CD25/Foxp3 (Tregs); CD3/CD69 Activation antigen (NK cells, T cells); GR-1/Mac1 (granulocyte/macrophages);CD36/MHCII.

3. pDC depletion and dysfunction study (diergroup 3-17):

To study the tolerogenic role of immature and mature pDC in advanced atherosclerotic lesions (LDLR$^{-/-}$ mice on a western type diet) we will perform a BMT in which LDLR$^{-/-}$ mice receive bone marrow from BDCA2-DTR or CD11c-DTR transgenic mice. Two weeks after collar placement diphtheria toxin (DT) (100-120ng/mouse) or PBS as control reagent will be administered every two days intraperitoneally (i.p.) for a period of three weeks to deplete either immature pDC (use of BDCA2-DTR model) or mature pDC (use of CD11c-DTR model) systemically. Additionally, we will perform a mixed BMT to establish double chimerisms. LDLR$^{-/-}$ mice receive bone marrow from BDCA2-DTR or CD11c-DTR transgenic mice mixed with IDO$^{-/-}$ bone marrow, ICOS-L$^{-/-}$ bone marrow, PD-L1$^{-/-}$ bone marrow, IFNγR II$^{-/-}$ bone marrow, SiglecH$^{-/-}$ bone marrow or IL-10$^{-/-}$ bone marrow in a ratio of 1:10. Mice will be sacrificed at the end of the experiment. This study will enable us to see if immature and mature pDC function in a

tolerogenic way in atherosclerotic lesions and through which mechanisms they induce tolerance.



Recipient mice:

LDLr$^{-/-}$ mice + WT BMC's (20 females)
LDLr$^{-/-}$ mice + BDCA2-DTR BMC's (20 females)
LDLr$^{-/-}$ mice + CD11c-DTR BMC's (20 females)
LDLr$^{-/-}$ mice + BDCA2-DTR + IDO$^{-/-}$ BMC's (20 females)
LDLr$^{-/-}$ mice + BDCA2-DTR + ICOS-L$^{-/-}$ BMC's (20 females)
LDLr$^{-/-}$ mice + BDCA2-DTR + IFNyRII$^{-/-}$ BMC's (20 females)
LDLr$^{-/-}$ mice + BDCA2-DTR + IL10$^{-/-}$ BMC's (20 females)
LDLr$^{-/-}$ mice + BDCA2-DTR + PD-L1$^{-/-}$ BMC's (20 females)
LDLr$^{-/-}$ mice + BDCA2-DTR + SiglecH$^{-/-}$ BMC's (20 females)
LDLr$^{-/-}$ mice + CD11c-DTR + ICOS-L$^{-/-}$ BMC's (20 females)
LDLr$^{-/-}$ mice + CD11c-DTR + IFNyRII$^{-/-}$ BMC's (20 females)
LDLr$^{-/-}$ mice + CD11c-DTR + IL10$^{-/-}$ BMC's (20 females)
LDLr$^{-/-}$ mice + CD11c-DTR + PD-L1$^{-/-}$ BMC's (20 females)
LDLr$^{-/-}$ mice + CD11c-DTR + SiglecH$^{-/-}$ BMC's (20 females)
LDLr$^{-/-}$ mice + CD11c-DTR + IDO$^{-/-}$ BMC's (20 females)

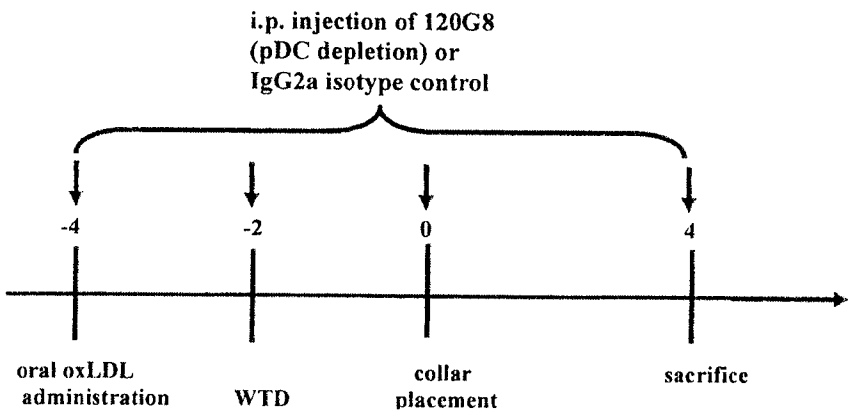
Week 1- :	Start of antibiotic treatment, up to week 6
Week 0:	Irradiation of animals with 10Gy
Week 0 + 1day:	Transplantation of bone marrow cells
Week 6:	Initiation of the western typ diet (0.25% cholesterol) till the end of the experiment
Week 8:	Collar placement (2 weeks after initiation of western typ diet)
Week 13:	diphtheria toxin injection for 3 weeks (i.p injection every two days)
Week 16:	Sacrifice of animals (8 weeks after collar placement) and extraction of carotid artery, heart, aortic tree, spleen, lymph nodes, blood.

At weeks 0, 6, 8 and at sacrifice, blood will be drawn via the vena saphena (100-200 microliters) to determine lipid levels and chimerism (indicated by arrow in flow-chart). At sacrifice, animals will be perfused with PBS-nitroprusside, carotid arteries and aortal tree will be excised and fixed. Excised carotid arteries will partly be used for ex vivo imaging by two photon laser scanning microscopy. Carotids, aortic arch and branch points will be cut and stained to evaluate atherosclerotic lesion area,

collagen content, IHC staining of macrophages, T cells, (CD4, CD8, CD25), staining of CTSC, smooth muscle actin, etc. All analyses will be evaluated blindly. In addition, blood, lymph node and spleen cells will be analyzed for the presence of CD3/CD4/CD8; CD4/CD25/Foxp3 (Tregs); CD3/CD69 Activation antigen (NK cells, T cells); GR-1/Mac1 (granulocyte/macrophages);CD36/MHCII.

4. Oral tolerance study (diergroup 27):

To determine whether pDC can mediate oral tolerance in atherosclerotic LDLR$^{-/-}$mice (early lesions), we will induce intragastric administration of modified lipids (oxidized LDL) three times per week over a period of 14 days before mice are put on a western type diet for further six weeks. Two weeks after starting the western type diet, **mice will receive collars** to develop atherosclerotic lesion. To see if pDC are involved in the mediation of oral tolerance we will perform the study in the presence (i.p. injection of an IgG2a isotype control) or absence (i.p. injection of 120G8 depletion antibody) of pDC (250µg/mouse). The antibody will be administered for 3 consecutive days the first week and then ones per week for the rest of the experiment. Since the antibody is not stable at 37°C, it cannot be replaced by an osmotic pump. Antibodies are diluted in 0.9% NaCl. Mice will be sacrificed at the end of the experiment.



Mice : **LDLr$^{-/-}$ mice + oral oxLDL administration +180G8 antibody** (10 female)
LDLr$^{-/-}$ mice + oral oxLDL administration + IgG2a isotype control (10 female)

Week -4: Start of oral oxLDL administration (three times/ week for 14 days) + i.p. injection of 120G8 antibody or IgG2a isotype control till the end of the experiment

Week -2 : Start of western type diet up till the end of the experiment, stop oral oxLDL administration

Week 0: Collar placement (2 weeks after initiation of western type diet)

Week 4: Sacrifice of animals

At weeks -4, -2, 0 and at sacrifice, blood will be drawn via the vena saphena (100-200 microliters) to determine lipid levels (indicated by arrow in flow-chart). **At sacrifice, animals will be perfused with PBS-nitroprusside, carotid arteries and aortal tree will be excised and fixed. Excised carotid arteries will be partly used for ex vivo imaging by two photon laser scanning microscopy. Carotids, aortic arch and branch points will be cut and stained to evaluate atherosclerotic lesion area, collagen content, IHC staining of macrophages, T cells, (CD4, CD8, CD25), staining of CTSC, smooth muscle actin, etc.** All

analyses will be evaluated blindly. In addition, blood, lymph node, spleen and intestinal cells will be analyzed for the presence of CD3/CD4/CD8; CD4/CD25/Foxp3 (Tregs); CD3/CD69 Activation antigen (NK cells, T cells); GR-1/Mac1 (granulocyte/macrophages);CD36/MHCII; PDCA1/ CCR9 (immature pDC).

In vitro experiments (diergroup 28):
A total amount of 15 $LDLR^{-/-}$ mice will be needed for the in vitro studies since the population of DCs and especially of pDCs consists only of a minor percentage of the total amount of leukocytes (± 5%). DCs will be isolated for several in vitro experiments: DC differentiation and activation, cytokine production, T cell activation, receptor expression, apoptosis.

9. Experimentele condities

9a. Anesthesie
Induction and maintenance of anesthesia is done with isoflurane administration (starting with 4% and decreasing till ± 2.5% depending on the mouse for maintaining anesthesia). During anesthesia, the eyes of the mice will be treated with eye ointment to prevent dryness.

9b. Pijnbestrijding
Pre-operative painkiller: Indomethacin (0.5mg/kg body weight), a non-steroidal antiinflammatory drug (NSAID) and temgesic (0.05 mg/kg body weight).
Post-operative painkiller: Indomethacin (0.5mg/kg body weight) or temgesic (0.05 mg/kg body weight), buprenorphine hydrochloride (0.1-0.05 mg/kg body weight (highest dose only used when necessary, because of side effects)). Applied when the mice wake up after anesthesia until following day.

9c. Euthanasie en Humane eindpunten
- Overdosis penthobarbital (115 mg/kg)
- The animals will be examined regularly and checked for activity, weight loss, food and water consumption, fur composition. If an animal looses more than 15% weight loss in one day or 20% weight loss in one week, it will be euthanized in agreement with the art. 14 functionaries. If an animal shows signs of peritonitis after repeated i.p. injections, circling after collar placement or wasting after BMT, it will be euthanized in agreement with the art. 14 functionaries.

10a. Ongerief

All mice will be housed in groups. They will be subjected to a high fat diet (=01). Mice in the NKT activation study, mice in the NKT depletion study and mice in the oral tolerance study will be on a high fat diet for 6 weeks. Mice that get a bone marrow transplantation will be on a high fat diet for 10 weeks. To evaluate the lipid contents (all mice) and check chimerism (mice that received a BMT), blood samples will be drawn several times via the vena saphena (=02). The discomfort for the animals that receive an intravenous injection will be moderate (=02). The discomfort for the animals that receive an oral administration of oxLDL by gastric intubation will moderate (=03).The suffering of the animals that receive bone marrow cells is moderate-serious (=04), since irradiation induces permanent damage to the immune system of the animals (6 weeks for recovery). Animals in all in vivo intervention studies will get a collar placement under anesthesia. The discomfort of the collar placement will be moderate-serious (=04) and will decrease after mice are recovered from surgery (maximum 1 week). The discomfort for the animals that receive intraperitoneal injections will be moderate-serious (=04). For the rest of the animals, the donor mice, the discomfort will be small (=01).

mouse group	**type**	**discomfort**	**duration**
mouse group 1	high fat diet	=01	6 weeks
	taking blood from the vena saphena	=02	2 times over the whole experiments (each time 5-15 minutes)
	intravenous injection	=02	2 times per week over 4 weeks
	intraperitoneal injection	=04	3 consecutive days in the first week + once per week for further three weeks (total 4 weeks)
	collar placement	=04	1 week for recovery
mouse group 2	high fat diet	=01	6 weeks
	taking blood from the vena saphena	=02	2 times over the whole experiments (each time 5-15 minutes)
	intraperitoneal injection	=04	2 times per week over 4 weeks
	collar placement	=04	1 week for recovery
mouse group 3-17	high fat diet	=01	10 weeks
	taking blood from the vena saphena	=02	4 times over the whole experiments (each time 5-15 minutes)
	intraperitoneal injection	=04	2 times per week over three weeks
	BMT	=04	6 weeks for recovery
	collar placement	=04	1 week for recovery
mouse group 28	high fat diet	=01	6 weeks
	taking blood from the vena saphena	=02	4 times over the whole

			experiments (each time 5-15 minutes)
	intraperitoneal injection	=04	3 consecutive days in the first week + once per week for further seven weeks (4 weeks in total)
	gastric intubation of oxLDL	=03	3 times per week for two weeks
	collar placement	=04	1 week for recovery

10b. Welzijnsevaluatie
From the experience in our group and that of other groups (Molecular Genetics) we know that collar placement only incidentally leads to complications. We know from the experience in our group that after a bone marrow transplantation in certain experiments we had high drop out numbers. We know from experience in our group that the systemic application of human diphtheria toxin is tolerated well.

11. Verzorging en huisvesting
The animal housing and environment complies in accordance with the Dutch regulations of animal welfare, with water and food ad libitum. Bone marrow recipients and transgenic mice are kept in filter-top cages and receive water with antibiotics (neomycin 100mg/l and polymixin B sulphate (60,000 U/l) for 7 weeks (ref SOP-19 with adaptation: the water will not be acidified anymore). The water will be refreshed 3 times per week.
The mice that will receive Diphteria toxin (DT) injections will be housed in filter top cages in a special housing area for 3 weeks. After that, they will be sacrificed.
Any problems, please contact the responsible researcher (, or (tel

12. Deskundigheid
All participating researchers have experience in the handling and operation of animals (art. 9 or art. 12).

13. Standard Operation Procedures (SOP)
Diet composition:
15% cocoa butter, 1% corn oil, 0.25% cholesterol, 40.5% sucrose, 10% corn starch, 5.95% cellulose (Hope Farms, Woerden, NL).
Bone marrow transplantation: SOP Gen-19-M (see attached)
Collar placement: see attached SOP
Taking blood from the vena saphena: SOP CPV-3-MR (see attached)
Diphteria toxin (DT) injection: see attached SOP
Intragastric oxLDL administration: see attached SOP
Sacrifice: The animals will be euthanized with an overdose penthobarbital (6:4)

Relevante literatuur

1. *Tangirala, R.K., E.M. Rubin, and W. Palinski, Quantitation of atherosclerosis in murine models: correlation between lesions in the aortic origin and in the entire aorta, and differences in the extent of lesions between sexes in LDL receptor-deficient and apolipoprotein E-deficient mice. J Lipid Res, 1995. 36(11): p. 2320-8.*

2. *von der Thusen, J.H., T.J. van Berkel, and E.A. Biessen, Induction of rapid atherogenesis by perivascular carotid collar placement in apolipoprotein E-deficient and low-density lipoprotein receptor-deficient mice. Circulation, 2001. 103(8): p. 1164-70.*

3. *Leijh, P.C., et al., Effect of thioglycolate on phagocytic and microbicidal activities of peritoneal macrophages. Infect Immun, 1984. 46(2): p. 448-52.*

SOP Gen-19-M
Bone marrow transplantation

Preparation of acceptor mice

Seven days before the bone marrow transfer:

1. Recipient mice are transferred to semi-sterile conditions (e.g. filter top cages).
2. Mice are fed a regular chow diet.
3. Mice are put on autoclaved drinking water (not acidified) to which neomycin (100 mg/l) and polymyxin B sulphate (60,000 U/l) are added (after autoclaving the water), until 6 weeks after the transfer. Minimum twice a week new drinking water is made and provided to the mice in clean bottles.

One day before the bone marrow transfer:

4. In a cross flow, mice are transferred from their filter-top cages to an irradiation box (which can be a plastic box suitable for 5-10 mice).
5. Mice are subjected to a lethal dose of total body irradiation using a röntgen source (10 Gy) (note 1).
6. After the irradiation, mice are transferred, in the cross flow, back to their filter top cages.

Isolation of bone marrow cells (on the day of the transfer)

1. Sacrifice the donor mice by cervical dislocation after sedation or CO_2/ O_2 asphyxiation (note 2).
2. Cut off the hind legs after removing the skin.
3. Isolate femurs and tibia from the mice and remove most of the skeletal muscles (note 3).
4. Put the bones in PBS, keep on ice and take to flow cabinet.
5. Sterilize the bones by submerging them for 30 seconds in 70% ethanol in a petri dish.
6. Transfer to sterile PBS in a petri dish.
7. Take a bone between forceps, cut off the ends and flush the bone using PBS by inserting a 25G needle at one end.
8. Collect bone marrow in a 50ml tube.
9. Repeat previous steps for all the bones.
10. Make the bone marrow cell suspension single cell by first passing it through a 19G needle, transfer it to a new tube and then pass it through a 25G needle.
11. Count the cells.
12. Spin the cells down at 1000rpm, 5 min at 4°C.
13. Resuspend the cells in RPMI 1640 / 2% FCS / 5U/ml heparin at a density of $5x10^7$ cells/ml and keep on ice.

Transplantation of the bone marrow cells

1. Take the cells to cross flow and inject the irradiated mice that have been slightly pre-warmed (note 4), intravenously with 100 µl of cell suspension using a 27G needle.
2. Six weeks after transplantation, mice may be removed from the filter top cages, drinking water can be regular again. Experiments start 6 weeks after transplantation (note 5).

Notes

1. The dose for irradiation should be determined empirically; currently we are using a dose of 10 Gy. Testing several doses of irradiation followed either by no-transplantation and analysis of survival or by transplantation and analysis of chimerism (*see* **Note 5**) after 4 weeks will be essential.
2. On average we inject 5 x 10^6 cells/recipient mouse. We obtain 2-4 x 10^7 cells/donor mouse.
3. Skeletal muscle from the bones is easily removed by rubbing them with a paper tissue.
4. Warming the mice using a heated mat or infrared lights enhances the visibility of the tail vein, highly facilitating the intravenous injections.
5. Ideally, complete chimerism is established, meaning that all hematopoietic cells of the recipient are of donor-origin. However, sometimes chimerism is incomplete or donor cells have only replaced specific lineages of the hematopoietic system. Therefore, it is often important to establish the amount of chimerism in the recipients. This can be done, for example, by transplanting female recipient mice with male donor bone marrow and quantitatively genotyping the bone marrow after transplantation for the presence of Y-markers (by quantitative RT-PCR or Southern blotting). Also other genetic differences between donors and recipients can be used to quantify chimerism.

SOP Collar placement

De chirurgische ingreep vindt plaats onder aseptische condities.
- de muis wordt in een kamertje verzadigd met 4.0% isofluraan gemengd met perslucht onder anesthesie gebracht en met 2.5% isofluraan gemengd met perslucht onder anesthesie gehouden.
- de ogen van de muis worden behandeld met oogzalf om uitdroging te voorkomen
- de muis wordt gefixeerd en op 36.5 gradenC gehouden via een gethermostreerde verwarmingsplaat.
- de voorzijde van de nek wordt geschoren en de huid gedesinfecteerd (betadine). Een mediale longitudinale incisie wordt gemaakt van ca. 1 cm. Door het scheiden van de m.sternocleidomastoideus van de trachea komt de a.carotis aan de rechter zijde van de muis bloot te liggen.
- de carotis communis wordt vrijgeprepareerd van het omringende bindweefsel waarbij erop gelet wordt de nervus vagus niet te beschadigen. Onder de carotis communis worden drie ligaturen (hecht-zijde 6-0) klaargelegd, waarmee later de collar kan worden bevestigd.
- een 2 mm lange collar (Silastic tubing met een binnendiameter van 0.3 mm, overlangs doorgesneden) wordt om de a. carotis communis gelegd en met de drie ligaturen vastgelegd.
- de m.sternocleidomastoideus wordt teruggeplaatst en de huid over de wond wordt gesloten (synthetisch hechtdraad 5-0).
- de muis komt bij een een temperatuur gereguleerde kamer (30 gradenC).

CPV-3-MR
Bloedafname via vena saphena

Doel:
Het herhaaldelijk afnemen van kleine hoeveelheden bloed zonder anesthesie via de vena saphena.

Benodigdheden:

- muis – naald (oranje): 0.5 mm x 16 mm, 25G x 5/8"
  rat – naald (blauw): 0.5 mm x 25 mm, 23 G)
- Fixatiedoek (om het dier in the fixeren, bijv. washandje of handdoek)
- Gaasjes of tissues
- Warmtebron: infrarode lamp (150 Watt) of Warmtebox (Beta Corp)
- Scheerapparaat
- Cupjes (al dan niet geheparinisеerd)

Voorbereiding:

- Het dier indien nodig verwarmen m.b.v. een waterlamp of –box, om de vena saphena goed zichtbaar te maken. Gedurende het opwarmen bij het dier blijven.
  - Warmtelamp: minimale afstand tot het dier 50 cm
    Duur: minimaal 5 tot maximaal 15 minuten.
  - Warmtebox:
    Temperatuur instellen op ongeveer 37°C
    Duur: minimaal 5 tot maximaal 15 minuten.
- De linker of rechter achterpoot wordt geschoren ter hoogte van de hak aan de laterale zijde.
- Het dier wordt gefixeerd door het tot het bekken in een speciaal daarvoor gemaakte dubbelgestikte washand (CPV) te laten lopen (of kies een andere manier van restraining).
- Het dier wordt vervolgens met de volle hand over de rug stevig rond het bekken vast gepakt.
- De rechter of linker achterpoot wordt gefixeerd tussen duim en wijsvinger zodat de poot gestrekt wordt.
- De poot wordt gestuwd door meer of minder druk op de binnenzijde van het bovenbeen (liesgebied) uit te oefenen.

Bloedafname:

- Zodra de vene goed zichtbaar is, wordt deze aangeprikt in het midden van het vat loodrecht op de huid.
- Door meer of minder te stuwen kan de uitstromende hoeveelheid beïnvloedt worden.
- Het bloed wordt opgevangen in een cupje.
- Per dier wordt een nieuwe naald gebruikt.

Nazorg:

- Tijdens en na afloop van de bloedafname wordt gecontroleerd of het dier ongewenste effecten van de handeling ondervindt.
- Na de bloedafname wordt gedurende enkele seconden de aanprikplek met een gaasje dichtgedrukt. Om het bloeden te verminderen kan ook de voet geflexed worden.

Voor de methode van bloedafname via de 'saphenous vein puncture' wordt ook verwezen naar het artikel van Hem et al. (1998).

Zie verder: http://www.uib.no/vivariet/mou_blood/Blood_coll_mice_.html

Literatuur:

Hem A., Smith A.J. and Solberg P. (1998) Saphenous vein puncture for blood sampling of the mouse, rat, hamster, gerbil, guinea pig, ferret and mink. Laboratory Animals 32, 364-368.

SOP Diphteria toxin (DT) Injection

Preparation of diphtheria toxin

1) preparation of diphtheria toxin: work always under a sterile hood
2) reconstitute a single vial of diphtheria toxin powder (1mg) with 1ml of sterile H_20 dest. or 0.01M Tris/ 0.001M Na_2EDTA (pH7.5) (use a syringe and a needle to plunge through the vial)
3) aliquot diphtheria toxin solution and store at -20°C just until use in a box 'DT-Hazardous'

Preparation of mice for diphtheria toxin injection

1) label cage cards 'DT-Hazardous'; if working in a procedure room, bring appropriate protective equipment and biohazard bags with you
2) thaw the amount of diphtheria toxin that is needed and dilute further in PBS (final concentration: 100-120ng DT/mouse)
3) diluted DT is loaded into a tuberculin syringe
4) inject 100µl of DT or PBS as control intraperitoneally into the mouse using a 27G needle (inject on an absorbent pad on the bench top)
5) dispose the used needle directly after use into sharps container
6) after injection mice are transferred into filter top cages in a special housing area (label area 'DT treatment' where animals are stored during the procedure)

Precautions:

1) Hazards

- Diphtheria toxin (DT) is a potent lethal toxin in humans, the minimum lethal dose is 100ng/kg (6-10 micrograms).
- the toxin is harmful in humans, if inhaled, injected or absorbed through the eyes and skin
- the toxin has no effect, if administered orally as it is unstable at an acidic pH
- if inadvertent skin pricking occurs, encourage bleeding and perform vigorous flushing of the area with copious amounts of water and/ or saline
- if intravenous injection occurs, seek a physician immediately

2) Protection

- individual that perform experiments using diphtheria toxin should have a recent Diphteria/ Tetanus booster vaccination (within the last five years)
- don't work with the toxin in the dried state, work only with reconstituted material
- work in a well-ventilated area and avoid inhalation of the product
- wear protective double gloves, a surgical mask and lab coat
- avoid contact with open wounds
- the amount of excreted unmetabolized DT by the injected mice is likely to be extremely low, cleaning of the cages can be disposed as usual two days after DT-injection

3) Waste/ clean-up

- contaminated pipettes, lab ware, etc. should be disposed of as hazardous waste (i.e. put in an appropriately labelled red biohazard bag and autoclave)
- decontaminate all objects in spill area with bleaching solution (allow 20-30 minutes of contact time)
- wipe area with disinfectant
- autoclave everything

SOP intragastric intubation

Aim: Controlled administration of a specific component through the oesophagus into the stomach

Materials:
- a 18G stainless steel, ball tipped needle
- gloves
- component (liquid) + 1ml syringe

Preparation:
- prepare component for intragastric intubation by taking it up with a 1ml syringe
- grasp the loose skin on the back of the mouse and restrain the tail with the ring finger and the little finger
- introduce the feeding needle carefully and gently from the pharynx into the esophagus when the mouse is in the act of swallowing
- administer component by pressing carefully and not too fast the plunger of the syringe (feeding amount limited to 1% of the body weight)

Risks/ complications:
- damage to the esophagus when needle is introduced too fast
- administration of the component into the trachea

University Maastricht

Faculty of Health, Medicine and Life Sciences

Dierexperimenten Commissie

DEC

voorzitter
p/a Secretariaat DEC-UM
Postbus 616
NL-6200 MD Maastricht
Telefoon:

Aan:

Uw referentie: Onze referentie : Maastricht, 01-03-2011

Geachte Onderzoeker,

Uw projectaanvraag: "*Studying the atheroprotective role of plasmacytoid dendritic cells in atherosclerosis*", is op de DEC vergadering van 25 februari 2011 besproken.

De DEC heeft een aantal vragen en opmerkingen:

- De duur van de proef op het voorblad is niet juist (dit is de langste periode binnen één project dat één dier in proef is). De DEC verzoekt dit aan te passen.
- Bij punt vraagt de DEC zich af wat de noodzaak is om een collar te plaatsen, terwijl de metingen worden gedaan aan de aortaboog?

Gelieve eventuele vragen te beantwoorden in **een brief en indien noodzakelijk Uw project aan te passen en duidelijk de aanpassingen grijs te markeren.**
Uw project staat bij de DEC geregistreerd onder nummer **2011-038**, gelieve dit nummer in verdere correspondentie te vermelden.

Hoogachtend,

Voorzitter DEC-UM

Aan:
DEC
. voorzitter
p/a Secretariaat DEC-UM
Postbus 616
NL-6200 MD Maastricht Telefoon: i

Dear DEC-UM,

I received your letter with questions and remarks about my project inquiry: "*Studying the atheroprotective role of plasmacytoid dendritic cells in atherosclerosis*". The project is registered under the DEC number **2011-038**.
I marked your remarks/ questions in grey and adapted the questions (also marked in grey).

The DEC was asking me if it is necessary to place collars in all four in vivo studies while final investigations will be done at the aortic arch (point 8. experiments: in vivo experiments). In these four studies the main aim is to analyse atherosclerotic lesion formation not only in the aortic arch and branch points but particularly in the carotid arteries, because we would like to compare the experimental outcome with results we obtained in earlier studies (compare experiments that have been done in the DEC 2009-080). Furthermore we are also interested in local intervention studies of the carotids with atherosclerotic lesions (ex vivo imaging). For these reasons we will use the collar model.

Kind regards,

Maastricht University

Faculty of Health, Medicine and Life Sciences

Aan:

Ons kenmerk	*Doorkiesnummer*	*Maastricht*
RH/MQ- 155-11	043-	07-04-2011

Project: *Studying the atheroprotective role of plasmacytoid dendritic cells in atherosclerosis.*

DEC-UM
Voorzitter DEC-UM

p/a secretariaat DEC-UM

Secretariaat DEC-UM

Bezoekadres

Postadres
Postbus 616
6200 MD Maastricht

Verantwoordelijk onderzoeker (VO):

Hierbij delen wij U mede dat voornoemd project aan de ethische toetsingscriteria voor proefdiergebruik voldoet.
De DEC maakt geen bezwaar tegen uitvoering van dit project zoals aangevraagd en geeft een **positief advies**.

Projectnummer:	**2011-038**
Diersoort:	**muis**
Aantal dieren:	**476**
Einddatum:	**07-04-2015**

Uw project staat bij de DEC en CPV geregistreerd onder bovenstaand nummer. Gelieve dieren, die voor dit project bestemd zijn, ook onder dit nummer aan te vragen.

Voorzitter DEC-UM

Vice-Voorzitter DEC-UM