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Studying the interaction of dendritic cell subsets and T-cells in atherosclerotic lesions

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	Naam	Tel (+ Tel privé enkel VO, VVO en VM)	E-mailadres	Bevoegdheid[5]	Cap. groep /afdeling
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. Principle Investigator (PI)				Art.9	

Diergroep	**1**	**2**	**3.**	**4.**	**5.**	**6.**	**7.**	**8.**
ctrl/exp/sham	exp	exp	exp	exp	exp	exp	exp	exp
Diersoort	01	01	01	01	01	01	01	01
Stam	C57Bl6	C57Bl6	C57Bl6	C57Bl6	C57Bl6	C57Bl6	C57Bl6	C57Bl6
Construct / mutatie ?	$LDLR^{-/-}$	$LDLR^{-/-}$	C57Bl6	C57Bl6	$LDLR^{-/-}$	$LDLR^{-/-}$	C57Bl6	$LDLR^{-/-}$
Herkomst (leverancier) *	01	01	02	02	01	01	01	01
Aantal	100	32	23	7	20	40	20	15
Geslacht	V	V	M	M	V	V	M	M/V
Dieren immuuncompetent ?	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
Doel van de proef *	31	31	31	31	31	31	31	31
Belang van de proef *	01	01	01	01	01	01	01	01
Toxicologisch onderzoek *	01	01	01	01	01	01	01	01
Bijzondere technieken *	01	01	02	02	01	01	01	02
Anesthesie *	04	01	01	01	01	01	01	01
Pijnbestrijding *	04	01	01	01	01	01	01	01
Mate ongerief *	04	04	01	01	03	04	04	01
Toestand dier einde exp*	01	01	01	01	01	01	01	01

* VHI-coderingen zie bijlage

1 Verantwoording

Aanvraag dierproef DEC-UM **(kaders zijn licht flexibel, maar het geheel is max. 5 pag. versie 2006)**

Titel: Studying the interaction of dendritic cell subsets and T-cells in atherosclerotic lesions

1. Doel van de proef.

Cardiovascular disease (CVD), principally heart disease and stroke, is the world's leading killer for both men and women among all racial and ethnic groups. Though statins and hypotensive drugs represent one of the major therapeutic revolutions in the modern era of cardiovascular medicine, the advent of these drugs has not afforded complete solutions for the treatment and prevention of cardiovascular disease. This demands a detailed study of the process and background involved in vascular lesions or plaque development instead of alleviating the risk factors with the presently available drugs. Inflammation is a natural biological response to injury that promotes healing and repair. It is deemed to contribute considerably to the progression and clinical manifestation of atherosclerosis. Dendritic cells (DC) are one subset of immune cells. They are specialized in decoding and integrating signals from the innate immune system and ferrying the information to the adaptive immune system (T and B cells). DC can be divided into several subsets.

Our focus is on plasmacytoid dendritic cells (pDC) and their involvement in atherosclerosis. pDC are derived from precursors in the bone marrow. Once activated by viral or bacterial stimuli they produce large amounts of type I interferons (IFN-α, IFN-β). 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. We recently have shown in a loss-of function approach that pDC in $LDLR^{-/-}$ mice on high fat diet induce tolerance by dampening the proliferation and activation of $CD4^+$ T-cells which leads to less atherosclerotic plaque formation [3]. How pDC T-cell interactions take place (systemically or in the atherosclerotic plaques and the neighbouring lymph nodes) and which markers/ receptors are involved needs still to be investigated.

Another DC-subset we are interested in, are the $CD8a^+$ DC. $CD8a^+$ DC are able to take up necrotic cell material, process it and display it as antigens on their MHCI molecules. This process, referred to as cross-presentation, results in activation of cytolytic CD8 T-cells, which will then mount an acute inflammatory response. In the later stages of atherosclerosis, where plaques with large necrotic cores are flooded with inflammatory cells, it is highly likely that this cascade of events destabilizes advanced atherosclerotic plaques.

In these studies we want to study the interaction of pDC and $CD8^+$ DC with T-cells (polarization, proliferation of T-cells) in $LDLR^{-/-}$ mice on high fat diet to see if hypercholesterolemia has any influence on the activation of different DC-subsets. This will enable us to determine if high cholesterol levels in the plasma can influence the tolerogenic status of pDC. Additionally these studies will tell us to which extent cross-presentation occurs (involvement of $CD8^+$ DC) under high cholesterol levels.

To study the function of pDC they will be pulsed ex vivo with modified lipids. Adoptive transfer of pulsed pDC into new $LDLR^{-/-}$ recipient mice on high fat diet (for different periods of time) will be performed after. pDC T-cell interactions (polarization and proliferation of T-cells in vivo and in vitro), and the development of atherosclerosis will be investigated.

In another study we want to isolate naïve $CD4^+$ T-cells from tolerogenic versus non-tolerogenic mice (oral oxLDL administration in the presence or absence of pDC) and perform in vitro polarization experiments.

To study the function of $CD8a^+$ DC (cross-presentation), $LDLR^{-/-}$ mice will be placed on high fat diet for different periods of time. Mice will receive an intravenous injection of necrotic Jurkat cells plus chicken Ovalbumine. When cross-presentation occurs, necrotic cell material is taken up and presented by $CD8a^+$ DC. This study will enable us to determine if high cholesterol levels in the

plasma can influence the extent in which cross-presentation occurs.
These studies will offer important insights in understanding the relationship between high cholesterol levels and the activation/ behaviour of different DC-subsets. Finally results will clarify, if DCs are good therapeutic targets for future therapeutic design in atherosclerosis.

2. Maatschappelijke relevantie en/of wetenschappelijk belang

According to the World Health Organisation (WHO), cardiovascular diseases (CVDs) are the number one cause of death globally. More people die annually from CVDs than from any other cause. By 2030, almost 23.6 million people will die from CVDs, mainly from heart disease and stroke. Progress in the treatment is mainly hampered by the incomplete understanding of the mechanism of the disease process and its major players. Inflammation plays a crucial role in the initiation and development of atherosclerosis. Extensive evidence supports inflammatory/immune activation of plaques as a cause of acute coronary syndromes.
Recent studies have shown that Dendritic cell subsets (DCs) are present in both, in human and mouse atherosclerotic lesions, suggesting a role of this cell subset in cardiovascular disease. In this study we will focus on the contribution of the different DC-subsets in atherosclerotic lesion formation. We expect that this study will not only contribute to the understanding of lesion development and progression but also will lead to new therapeutic strategies to treat the disabling disease of symptomatic atherosclerosis.

3. Alternatieven

In vitro methods using cell cultures represent important tools in the study of the role of DC-subsets 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 DC 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 and morbidity in the world, despite current advance in therapeutic intervention. Cardiovascular disease thus not only puts a sizable burden to health care expenditure but also is accompanied by great losses for economy, and personal wellbeing due to its disabling nature. This justifies further efforts to study the mechanism of disease progression and for the identification of new targets for therapy or prevention that add to the currently available modalities. It also renders mouse models for atherosclerosis mandatory for the benefit of the society. In this study we will investigate the contribution of specific DC-subsets (pDC and $CD8a^+$ DC) in the development and progression of the disease and determine their relevance as therapeutic targets. Given the complex, multifactor character of the disease, it is essential that these studies will be executed in the complex context of

atherosclerosis using a mouse model, representative for human disease.

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.

It has been shown by several groups that several dendritic cells (DC) subsets are present not only in human atherosclerotic lesions, but also in mouse lesions. The functional input of DCs to atherosclerotic lesion formation is still in its beginnings. We could show in a recent study that plasmacytoid dendritic cells (pDC), a specific subset of DC, act tolerogenic in $LDLR^{-/-}$ on a high fat diet and led to reduced plaques by dampening the activation of $CD4^+$ T-cells. Which signal is triggering pDC to act tolerogenicand where the pDC T-cell interactions take place needs still to be investigated. Furthermore it is still not known which role $CD8a^+$ DC are playing in the process of atherogenesis and if they are involved in plaque (de)stabilization.

To study a direct tolerogenic function of pDC in the process of atherosclerosis, pDC will be isolated from spleens of C57Bl6 mice and will be pulsed ex vivo with modified lipids (oxidized LDL) or with PBS only (control). After, pDC will be transferred intravenously into $LDLR^{-/-}$ recipient mice that will be placed on high fat diet for different periods of time. Mice will be boosted two times with pulsed pDC before they are put on high fat diet. The number of pDC are very low in blood and lymphoid organs of C57Bl6 mice (~0.1-0.3%). To have enough cells for adoptive transfer and in vitro experiments a lot of mice need to be sacrificed. To avoid unnecessary sacrifice of animals we will use a Flt3L-secreting B16 melanoma cell line (available in our Department). Flt3L is one of the most important growth factors responsible for the growth of several DC-subsets. The injection of the melanoma cell line that is constantly secreting this growth factor will lead to a high expansion of DC without activating them. 10-14 days after subcutaneous injection of the B16 cells into C57Bl6 mice, pDC can be isolated from spleens, pulsed ex vivo under sterile conditions and transferred into new recipient mice. Boosting of pulsed pDC will be done for two times before mice are placed on high fat diet for different periods of time. Mice that are placed for six weeks on high fat diet will receive collars to study the development of plaque formation after pDC transfer. In all groups polarization (by flow cytometry) and proliferation (by BrdU injection) of T-cell subsets will be investigated in different organs.

In a second study we will investigate the influence of pDC on the proliferation of CD4 and CD8 T-cells in $LDLR^{-/-}$ mice on a high fat diet. We will perform the study in the presence or absence of pDC (use of a specific pDC depletion antibody 120G8). $LDLr^{-/-}$ recipient mice on a high-fat diet will receive fluorescently-labeled Ovalbumin- specific OT-I (CD8 T-cells) or OT-II (CD4 T-cells) cells together with Ovalbumin- pulsed necrotic splenocytes three days before sacrifice. At sacrifice T-cell proliferation and expansion of the OT-cell subsets will be measured by flow cytometry. The results of both studies will clarify if pDC are involved in suppression of T-cell proliferation in hypercholesterolemic mice. The OT-I/ OT-II model allows us to study proliferation and expansion of specific CD4 and CD8 T-cells. OT-I and OT-II cells are from mice transgenic for a specific T-cell receptor designed to recognize a specific chicken ovalbumin epitope when it is presented by DC-subsets. OT-I and OT-II cells will react to the ovalbumin and proliferate, a reaction that is measurable by flow cytometry. OT-I and OT-II mice will be provided by our

In another study we will culture naive $CD4^+$ Tcells, isolated from mice that received oxLDL via oral gavage and are placed on high fat diet afterwards. Oral administration of oxLDL will take place in

the presence or absence of pDC (pDC-depletion by use of a depletion antibody). Isolated $CD4^+$ T-cells will be cultured in vitro under different stimulation conditions (addition of modified lipids, etc.) to investigate T-cell polarization.
In the 4th study we want to study the effect of hypercholesterolemia on the efficiency of cross-presentation (by $CD8a^+$ DC). Herefore, $LDLR^{-/-}$ mice will be placed for different periods of time on high fat diet. Three days before sacrifice all mice will receive necrotic Jurkat cells pulsed with Ovalbumin and fluorescently-labeled OT-I cells. This study will show us if cross-presentation via $CD8a^+$ DC occurs in mice with high cholesterol levels in the plasma and if these $CD8a^+$ DC support activation/ proliferation of T-cells which would finally lead to an activation of the immune system, a process that is occuring in the atherosclerotic lesion.
All studies will clarify if different DC-subsets get activated by modified lipids/ high cholesterol levels and to which content they contribute to the progress of atherosclerosis (specific interaction with T-cells, T-cell polarization and proliferation). Furthermore this clarifies if these cells are good targets for future therapeutic interventions.

6. Wetenschappelijke beoordeling
The principle investigator (PI) of the has evaluated and approved the scientific content of this DEC proposal.

6 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.

OT-I and OT-II (Ovalbumine T-cell receptor specific transgenic mice) mice will be used to study the cross-presenting capacity of $CD8a^+$ DC in $LDLR^{-/-}$ mice on a high fat diet and to study the function of pDC in atherosclerotic mice. These mice will be provided by

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 in vitro experiments, both male and female mice will be used as there are no indications of gender differences.

7.c. Aantallen

We will perform four major in vivo experiments to investigate the role of pDCs and $CD8a^+$ DC in atherosclerosis and to clarify if these cells are good therapeutic targets for cardiovascular disease: 1) studying the effect of pulsed pDC on T-cell polarization, proliferation and plaque development (adoptive transfer of pDC), 2) T-cell proliferation in the presence or absence of pDC, 3) in vitro polarization of primed $CD4^+$ T cells, isolated from oxLDL-pulsed mice and 4) studying the effect of $CD8a^+$ DC on hypercholesterolemia (cross-presentation).

For the expansion of pDC/ $CD8a^+$DC, we will inject the Flt3L-secreting B16 melanoma cell line into C57/Bl6 mice. Expanded pDC and $CD8a^+$ DC will be used either for the above mentioned major in vivo experiments or for in vitro studies.

1. **Studying the effect of pulsed pDC on T-cell polarization, proliferation and plaque development (adoptive transfer of pDC) (diergroup 1)**

In this intervention study we will investigate if pDC, pulsed ex vivo with modified lipids (oxLDL) can induce tolerance when injected into new female $LDLR^{-/-}$ recipient mice that are placed on high fat diet for different time periods (adoptive pDC transfer). To have enough pDC for adoptive transfer pDC will be isolated from spleen of C57Bl6 mice that are treated with Flt3L-secreting B16 melanoma cells. Here we will pulse pDC with oxLDL or with PBS (control). Recipient $LDLR^{-/-}$ mice will be boosted two times with oxLDL- or PBS-pulsed pDC before they are placed on a high fat diet. $LDLR^{-/-}$ recipients that are on high fat diet for six weeks will receive a collar. For this intervention study we will need 100 female $LDLR^{-/-}$ mice. Mice will be divided over 10 groups (n= 10 mice/ group).

Here we will place mice for either 0.5 week, 1 week, 1.5 weeks, 3 weeks or 6 weeks on high fat diet. WE have chosen these different time points to investigate if pDC are able to induce T-cell polarization into an anti-inflammatory direction (induction of regulatory T-cells, Th2 T-cells) already in the early beginning of high fat diet (already after 0.5 or 1 week) or just at later time points (3 weeks, 6 weeks). Furthermore we want to know if pDC stay tolerogenic over the whole time of high fat diet or if (and when) they switch to a proinflammatory status, supporting lesion formation.

Results of previous studies have shown us already that pDC in mice that are only for 0.5 weeks to 1 week on high fat diet start upregulating tolerogenic markers. In these studies we did not investigate T-cell polarization. We also want to know what is happening after 1.5, 3 and 6 weeks.
The results of this study will help us to decide if pulsed pDC are possible therapeutic tools dampening atherosclerotic lesion progression and further on to which time point it would be best to inject them (early versus later time points).

groups	treatment/ plaque development	animal number
A	oxLDL pulsed pDC, 0.5 weeks on HFD	10
B	PBS pulsed pDC (control), 0.5 weeks on HFD	10
C	oxLDL pulsed pDC, 1 week on HFD	10
D	PBS pulsed pDC (control), 1 week on HFD	10
E	oxLDL pulsed pDC, 1.5 weeks on HFD	10
F	PBS pulsed pDC (control), 1.5 weeks on HFD	10
G	oxLDL pulsed pDC, 3 weeks on HFD	10
H	PBS pulsed pDC (control), 3 weeks on HFD	10
I	oxLDL pulsed pDC, 6 weeks on HFD	10
J	PBS pulsed pDC (control), 6 weeks on HFD	10
	Total	**100**

2. Studying the effect of T-cell proliferation in the presence or absence of pDC (diergroup 2-4)
In this intervention study we will investigate the influence of pDC on proliferation of CD4 and CD8 T-cells in $LDLR^{-/-}$ mice on high fat diet. We will perform the study in the presence (IgG2a isotype control) or absence of pDC (use of the pDC depletion antibody 120G8). 3 days before sacrifice OT-I (CD8 T-cells) or OT-II (CD4 T-cells) will be injected together with Ovalbumin- pulsed necrotic splenocytes. At sacrifice T-cell proliferation of the T-cell subsets (OT-I and OT-II) will be measured by flow cytometry. The study will give insight into the involvement of pDC in suppression of T-cell proliferation. For this intervention study we will need 32 female $LDLR^{-/-}$ mice. Mice will be divided over 4 groups (n=8 mice/ group).
We will furthermore need 7 **OT-I** mice and 7 **OT-II** mice to have enough OT-I and OT-II cells (isolated from spleen and lymph nodes) for the injection into $LDLR^{-/-}$ mice.

groups	treatment/ plaque development	animal number
A	injection of OT-I cells + IgG2a isotype control	8
B	injection of OT-I cells + 120G8	8
C	Injection of OT-II cells + IgG2a isotype control	8
D	Injection of OT-II cells + 120G8	8
	Total	**32**

3. In vitro polarization of naive $CD4^+$ T cells, isolated from oxLDL-fed mice (diergroup 5)

In this intervention study we will isolate naive $CD4^+$ T-cells from $LDLR^{-/-}$ mice, that received oxLDL or PBS via oral gavage. Oral administration of oxLDL or PBS will be done in the presence (IgG2a isotype control) or absence of pDC (use of the pDC depletion antibody 120G8). $CD4^+$ T-cells will be cultured in vitro to investigate T-cell polarization. For this intervention study we will need 20 female $LDLR^{-/-}$ mice. Mice will be divided over 4 groups (n=5 mice/ group).

groups	treatment/ plaque development	animal number
A	T-cells from oxLDL pulsed mice + 120G8	5
B	T-cells from oxLDL pulsed mice + IgG2a isotype control	5
C	T-cells from PBS pulsed mice + 120G8	5
D	T-cells from PBS pulsed mice + IgG2a isotype control	5
	Total	**20**

4. Studying the effect of $CD8a^+$ DC on hypercholesterolemia (cross-presentation) (diergroup 6+3)

In this intervention study we will determine if high cholesterol levels in the plasma can influence the extent in which cross-presentation (by $CD8a^+$ DC) occurs. Herefore we will use the OT-I T-cell model. For this study we will need 40 female $LDLR^{-/-}$ mice that will be placed for different time periods on high fat diet (3 weeks, 1.5 weeks, 1 week, 0.5 weeks) or on normal chow diet. The 40 mice will be divided over 5 groups. We will furthermore need **16 OT-I mice** to have enough OT-I (isolated from spleen and lymph nodes) for the injection into $LDLR^{-/-}$ mice.
Here we will place mice for either 0.5 week, 1 week, 1.5 weeks, 3 weeks or 6 weeks on high fat diet. WE have chosen these different time points to investigate if cross-presentation occurs already in the early beginning of high fat diet or just at later time points. Previous studies in our group have shown that other cell subsets (monocytes) get activated already at a very early time point in mice fed a high fat diet and can by this support lesion progression. Here we are focusing on the $CD8a^+$ DC and there interaction with T-cells. It is important for us to know if T-cells get already activated at an early time point and if they stay activated over the whole time of high fat diet. Results are important for us to decide to which time point we can interfere and by this suppress lesion development.

groups	treatment	animal number
A	no treatment (animals on chow diet)	8
B	high fat diet for 3 weeks	8
C	high fat diet for 1.5 weeks	8
D	high fat diet for 1 week	8
E	high fat diet for 0.5 weeks	8
	Total	**40**

5. **Expansion of DC-subsets using the Flt3L-secreting B16 melanoma cell line (diergroup 7)**

For the adoptive transfer of pDC (experiment 1) $100x10^6$ pDC are needed in total. From one spleen of a C57/Bl6 mouse that is injected with the Flt3L-secreting B16 melanoma cell line we will receive $5x10^6$ pDC. This means that we will need 20 C57Bl6 mice in total for the in vivo experiments.

In vitro studies (diergroup 8)

15 $LDLr^{-/-}$ mice will be needed to be able to perform DC culture studies in vitro (pulsing of different DC-subsets with modified lipids, control reagents).

The number of animals requested is 192 for the in vivo studies (female $LDLr^{-/-}$ recipients). 23 OT-I and 7 OT-II mice are needed. 20 male C57Bl6 are necessary for the isolation of pDC.
15 LDR-/- mice are needed for in vitro experiments.
The **total amount** of mice that are needed is **257**.

Powerberekening:

1. **Studying the effect of pulsed pDC on T-cell polarization, proliferation and plaque development (adoptive transfer of pDC) (diergroup 1)**

From previous studies in our group we know that the average is σ= 31.5 and δ is 42 (plaque content as parameter to get significant differences). 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 study. In total we will need 10 animals/groups * 10 groups = 100 mice.

2. **Studying the effect of T-cell proliferation in the presence or absence of pDC (diergroup 2-4)**

In this study we will investigate the effect of T-cell proliferation in the presence or absence of pDC. From previous studies in our group we know that the average is σ= 28 and δ is 40 (necessary to calculate possible significant differences). 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 $n = 15.7\ (28/40)^2$ mice, which corresponds to a total number of animals (n) of 7.693. We calculate that n=8 mice per treatment group should be enough for the planned study. In total we will need 8 animals/groups * 4 groups = 32 mice.

Additionally we need OT-I mice and OT-II mice to isolate OT-I and OT-II T-cells for in vivo injection into the $LDLR^{-/-}$ recipients. $2x10^6$ OT-I or OT-II cells will be injected in 1 mouse. We have 16 $LDLR^{-/-}$ mice that receive OT-I and 16 $LDLR^{-/-}$ mice that receive OT-II cells. In total we need $32x10^6$ OT-I and $32x10^6$ OT-II cells. From 1 OT-I or OT-II mouse we can isolate $5x10^6$ OT-I or OT-II cells.
Than we need 7 OT-I mice ($5x10^6$ x 7 = $35x10^6$) and 7 OT-II mice ($5x10^6$ x 7 = $35x10^6$) in total.

3. **In vitro polarization of naive $CD4^+$ T cells, isolated from oxLDL-fed mice (diergroup 5)**

In this study we will perform in vitro experiments with naïve $CD4^+$ T-cells that are isolated from oxLDL- or PBS-pulsed mice (via oral gavage). To have a biological significance in vitro, n=5 per group is necessary. In total we will need 5 animals/ group* 4 groups = 20 mice.

4. Studying the effect of CD8a$^+$ DC on hypercholesterolemia (cross-presentation) (diergroup 6+3)

In this study we will investigate the effect of hypercholesterolemia on the efficiency of cross-presentation. From previous studies in our group we know that the average is σ= 28 and δ is 40 (necessary to calculate possible significant differences). 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 $n = 15.7\ (28/40)^2$ mice, which corresponds to a total number of animals (n) of 7.693. We calculate that n=8 mice per treatment group should be enough for the planned study. In total we will need 8 animals/groups * 5 groups = 40 mice.

Additionally we need OT-I mice to isolate OT-I T-cells for in vivo injection into the LDLR$^{-/-}$ recipients. $2x10^6$ OT-I cells will be injected in 1 mouse. We have 40 LDLR$^{-/-}$ mice in total that receive OT-I cells. In total we need $80x10^6$ OT-I cells. From 1 OT-I mouse we can isolate $5x10^6$ OT-I cells.
Than we need 16 OT-I mice ($5x10^6$ x 16 = $80x10^6$) in total.

5. Expansion of DC-subsets using the Flt3L-secreting B16 melanoma cell line (diergroup 7)

For the adoptive transfer of pDC (experiment 1) $100x10^6$ pDC are needed in total. From one spleen of a C57/Bl6 mouse that is injected with the Flt3L-secreting B16 melanoma cell line we will receive $5x10^6$ pDC. This means that we will need 20 C57Bl6 mice that will be injected with the Flt3L-secreting B16 melanoma cell line in total for the in vivo experiments.

In vitro experiments (diergroup 8)
Taking into account the amount of cells obtained per animal (dendritic cells), the amount of experimental procedures (pulsing of cells, coculture assays) and repetition of experiments (previous experiments have shown that the variance is on average 35%, 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.

Experiment	Intervention	Genotype	N	Origin
Adoptive transfer of pDC **(diergroep 1)**	oxLDL pulsed pDC, 0.5 weeks on HFD	LDL-r$^{-/-}$	10	PATH breeding (CPV)/ Jackson
	PBS pulsed pDC, 0.5 weeks on HFD	LDLr$^{-/-}$	10	PATH breeding (CPV)/ Jackson
	oxLDL pulsed pDC, 1 week on HFD	LDLR$^{-/-}$	10	PATH breeding (CPV)/ Jackson
	PBS pulsed pDC, 1 week on HFD	LDLR$^{-/-}$	10	PATH breeding (CPV)/ Jackson
	oxLDL pulsed pDC, 1.5 weeks on HFD	LDL-r$^{-/-}$	10	PATH breeding (CPV)/ Jackson
	PBS pulsed pDC, 1.5 weeks on HFD	LDLr$^{-/-}$	10	PATH breeding (CPV)/ Jackson
	oxLDL pulsed pDC, 3 weeks on HFD	LDLR$^{-/-}$	10	PATH breeding (CPV)/ Jackson
	PBS pulsed pDC, 3 weeks on HFD	LDLR$^{-/-}$	10	PATH breeding (CPV)/ Jackson
	oxLDL pulsed pDC, 6 weeks on HFD	LDLR$^{-/-}$	10	PATH breeding (CPV)/ Jackson
	PBS pulsed pDC, 6 weeks on HFD	LDLR$^{-/-}$	10	PATH breeding (CPV)/ Jackson
T-cell proliferation +/- pDC **(diergroep 2)**	+ OT-I T-cells + 120G8 antibody	LDLR$^{-/-}$	8	PATH breeding (CPV)/ Jackson
	+ OT-I T-cells + isotype IgG2a control	LDLR$^{-/-}$	8	PATH breeding (CPV)/ Jackson
	+ OT-II T-cells + 120G8 antibody	LDLR$^{-/-}$	8	PATH breeding (CPV)/ Jackson
	+ OT-II T-cells + isotype IgG2a control	LDLR$^{-/-}$	8	PATH breeding (CPV)/ Jackson
OT-I cells **(diergroup 3)**	adoptive transfer of OT-I T-cells	OT-I T-cells	7	
OT-II cells **(diergroup 4)**	adoptive transfer of OT-II T-cells	OT-II T-cells	7	
Polarization of naive CD4^{+} T-cells **(diergroep 5)**	+ oxLDL + 120G8 antibody	LDLR$^{-/-}$	5	PATH breeding (CPV)/ Jackson
	+ oxLDL + isotype IgG2a control	LDLR$^{-/-}$	5	PATH breeding (CPV)/ Jackson
	+ PBS + 120G8 antibody	LDLR$^{-/-}$	5	PATH breeding (CPV)/ Jackson

	+ PBS + isotype IgG2a control	LDLR$^{-/-}$	5	PATH breeding (CPV)/ Jackson
Cross-presentation/ hypercholesterolemia **(diergroep 6)**	high fat diet	LDLR$^{-/-}$	40	PATH breeding (CPV)/ Jackson
(diergroup 3)	adoptive transfer of OT-I T-cells	OT-I T-cells	16	
Expansion of pDC **(diergroup 7)**	+ Flt3L-secreting B16 melanoma cells	C57Bl6	20	PATH breeding (CPV)/ Jackson
In vitro experiment **(diergroep 8)**	pDC isolation	LDLR$^{-/-}$	15	PATH breeding (CPV)/ Jackson
Total amount of mice			**257**	

8. Experiment

We have planned to perform four major in vivo experiments to investigate the role of pDCs and $CD8a^+$ DC in atherosclerosis and to clarify if these cells are good therapeutic targets for cardiovascular disease: 1) studying the effect of pulsed pDC on T-cell polarization, proliferation and plaque development (adoptive transfer of pDC), 2) studying the effect of T-cell proliferation in the presence or absence of pDC, 3) in vitro polarization of naïve $CD4^+$ T cells, isolated from oxLDL-fed mice and 4) studying the effect of $CD8a^+$ DC on hypercholesterolemia.

For the expansion of pDC, we will inject the Flt3L-secreting B16 melanoma cell line into C57Bl6 mice. Expanded pDC will be used either for the above mentioned major in vivo experiments or for in vitro studies.

In study 1 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 this study we will investigate plaque development 4 weeks after collar placement. Here, 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, specifically T-cells (polarization and proliferation status). All data will be analyzed blindly.

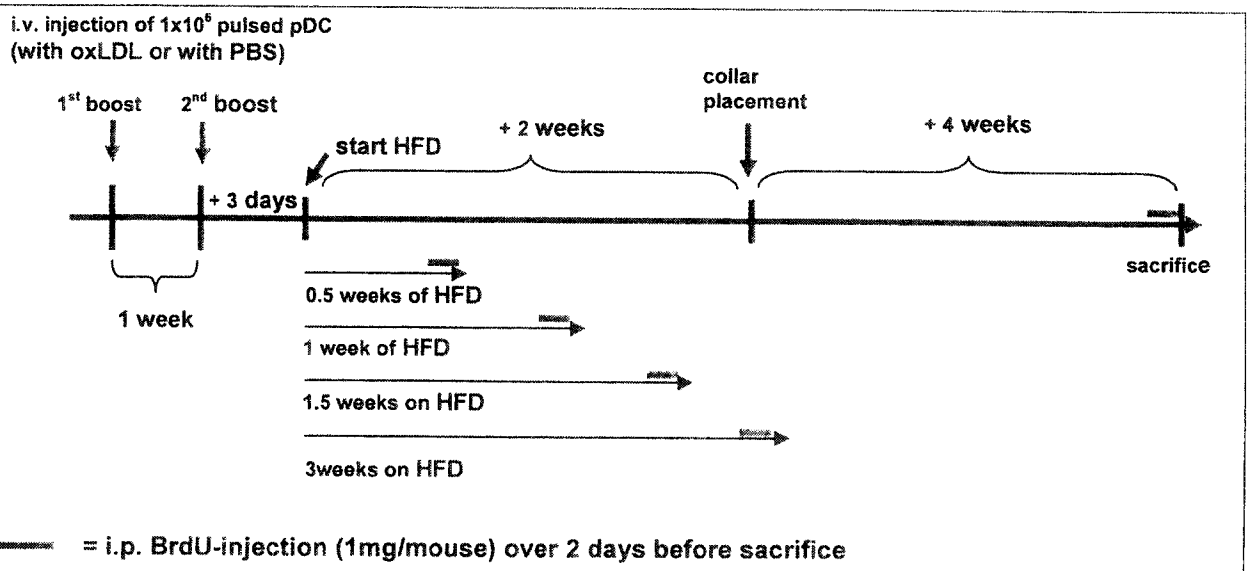
In study 2 we will investigate the influence of pDC on proliferation of CD4 and CD8 T-cells in $LDLR^{-/-}$ mice on a high fat diet. In study 3 we will investigate the in vitro polarization of isolated naïve $CD4^+$ T-cells from mice that will be fed before with oxLDL (oral gavage).

In study 4 we will investigate the effect of hypercholesterolemia on the efficiency of cross-presentation.

1. **Studying the effect of pulsed pDC on T-cell polarization, proliferation and plaque development (adoptive transfer of pDC) (diergroup 1)**

In this experiment pDC will be isolated from spleens of C57/Bl6 mice that were injected with Flt3L-secreting B16 melanoma cells. Isolated pDC will be cultured in vitro for 4-8 hours with PBS (control) or 30 ug oxidized LDL (oxLDL) (=ex vivo pulsing) before being administered intravenously to $LDLR^{-/-}$ mice ($1x10^6$ pDCs per mouse). $LDLR^{-/-}$ recipient mice will be boosted 1 week later with the same antigen-loaded pDC-subsets ($1x10^6$ DCs per mouse).

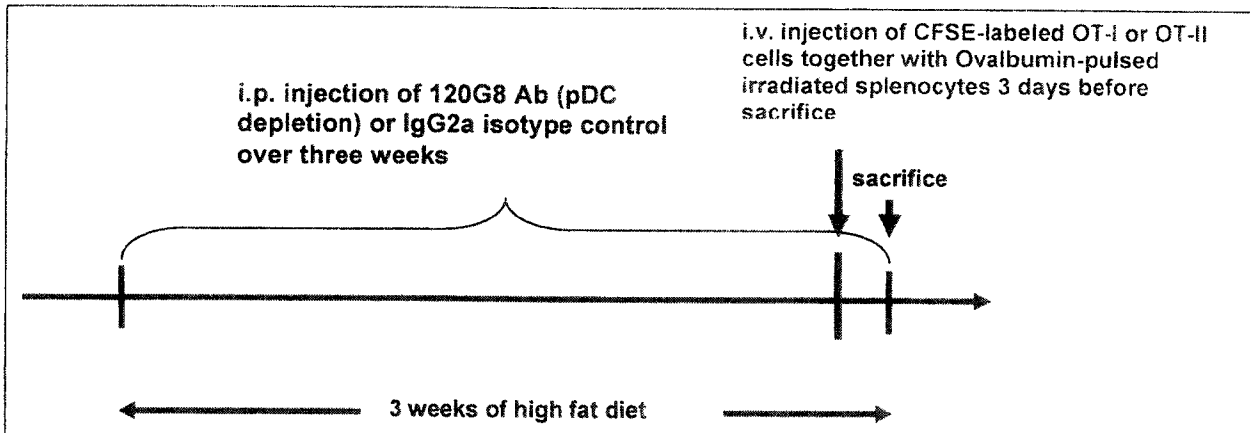
$LDLR^{-/-}$ mice will be put 3 days after the 2^{nd} injection on a high fat diet for several time points (0.5 weeks, 1 week, 1.5 weeks, 3 weeks and 6 weeks). $LDLR^{-/-}$ recipiënt mice that are placed on high fat diet for six weeks will receive collars two weeks after starting the high fat diet. All mouse groups will receive intraperitoneal BrdU-injections (1mg/mouse) 2 days before sacrifice for every 8 hours (six injections in total) to study T-cell proliferation. BrdU will intercalate in the DNA of proliferating cells.



At the beginning of the experiment blood will be drawn from the vena saphena (100-200ul) to analyze lipid levels in the plasma. At sacrifice, blood, lymph node, spleen, liver, thymus and intestine cells will be analyzed for the presence of different T-cell subsets (Th1, Th2, Treg) and T-cell proliferation by flow cytometry.
Mice will be perfused with PBS-nitroprusside, carotid arteries and aortal tree will be excised and fixed. 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), smooth muscle actin, etc. All analyses will be evaluated blindly.

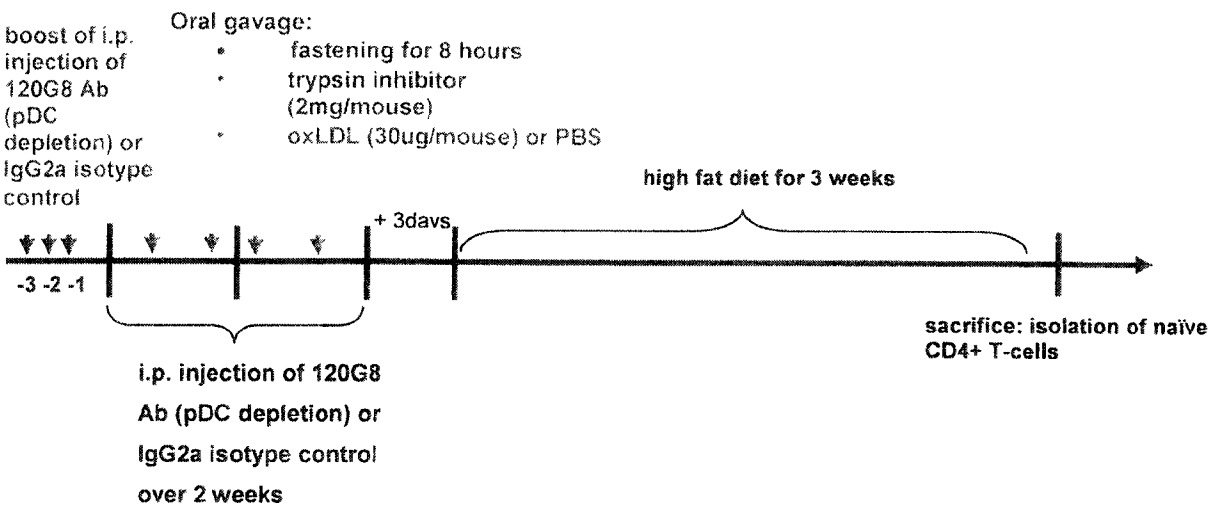
2. **Studying the effect of T-cell proliferation in the presence or absence of pDC (diergroup 2-4)**

In this intervention study we will investigate the influence of pDC on proliferation of CD4 and CD8 T-cells in $LDLR^{-/-}$ mice on a high fat diet for three weeks. Mice will be treated with either a pDC specific depletion antibody (120G8) or an IgG2a isotype control (200µg/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. $LDLR^{-/-}$ mice on HFD will receive an intravenous injection of CFSE-labeled Ovalbumin-specific OT-I (CD8 T-cells) ($2x10^6$ cells/ 200ul PBS) or OT-II (CD4 T-cells) ($2x10^6$ cells/ 200ul PBS) cells together with Ovalbumine-pulsed necrotic splenocytes (irradiation of cells with 1500rad) ($1x10^5$ necrotic cells/ 200ul PBS) three days before sacrifice. At sacrifice, blood, lymph node, spleen, and thymus will be analyzed for the T-cell proliferation and expansion (OT-I and OT-II T-cells). The results will clarify if pDC are involved in suppression of T-cell proliferation in hypercholesterolemic mice.



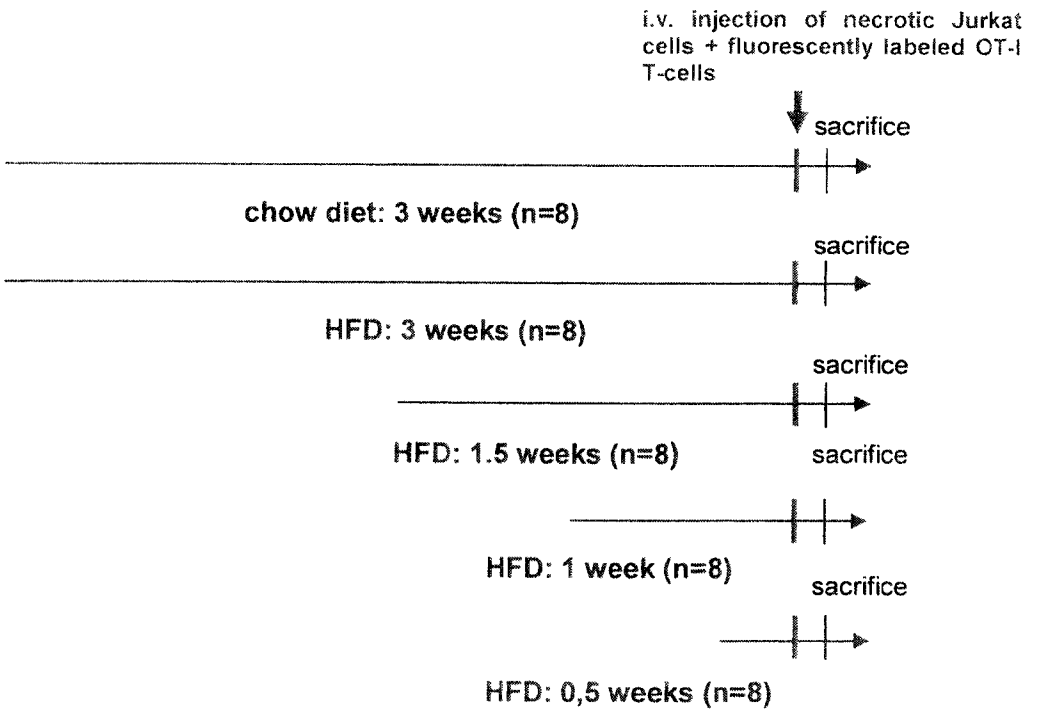
3. In vitro polarization of naïve $CD4^+$ T cells, isolated from oxLDL-fed mice (diergroup 5)

In this intervention study we will analyse in vitro polarization of in vivo primed naïve $CD4^+$ T-cells, isolated from $LDLR^{-/-}$ mice that are placed for three weeks on high fat diet. Before starting the high fat diet, mice will receive an oral gavage of oxLDL (30ug/ 100ul of PBS) or PBS alone. 10 minutes before administration of oxLDL or PBS mice receive via oral gavage a trypsin inhibitor to avoid degradation of the administered compound (2mg/ mouse diluted in 200ul of PBS). oxLDL or PBS will be administered in total 4 times over a period of 2 weeks Before each oral gavage animals will be fasted for 8 hours. After the oral gavage animals receive food ad libitum.
Mice will also be treated with either a pDC specific depletion antibody (120G8) or an IgG2a isotype control (200µg/mouse) over the two weeks of oral gavage. The antibody will be administered for 3 consecutive days before starting the oral gavage and then ones per week during oral gavage by intraperitoneal injection. Since the antibody is not stable at 37°C, it cannot be replaced by an osmotic pump. Antibodies are diluted in 0.9% NaCl. At start of the high fat diet a blood sample will be drawn via the vena saphena (100-200 microliters) to determine lipid levels.At sacrifice naïve $CD4^+$ T-cells will be isolated from spleen and lymph nodes and used for further in vitro culture experiments.



4. Studying the effect of CD8a$^+$ DC on hypercholesterolemia (cross-presentation) (diergroup 6+3)

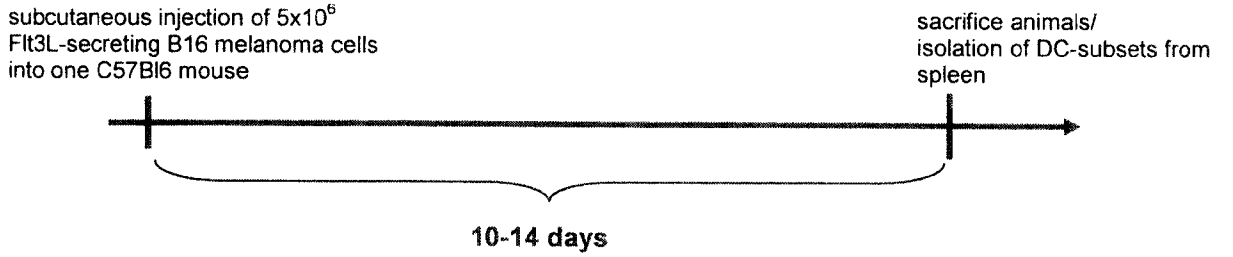
In this intervention study we will investigate if high cholesterol levels in the plasma can influence the extent in which cross-presentation occurs. We will make use of female LDLR$^{-/-}$ mice that are placed on chow diet for 3 weeks or on high fat diet for 3, 1.5, 1 or 0.5 weeks. Each mouse will receive an intravenous injection of necrotic Jurkat cells that are pulsed with ovalbumin (irradiation of cells with 1500rad) (1x10^5 necrotic cells/ 200ul PBS), as well as an injection with fluorescently (CFSE) labeled OT-I T-cells (2x10^6 cells/ 200ul PBS) three days before sacrifice. OT-I T-cells are isolated from mice transgenic for a specific T-cell receptor designed to recognize a specific chicken ovalbumin epitope, when presented in a MHC-I (major histocompatibility complex) context. When cross-presentation occurs, necrotic cell material including the chicken ovalbumin epitopes are taken up and presented by CD8a$^+$ DC. OT-I T-cells will react to the specific ovalbumin epitopes and proliferate.
The results of this experiment will offer important insight in understanding the relationship between cross-presenting activity and high cholesterol levels.



At the beginning of the experiment a blood sample will be drawn via the vena saphena (100-200 microliters) to determine lipid levels. Animals that are for different time points on diet will be sacrificed by an overdosis penthobarbital (150-200 mg/kg BW) and blood, spleen, lymph nodes and aortic arch/ descending aorta will be taken out for isolation of the different cell subsets including the OT-I T-cells. The immune status of the cells and the proliferation of the OT-I T-cells will be measured by flow cytometry.

Expansion of DC-subsets (plasmacytoid and conventional dendritic cells) in vivo (diergroup 7)

pDC numbers are very low in C57Bl6 mice (0.1-0.3% in blood and spleen). To perform transfer of isolated pDC into recipiënt mice $1x10^6$ cells are needed minimally for one intravenous injection (Experiment 1: adoptive transfer of pDC). The expansion of DC-subsets can be induced by injection of the growth factor Flt3L. Mouse in vivo injections of pure Flt3L are really expensive. For this reason there is a Flt3L-secreting B16 melanoma cell line available (available in our Department) that promotes the expansion of DC-populations in vivo. To isolate Flt3L-expanded DC-populations, C57/Bl6 mice will be injected subcutaneously with $5x10^6$ Flt3L-secreting B16 melanoma cells. After 10-14 days, spleens of these mice will be taken out to isolate DC-populations. After injection of Flt3L-secreting B16 melanoma cell into C57Bl6 mice, we will receive $5x10^6$ pDC from one spleen.



In vitro experiments (diergroup 8):
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 (150-200 mg/kg BW)
- 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 in one day or 20%

weight 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, it will be euthanized in agreement with the art. 14 functionaries.

Zorg

10a. Ongerief

Animals will be subjected to a high fat diet (=01). To evaluate the lipid contents (group 1 and 4) blood samples will be drawn 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 or PBS by oral gavage will be moderate (=03). Collar placement will be done 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). The discomfort for the animals that receive subcutaneous injections will be moderate (=02). For the rest of the animals, the donor mice, the discomfort will be small (=01). All animals will be sacrificed by an overdosis pentobarbital (150-200 mg/kg BW) at the end of experiment (=03).

mouse group	**type**	**discomfort**	**duration**
mouse group 1	high fat diet	=01	0.5, 1, 1.5, 3 or 6 weeks
	taking blood from the vena saphena	=02	1 time over the whole experiments (each time 5-15 minutes)
	intravenous injection	=02	2 times over 1 week
	intraperitoneal injection (BrdU)	=04	6 times over 2 days
	collar placement	=04	1 week for recovery
	sacrifice of animals (overdosis pentobarbital: 150-200 mg/kg BW)	= 03	end of experiment
mouse group 2	high fat diet	=01	3 weeks
	intraperitoneal injection	=04	3 consecutive days in the first week + once per week for further three weeks (total 4 weeks)
	intraperitoneal injection (OT-I or OT-II cells + Ovalbumine)	=04	1 time
	sacrifice of animals (overdosis pentobarbital: 150-200 mg/kg BW)	= 03	end of experiment
mouse group 3	sacrifice of animals (overdosis pentobarbital: 150-200 mg/kg BW), after sacrifice isolation of OT-I T-cells from spleen	= 03	end of experiment
mouse group 4	sacrifice of animals (overdosis pentobarbital: 150-200 mg/kg BW), after sacrifice isolation of OT-II T-cells from spleen	= 03	end of experiment
mouse group 5	high fat diet	=01	1 week
	fastening of the animals for 8 hours + gastric intubation of oxLDL or PBS (+ trypsin inhibitor 10 minutes before oxLDL or PBS administration)	=03	4 times over 14 days
	intraperitoneal injection	=04	3 consecutive days before oral gavage + once per week over 2 weeks

	taking blood from the vena saphena	=02	1 time over the whole experiments (each time 5-15 minutes)
	sacrifice of animals (overdosis pentobarbital: 150-200 mg/kg BW)	= 03	end of experiment
mouse group 6	high fat diet	=01	0.5, 1, 1.5 or3 weeks
	taking blood from the vena saphena	=02	1 time over the whole experiments (each time 5-15 minutes)
	intraperitoneal injection (OT-I cells + ovalbumine)	=04	1 time
	sacrifice of animals (overdosis pentobarbital: 150-200 mg/kg BW)	= 03	end of experiment
mouse group 7	subcutaneous injection (Flt3L-secreting B16 cell line)	=02	1 time
	injection of tumour cell line	=04	10-14 days of growth
	sacrifice of animals (overdosis pentobarbital: 150-200 mg/kg BW)	= 03	end of experiment
mouse group 8	sacrifice of animals (overdosis pentobarbital: 150-200 mg/kg BW), after sacrifice isolation of cells for in vitro experiments	= 03	end of experiment

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.

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.
Any problems, please contact the

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).
Collar placement: see attached SOP
Taking blood from the vena saphena: SOP CPV-3-MR (see attached)
Intragastric oxLDL administration: see attached SOP
Sacrifice: The animals will be euthanized with an overdose pentobarbital (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. *DaissormontI., Christ A., Biessen E., Plasmacytoid Dendritic cells protect against atherosclerosis by tuning T-cell proliferation and activity. Circ.Res., 2011*

SOP Collar placement

The surgical intervention takes place under aseptic conditions.

- the mouse is placed into a chamber
  → to start anesthesia: animal receives 4.0% isofluran mixed with air
  → to maintain anesthesia: animal receives 2.5% isofluran mixed with air
- eyes of the animal are treated with eye salve to avoid drying-out
- the animal is fixed on a thermo controllable heating plate (here: 36.5°C)
- the front side of the neck is shaved and skin is desinfected (betadine).
- a medial longitudinal incision of circa 1cm is made
- due to cutting of the m.sternocleido mastoideus of the trachea the right a.carotis of the animal is exposed
- the carotis communis is dissected from the surrounding connective tissue whereat the nervus vagus should not be damaged
- three threats (pike silk 6-0) are placed under the carotis communis wherewith the collar will be fixed
- a 2mm long collar (silastic tubing with an inside diameter of 0.3mm, longitudinal cut) is placed around the a.carotis communis and fixed with the three threads
- the m. sternocleido mastoideus is placed back and the skin is sutured (synthetical pike silk 5-0)
- the animal is placed in a thermo controllable chamber (30°C)

CPV-3-MR
Bloedafname via vena saphena

SOP Oral gavage

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

Aan:

voorzitter
p/a Secretariaat DEC-UM
Postbus 616
NL-6200 MD Maastricht
Telefoon: 043

Uw referentie: Onze referentie : Maastricht, 19-12-2011

Geachte Onderzoeker,

Uw projectaanvraag: "*Studying the interaction of dendritic cell subsets and T-cells in atherosclerotic lesions*", is op de DEC vergadering van 16 december 2011 besproken.

De DEC heeft een aantal vragen en opmerkingen:

1) De DEC verzoekt de code voor bijzonder technieken van groep 7 aan te passen in code 01.
2) De DEC verzoekt de naam van de PI bij punt 6 te verwijderen en op het voorblad te vermelden. Het is niet de bedoeling dat verwezen wordt naar personen in de aanvraag, in verband met de Wet Openbaarheid van Bestuur. Tevens verzoekt de DEC één van de overige uitvoerende als vervangend verantwoordelijke onderzoeker te vermelden (je kunt namelijk als vervangend verantwoordelijke onderzoeker niet de eigen aanvraag ( ) wetenschappelijk beoordelen).
3) Punt 7c- De DEC wenst voor studie 1 en 4 een toelichting over de noodzaak van de verschillende tijdstippen. Voor studie 2 en 4 wenst de DEC ook een powerberekening. De DEC vraagt zich af bij studie 5, waarom 5 extra muizen nodig zijn. De DEC wenst een onderbouwing hiervoor.
4) Bij punt 10a mist de DEC de vermelding van groep 3, 4 en 8 en verzoekt deze nog toe te voegen en het totaal aan te geven per groep (sommeren).

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 **2011-166**, gelieve dit nummer in verdere correspondentie te vermelden.

De DEC-UM wenst u en uw familie fijne feestdagen en een voorspoedig en vooral gezond 2012!

Hoogachtend,

Voorzitter DEC-UM

Dear DEC commissie,

You asked me in my DEC project: "*Studying the interaction of dendritic cell subsets and T-cells in atherosclerotic lesions*" (2011-166) several questions that I will answer by this letter. Please find the changings also marked in grey in the DEC proposal.

1) ***De DEC verzoekt de code voor bijzonder technieken van groep 7 aan te passen in code 01.***

→ I changed the code here on the front page. Please find the changement marked in grey.

2) ***De DEC verzoekt de naam van de PI bij punt 6 te verwijderen en op het voorblad te vermelden. Het is niet de bedoeling dat verwezen wordt naar personen in de aanvraag, in verband met de Wet Openbaarheid van Bestuur. Tevens verzoekt de DEC één van de overige uitvoerende als vervangend verantwoordelijke onderzoeker te vermelden (je kunt namelijk als vervangend verantwoordelijke onderzoeker niet de eigen aanvraag (E. Biessen) wetenschappelijk beoordelen).***

→ I added the name of the PI, on the front page of the DEC and removed it at point 6. Additionally I included of Maastricht

3) ***Punt 7c- De DEC wenst voor studie 1 en 4 een toelichting over de noodzaak van de verschillende tijdstippen. Voor studie 2 en 4 wenst de DEC ook een powerberekening. De DEC vraagt zich af bij studie 5, waarom 5 extra muizen nodig zijn. De DEC wenst een onderbouwing hiervoor.***

→ *In study 1* we will place mice for either 0.5 week, 1 week, 1.5 weeks, 3 weeks or 6 weeks on high fat diet. We have chosen these different time points to investigate if pDC are able to induce T-cell polarization into an anti-inflammatory direction (induction of regulatory T-cells, Th2 T-cells) already in the early beginning of high fat diet (already after 0.5 or 1 week) or just at later time points (3 weeks, 6 weeks). Furthermore we want to know if pDC stay tolerogenic over the whole time of high fat diet or if (and when) they switch to a proinflammatory status, supporting lesion formation.
Results of previous studies have shown us already that pDC in mice that are only for 0.5 weeks to 1 week on high fat diet start upregulating tolerogenic markers. In these studies we did not investigate T-cell polarization. We also want to know what is happening after 1.5, 3 and 6 weeks.
The results of this study will help us to decide if pulsed pDC are possible therapeutic tools dampening atherosclerotic lesion progression and further on to which time point it would be best to inject them (early versus later time points).

→ *In study 4* we will place mice for either 0.5 week, 1 week, 1.5 weeks, 3 weeks or 6 weeks on high fat diet. We have chosen these different time points to investigate if cross-presentation occurs already in the early beginning of high fat diet or just at later time points. Previous studies in our group have shown that other cell subsets (monocytes) get activated already at a very early time point in mice fed a high fat diet and can by this support lesion progression. Here we are focusing on the $CD8a^+$ DC and their interaction with T-cells. It is important for us to know if T-cells get already activated at an early time point and if they stay activated over the whole time of high fat diet. Results are important for us to decide to which time point we can interfere and by this suppress lesion development.

→ For study 2 and 4 the DEC commissie asked for a power calculation. I included the power calculation in the DEC proposal (marked in grey).

→In study 5 I excluded the 5 extra mice. I calculated wrong and corrected for this. Animal numbers are downscaled to n=20.

4) ***Bij punt 10a mist de DEC de vermelding van groep 3, 4 en 8 en verzoekt deze nog toe te voegen en het totaal aan te geven per groep (sommeren).***

→ Here I included mouse groups 3,4 and 8 in the table and mentioned the discomfort for these animals.

All changings are marked in grey in the DEC proposal!

From:
Sent: dinsdag 7 februari 2012 11:02
To:
Subject: FW: Project 2011-166-w
Attachments: DEC aanvraag formulier_pDC.doc; Voorblad aanvraag dierproef DEC_pDC.doc

Geachte onderzoeker, beste

Ik heb de herziene versie met de DEC besproken en zij hebben nog ene vraag:

- De DEC wil graag een referentie zien waarbij het verschil tussen 0.5 en 1 week goed zichtbaar wordt of in de upregulatie van telerogene markers of in de de monocyte activatie. Dus graag referenties (of plaatjes) aanleveren die bij die 2 opmerkingen passen.

Graag je reactie zodat ik deze aanvraag kan afhandelen.

Met vriendelijke groet namens DEC-UM:

Ambtelijk secretaris

Postbus 616-UNS 50-Box 48, 6200 MD Maastricht T 043 388 1108
E-mail:

Werktijden: Ma-Di-Wo-Don van 08.00 uur tot 16.00 uur

From:
Sent: dinsdag 24 januari 2012 13:24
To:
Subject: RE: Project 2011-166-w

Dear

please find attached the changements in DEC project 2011-166 that you asked for. Changings are marked in grey.

I have to apologize that I am sending the changings not in the mentioned deadline (13th of January). Due to important experiments (animal sacrifices) I just forgot to send it in time and I just realized today. I hope you can consider it.

Kind regards,

Maastricht University Medical Center

phone: +3143

Van:

7-2-2012

Dear

The DEC commissie had the following remarks on my DEC project **2011-166**:

De DEC wil graag een referentie zien waarbij het verschil tussen 0.5 en 1 week goed zichtbaar wordt of in de upregulatie van telerogene markers of in de de monocyte activatie. Dus graag referenties (of plaatjes) aanleveren die bij die 2 opmerkingen passen.

1)

In study 1 '**Studying the effect of pulsed pDC on T-cell polarization, proliferation and plaque development (adoptive transfer of pDC)**' we want to investigate if pDC, pulsed ex vivo with modified lipids (oxLDL) can induce tolerance when injected into new female $LDLR^{-/-}$ recipient mice that are placed on high fat diet for different time periods (adoptive pDC transfer). Here we will place mice for either 0.5 week, 1 week, 1.5 weeks, 3 weeks or 6 weeks on high fat diet. We have chosen these different time points to investigate if pDC are able to induce T-cell polarization into an anti-inflammatory direction (induction of regulatory T-cells, Th2 T-cells) already in the early beginning of high fat diet (already after 0.5 or 1 weeks) or just at later time points (3 weeks, 6 weeks).
Results of previous studies have shown us already that pDC in mice that are only for 0.5 weeks on high fat diet start upregulating tolerogenic markers.

→ **here the DEC asked to get a reference in which we have shown the upregulation of tolerogenic markers by plasmacytoid dendritic cells at 0.5 weeks of high fat diet.**

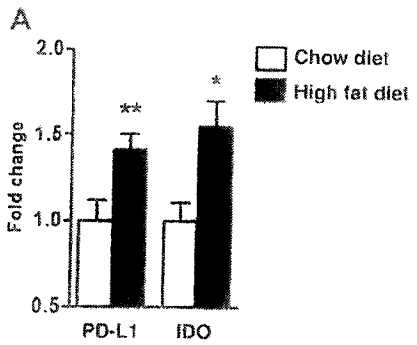


Figure 1a: Upregulation of tolerogenic markers (PD-L1 and IDO) by pDC from $LDLR^{-/-}$ mice that were on high fat diet for 0.5 weeks.

2)

In study 4 of the DEC project 2011-166 '**Studying the effect of CD8a$^+$ DC on hypercholesterolemia (cross-presentation)**' we want to investigate if high cholesterol levels in the plasma can influence the extent in which cross-presentation (by CD8a$^+$ DC) occurs. Here we will place mice for either 0.5 week, 1 week, 1.5 weeks, 3 weeks or 6 weeks on high fat diet. We have chosen these different time points to investigate if cross-presentation occurs already in the early beginning of high fat diet or just at later time points.

Previous studies in our group have shown that other cell subsets (monocytes) get activated already at a very early time point in mice fed a high fat diet and can by this support lesion progression.

→ here the DEC asked to get a reference in which we have shown the activation of monocytes at really early time points (0.5 weeks and 1 week) of high fat diet.

Please find below a graph that shows an increase in the number of monocytes in spleen (monocytosis) (Figure 2a). This actually indicates an increase in monocyte proliferation already after 0.5 and 1 week of high fat diet. Additionally we measured the concentration of proinflammatory cytokines (IL-1beta, IL-12p70 and IL-6) released by monocytes. These graphs also clearly show an increase in proinflammatory cytokines at really early time point of high fat diet pointing to an activation of monocytes.

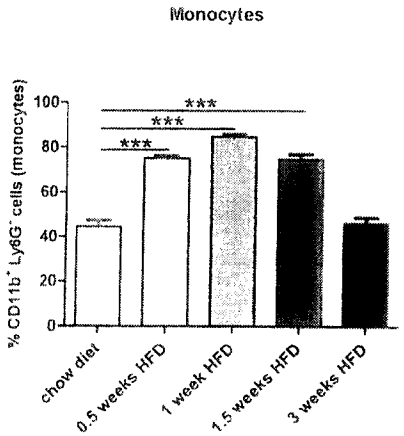
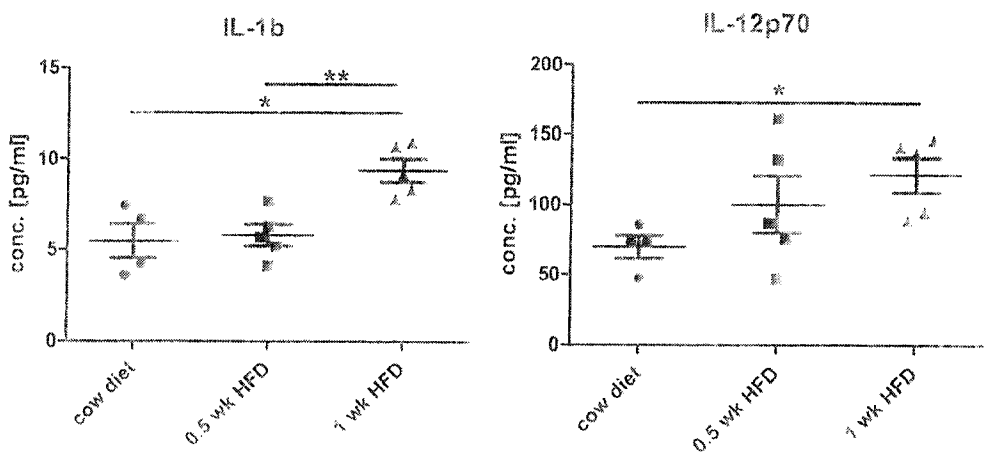


Figure 2a: Number of monocytes in spleen of LDLR$^{-/-}$ mice that are for different time points on high fat diet or on chow diet.



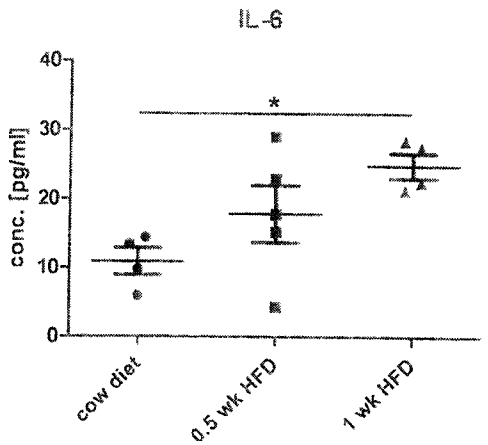


Figure 2b: Concentration of proinflammatory cytokines released by activated monocytes.

Maastricht University

Faculty of Health, Medicine and Life Sciences

Aan:

Ons kenmerk	*Doorkiesnummer*	*Maastricht*
	043-	13-02-2012

Project: *Studying the interaction of dendritic cell subsets and T-cells in atherosclerotic lesions.*

Verantwoordelijk onderzoeker (VO):

Namens de Vergunninghouder van de DEC-UM, 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:	**2011-166**
Diersoort:	**muis**
Aantal dieren:	**257**
Einddatum:	**13-02-2016**

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

Vicevoorzitter DEC-UM

DEC-UM
Voorzitter DEC-UM

p/a secretariaat DEC-UM

Secretariaat DEC-UM
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