Mast cells as immune regulators in atherosclerosis

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Chapter 5

Disruption of a CD1d-mediated interaction between mast cells and NKT cells aggravates atherosclerosis

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Abstract

The development of atherosclerosis is tightly regulated by the innate and adaptive immune system. Communication between these two compartments occurs, among others, also through lipid-antigen presentation by CD1d, a protein found on antigen presenting cells, to the NKT cell population. Recent evidence states that also mast cells express CD1d and directly communicate with NKT cells. However, no such relationship has been reported in atherosclerosis. Here, we aimed to elucidate the CD1d-mediated interaction between mast cells and NKT cells upon atherosclerosis progression *in vivo*. We adoptively transferred $CD1d^{-/-}$ or control mast cells to mast cell deficient $apoE^{-/-}/Kit^{W\text{-}sh/W\text{-}sh}$ mice and subsequently placed these animals on a Western-type diet for 10 weeks. In the course of the study, $CD1d^{-/-}$ mast cell restoration resulted in a mild increase in total serum cholesterol levels. At the endpoint, the aortic root of the $CD1d^{-/-}$ mast cell reconstituted mice showed increased plaque size with less collagen deposition and more intimal $CD4^{+}$ T cells, as compared to control mice. The T cell population in the spleen and circulation had higher T-bet expression and elevated pro-inflammatory cytokine production inside the aortic arch, in the form of IFNγ, TNFα and IL-17. This study is the first to illustrate that disruption of the CD1d communication pathway between mast cells and NKT cells aggravates atherosclerosis through a shift towards inflammatory T cell responses. This newly found ability of mast cell action during plaque progression sheds new light on their role in atherosclerosis.

1. Introduction

The accumulation and modification of lipids within large arteries is the essential step for the onset of atherosclerosis[1]. Yet, it is the activation of the innate and adaptive immune system that establishes the disease development, in the form of macrophage foam cells taking up these modified lipids and forming an atherosclerotic plaque[2]. Along with foam cell formation, macrophages process and present digested lipoprotein antigens to various T cell populations, through their MHC-machinery[3]. However, lipid fragments can also be presented through an MHC-I like protein named CD1d[4]. Presentation through CD1d is designed to specifically target and activate NKT cells, an adaptive cell population with unique T cell receptor (TCR) chains, tailored for endogenous and exogenous lipids[5]. In addition, these cells carry various markers of the innate NK cell population, therefore being the bridge between innate and adaptive immunity. Upon activation, NKT cells secrete vast amounts of T_{H1} and T_{H2} cytokines, such as IFNγ, TNFα or IL-4 and IL-13, respectively, depending on the quality and prevalence of their activating ligand[6,7]. Furthermore, their activation can be differentially shaped by co-stimulatory molecules, such as CD40L[8] and OX40[9]. However, it can also be cytokine-induced, independently of CD1d mediation[10,11]. NKT cells reside at high numbers in the liver[12], but have also been reported inside the atherosclerotic plaques of experimental models[13] and human atheromata[14], where they are thought to act in a proatherogenic manner[15]. NKT cells appear crucial mostly in the initial phase of the disease where they exacerbate plaque progression[13], until the adaptive response develops, whereupon primarily IFNγ producing $CD4^+$ T_{H1} cells[16] take over. However, NKT cells have also been described to influence advanced atherosclerosis, mainly by inducing plaque destabilization and necrotic core formation[17]. Previous studies have established that NKT cell activation through its most potent known activator, α-galactosylceramide (α-GalCer), increases atherosclerotic plaque progression in $apoE^{-/-}$ mice[17,18], whereas CD1d deficiency impairs neointima formation[19]. In addition, CD1d protein expression was elevated inside human atherosclerotic tissues in comparison to normal intima[20] and its role has been further confirmed in $CD1d^{-/-}LDLr^{-/-}$ mice, which upon high fat diet develop smaller plaques[17]. In fact, $CD1d^{-/-}$ mice completely lack NKT cells, indicating how essential CD1d molecules are for the thymic development of the NKT cell population[21]. NKT cells undergo maturation in the thymus and upon reaching stage 2, can migrate to the periphery[22]. Subsequently, these cells, whether in the periphery or in the thymus, may advance to stage 3 after glycolipid presentation through CD1d[23].

CD1d is expressed in various immune cells aside from macrophages. Professional antigen presenting cells such as dendritic cells and B cells are known activators of NKT cells through this receptor[24,25]. CD1d has also been reported to appear on the membrane of non-antigen presenting cell populations, such as the mast cells[26]. A recent

study suggested that CD1d expression on peritoneal mast cells can lead to NKT cell proliferation and cytokine release *in vitro*[27]. An indication of how this interaction may shape the immune response *in vivo* is given in allergic asthma where the CD1d-mediated action of mast cells on NKT cells aggravates airway inflammation by leading to more IgE production[26], a pathway known to trigger mast cell activation[28]. Mast cells are classical pro-inflammatory intermediaries in atherosclerosis[29], detected at an activated state inside atherosclerotic plaques[30]. Under the influence of modified lipids, mast cells secrete cytokines exacerbating disease progression[31], a characteristic that they share with NKT cells.

However, no direct interaction between these two cell types has been described in atherosclerosis before. Therefore, we aimed to examine whether an established communication, mediated by CD1d, between mast cells and NKT cells would affect disease progression *in vivo*. We adoptively transferred CD1d$^{-/-}$ or control mast cells into mast cell deficient apoE$^{-/-}$/Kit$^{W\text{-}sh/W\text{-}sh}$ mice and examined the progression of atherosclerosis in hyperlipidemic conditions.

2. Materials & Methods

2.1 Cell culture

Bone marrow isolated from female control LDLr$^{-/-}$ or from CD1d$^{-/-}$/LDLr$^{-/-}$ mice was cultured for 4 weeks at 37°C and 5% CO_2, in RPMI 1640 medium containing 25mM HEPES (Lonza) supplemented with 10% fetal calf serum (FCS), 60μM β-mercaptoethanol (Sigma), 100U/mL mix of penicillin/streptomycin (PAA), 1% non-essential amino-acids (NEAA; Gibco), 1% sodium pyruvate (Sigma) and 2% L-glutamine (Lonza) in the presence of cytokine IL-3 (5 ng/mL; Immunotools), to obtain mature mast cells. CD1d expression and mast cell purity, based on the expression of FcεRIα and CD117, were assessed at the adoptive transfer point (**Supplementary Figures 1A,B**).

2.2 Animals

All animal handling was executed in conformation with the guidelines of Directive 2010/63EU, as stated by the European Parliament and the experimental line was approved by the Leiden University Animal Ethics committee. Mice were bred and housed in the local facility with water and food supply *ad libitum*.

2.3 Atherosclerosis

Atherosclerosis-prone, mast cell deficient female apoE$^{-/-}$/Kit$^{W\text{-}sh/W\text{-}sh}$ mice with an average age of 15 weeks (n=10/grp) were adoptively transferred i.v. with $10*10^6$ fully maturated bone marrow derived mast cells, isolated from either LDLr$^{-/-}$ or CD1d$^{-/-}$LDLr$^{-/-}$ mice, hereafter referred to as control or CD1d$^{-/-}$ mast cells, respectively. After 4 weeks, mice were placed on a Western-Type diet (WTD) containing 0.25% cholesterol and 15% cocoa butter (SDS, Essex, UK) for a period of 10 weeks. The experimental set-up is described in **Supplementary Figure 1C**. During the study, tail blood was collected through the tail-vein, and serum was obtained upon

centrifugation at 8.000 rpm for 10 minutes. Total cholesterol levels were assessed for weeks 0, 3, 6 and 9 of the experiment, using an enzymatic colorimetric assay and compared to an internal Precipath control (Roche Diagnostics). Serum collected at the experimental endpoint was used for lipoprotein fraction separation (n=9 mice/grp). Samples were separated through a Superose 6 column (Smart Systems, Pharmacia) and the retrieved fractions were subsequently analysed for cholesterol levels. At the experimental end-point all animals were subcutaneous anesthetized with a mix of ketamine (40mg/mL), sedazine (6.25mg/mL) and atropine (0.05mg/mL).

2.4 Immunohistochemistry

The hearts of all mice were dissected below the atria and freshly frozen in OCT compound (Sakura). Hearts were sectioned, horizontally and towards the aortic arch. Upon identification of the trivalve leaflets of the aortic root, 10μm sections were collected. Mean plaque size was calculated for 7-8 sequential sections using an Oil-Red-O (ORO) staining (Sigma Aldrich). Macrophage content was assessed upon staining with a MOMA-2 antibody (1:1000, rat IgG2b, Serotec Ltd.). Intraplaque collagen was quantified using a Masson's Trichrome staining kit (Sigma Aldrich). The $CD4^+$ (1:90, clone RM4-5, BD Biosciences) and $CD8^+$ (1:100, clone Ly-2, BD Biosciences) T cell content in the aortic root was manually quantified. All morphometric analyses were performed in a blinded independent fashion on a Leica DM-RE microscope using a Leica QWin software (Leica Imaging Systems, UK).

2.5 Aortic arch cytokine stimulation

Aortic arches were digested in a mix of collagenase I (450u/mL; Sigma) and XI (250U/mL Sigma) and DNase (120U/mL; Sigma) and hyaluronidase (120 U/mL; Sigma). Single cells were obtained with a 70μm cell strainer. Subsequently cells were stimulated with a mix of PMA/Ionomycin (Sigma) in the presence of Brefeldin A (eBioscience) for 30 minutes. Cytokine production was measured using flow cytometry, after cell fixation and permeabilization (BD Bioscience).

2.6 Flow Cytometry

All blood and spleen samples were lysed with an erythrocyte lysis buffer (0.1mM EDTA, 10mM $NaHCO_3$, 1mM NH_4Cl, pH=7.2) to obtain white blood cells. Spleen and thymus samples were processed through a 70μm cell strainer for single cell selection. Single white blood (WB), thymus and spleen cells were stained with a viability dye solution as well as fluorescently labeled antibodies for extracellular proteins or permeabilized (Ebioscience) and subsequently stained with *intracellular* antibodies for transcription factor and cytokine determination (**Supplementary Table 1**). Measurements were performed on a FACS Canto II (Becton Dickinson, Mountain View, CA) and analysed using FlowJo software.

2.7 Statistics

All data are depicted as mean±SEM and analysed in GraphPad Prism 7. A 2-tailed Student *t*-test was used to compare between groups. Data were tested for normal distribution and Non-Gaussian distributed values were compared with a Mann-Whitney *U* test. Probability $P<0.05$ was considered significant.

3. Results

3.1 NKT cell levels are elevated upon CD1d$^{-/-}$ mast cell reconstitution

In this project, we aimed to examine the CD1d-mediated interaction between mast cells and NKT cells in atherosclerosis. We adoptively transferred CD1d$^{-/-}$ or control mast cells into mast cell deficient mice and subsequently fed both groups a WTD for 10 weeks. Upon 3 weeks of WTD, circulating CD1d-tetramer^{+} NKT cell levels were elevated in the CD1d$^{-/-}$ mast cell reconstituted group as compared to the control mast cell group (**Figure 1A.**, w3: CD1d$^{-/-}$: 3.04±0.5% vs control: 1.77±0.2%, P=0.0006). However, no difference was detected in the activated CD69^{+} circulating NKT cells (**Figure 1B**). At the experimental end-point the NKT cell population in the thymus was found increased upon CD1d$^{-/-}$ mast cell reconstitution, as compared to control mast cells (**Figure 1C**, CD1d$^{-/-}$: 0.65±0.05% vs control: 0.48±0.04%, P=0.014). Among the thymic NKT cells, particularly stage 2 CD24^{-}CD44^{+}NK1.1^{-} migrating NKT cells appeared elevated (**Figure 1D**, CD1d$^{-/-}$: 69.9±3.2% vs control: 59.2±2.8%, P=0.024) while stage 3, CD24^{-}CD44^{+}NK1.1^{+} NKT cells were slightly reduced in the CD1d$^{-/-}$ mast cell group, in comparison to control (**Figure 1E**, P=0.075). The liver NKT cell population did not differ between the groups (**Figure 1F**). However, the NKT cells of the CD1d$^{-/-}$ mast cell group were significantly less activated as compared to the control mast cell group (**Figure 1G**, CD1d$^{-/-}$: 55.9±1.8% vs control: 63.5±2.3%, P=0.018). It is worth noting that at the experimental endpoint we were able to detect a similar level of mast cells inside the peritoneal cavity in both groups of mice (**Supplementary Figure 2A**), whereas the CD40L levels on the NKT population of CD1d$^{-/-}$ reconstituted mice was found reduced as compared to control (**Supplementary Figure 2B**). The above findings indicate that lack of CD1d expression by the mast cells results in the migration of more stage 2 NKT cells from the thymus to the periphery, yet, in the liver these cells do not appear to get activated.

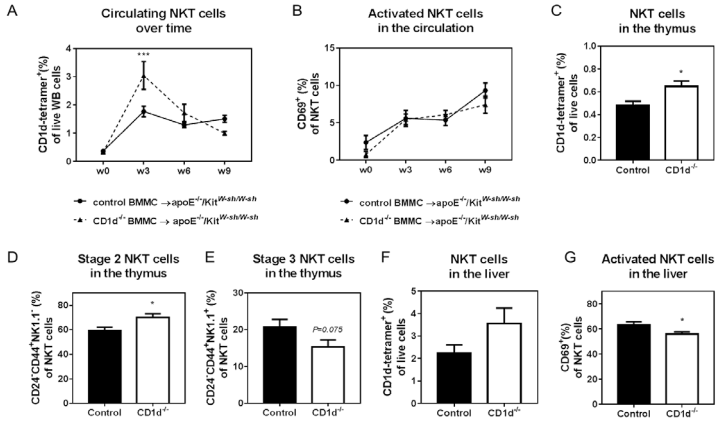

Figure 1: NKT cell levels are elevated but cells appear less active upon reconstitution of mast cell deficient mice with CD1d$^{-/-}$ mast cells. (**A**) Circulating levels of NKT cells increased at 3 weeks of WTD in the CD1d$^{-/-}$ reconstituted mice as compared to control. (**B**) No difference was detected on the NKT cell activation in the blood over time, between the groups. (**C**) Total thymic NKT cells as well as (**D**) migrating stage 2 NKT cells of mice carrying CD1d$^{-/-}$ mast cells were elevated, in comparison to NKT cells in mice reconstituted with control mast cells. (**E**) CD1d-dependent stage 3 NKT cells in the thymus, were slightly reduced in CD1d$^{-/-}$ reconstituted mice, as compared to control. (**F**) No difference in the total NKT cell populations of the liver between the two groups. (**G**) The NKT cells in the liver of CD1d$^{-/-}$ reconstituted mice showed decreased activation as compared to control mice. All values are depicted as mean±SEM. **P<0.05, ***P<0.001*

3.2 Serum cholesterol levels are elevated in the CD1d$^{-/-}$ mast cell reconstituted mice

In the course of the experiment, we analysed the total cholesterol levels in the serum of both groups of mice. We observed that after 3 weeks of WTD, total cholesterol was elevated in the circulation of CD1d$^{-/-}$ mast cell reconstituted mice, as compared to control mast cell repopulated mice (**Figure 2A**, w3: CD1d$^{-/-}$: 1258±74 mg/dL vs control: 1066±46 mg/dL, P=0.02). At the experimental endpoint, we further examined the lipoprotein fractions carried in the circulating cholesterol levels. We did not observe any difference among the (V)LDL and HDL fractions between the groups (**Figure 2B**).

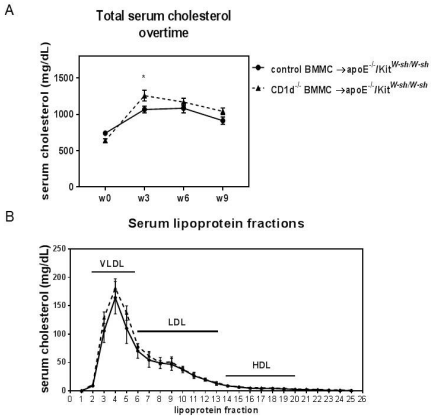

Figure 2: Cholesterol levels in the circulation show a mild increase in CD1d$^{-/-}$ mice at week 3. (**A**) Total cholesterol levels in the serum of CD1d$^{-/-}$ mast cell reconstituted mice showed a slight increase over time of WTD, as compared to control mast cell reconstituted mice. (**B**) No difference was observed in the lipoprotein fractions between the two groups of mice. **P<0.05*

3.3 CD1d-disruption on the mast cells increases atherosclerotic plaque size in the aortic root

After 10 weeks of WTD, the aortic root was analysed to determine atherosclerotic plaque size and morphology. Specifically, the atherosclerotic plaque size was analysed using an ORO staining (**Figure 3A**), the macrophage content in the area was detected using a MOMA-2 staining (**Figure 3B**) while the collagen composition of the plaque was established using a Masson's trichrome staining (**Figure 3C**). The atherosclerotic

plaque of the aortic root in CD1d$^{-/-}$ mast cell reconstituted mice was increased by 15% as compared to the plaque size of control mast cell reconstituted mice (**Figure 3D**, CD1d$^{-/-}$: 95*10^4±3.7*10^4 μm^2 vs control: 83*10^4±3.8*10^4 μm^2, P=0.033). Of note, the plaque development was elevated across the entire aortic root of the heart, measured from the start of the three-valve area up to the receding of the valves (**Supplementary Figure 3A**). Furthermore, the macrophage deposition in the plaque area of these mice was slightly elevated (**Figure 3E**, CD1d$^{-/-}$: 37*10^4±1.4*10^4 μm^2 vs control: 32*10^4±2.5*10^4 μm^2, P=0.066). In contrast, the plaques of the CD1d$^{-/-}$ mast cell reconstituted group showed reduced collagen content, as compared to control (**Figure 3F**, CD1d$^{-/-}$: 55.7±2% vs control: 61.1±1%, P=0.038).

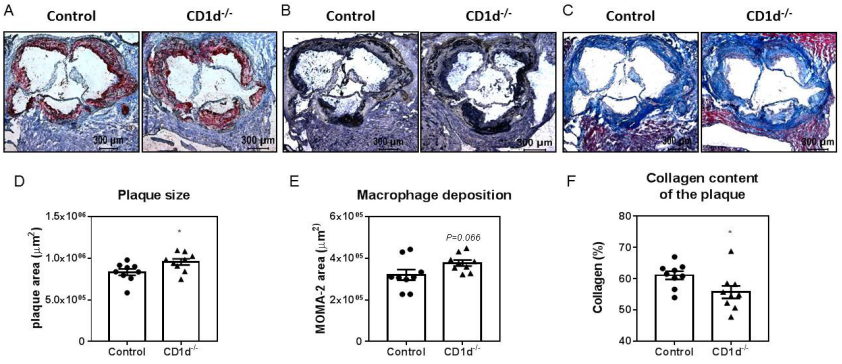

Figure 3: Mice reconstituted with CD1d$^{-/-}$ mast cells develop increased atherosclerosis in the aortic root, as compared to mice reconstituted with control mast cells. Representative pictures per group of mice for (**A**) ORO staining, (**B**) MOMA-2 staining and (**C**) Masson's trichrome staining (n=9/grp). The aortic root of mice reconstituted with CD1d$^{-/-}$ mast cells show (**D**) higher atherosclerotic plaques, (**E**) higher macrophage area and (**F**) lower collagen deposition, as compared to mice reconstituted with control mast cells. All values are depicted as mean±SEM. **P<0.05*

3.4 T cells within the atherosclerotic site produce pro-inflammatory cytokines

Upon further characterization of the aortic root area, we manually quantified the CD4$^+$ and CD8$^+$ T cell content. The number of CD4$^+$ T cells in the CD1d$^{-/-}$ mast cell mice showed a slight increase as compared to control (**Figure 4A**, CD1d$^{-/-}$: 14.9±4.2 cells/μm^2 vs control: 5.6±1.3 cells/μm^2, P=0.060). No difference was observed in the intimal CD8$^+$ T cells between the groups (**Figure 4B**). In addition, we analysed, using flow cytometry, the T cell content –as defined by Thy1.2$^+$ expression- in the aortic arch of these mice. The CD1d$^{-/-}$ mast cell bearing mice showed a mild reduction in the percentage of T cells in the area, in relation to control mice (**Figure 4C**, P=0.060). This was mainly attributed to a slight reduction in the CD8$^+$ T cell percentage (**Supplementary Figure 3B**). Therefore, the total T cell population present in the aortic arch of CD1d$^{-/-}$ mast cell reconstituted mice is characterized mainly by CD4$^+$ T cells (**Figure 4D**, CD1d$^{-/-}$: 32.2±2.8% vs control:

24.6±2.1%, P=0.041). Importantly, the T cells present in the aortic arch of these mice showed a substantial increase in pro-inflammatory cytokine production. Specifically, the aortic T cells of the CD1d$^{-/-}$ reconstituted group presented a significant increase in the production of cytokines IFNγ (**Figure 4E**, P=0.012), IL-17 (**Figure 4F**, P=0.025) and TNFα (P=0.0016) as opposed to the control group.

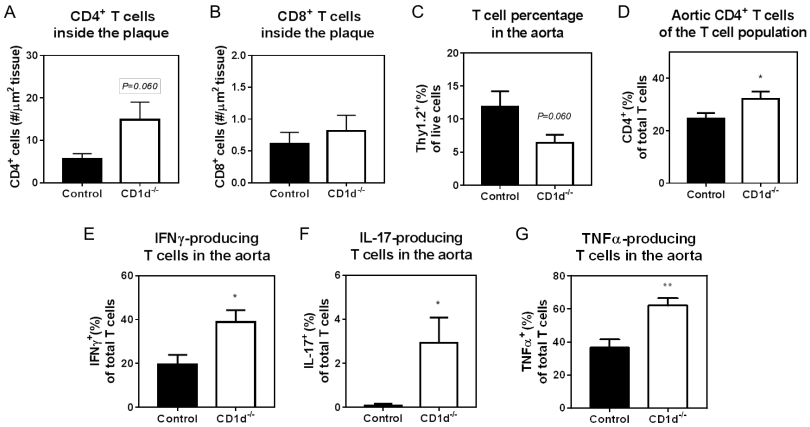

Figure 4: CD4$^+$ T cells are increased inside atherosclerotic sites of CD1d$^{-/-}$ mast cell reconstituted mice and produce higher amounts of pro-inflammatory cytokines. (**A**) CD4$^+$ T cells in the plaque intima of the aortic root appeared increased upon CD1d$^{-/-}$ mast cell reconstitution, as compared to control. (**B**) No difference was observed in the intimal CD8$^+$ T cell numbers between the groups. (**C**) Aortic T cells appear reduced in the aorta of the CD1d$^{-/-}$ reconstituted group as compared to control reconstituted mice. (**D**) The CD4$^+$ T cell levels of the total T cell population are elevated in the CD1d$^{-/-}$ mast cell group as compared to control. (**E**) IFNγ-producing, (**F**) IL-17-producing and (**G**) TNFα-producing T cells in the aorta of CD1d$^{-/-}$reconstituted mice are increased, in comparison to control mice. All values are depicted as mean±SEM. **P<0.05, **P<0.01*

3.5 T-bet expression is higher in the CD4$^+$ and CD8$^+$ T cell populations

Having in mind the close relation between NKT and T_{H1} cells, and upon observing the T cell responses in the atherosclerotic tissue, we aimed to analyse the systemic T cell response. We examined circulating and splenic T cells for the expression of transcription factor T-bet, which controls the secretion of pro-inflammatory cytokine IFNγ and CD4 T_{H1} cells [32]. In the blood of the CD1d$^{-/-}$ mast cell reconstituted mice we detected a substantial increase in T-bet expressing CD8$^+$ T cells, as compared to control (**Figure 5A**, CD1d$^{-/-}$: 8.2±0.9% vs control: 4.7±0.5%, P=0.004). Furthermore, while there was no difference in the percentage of T_{H1} cells (**Figure 5B**), the T-bet expression levels of the total CD4$^+$ T cell population were elevated in the CD1d$^{-/-}$ mast cell group in relation to control (**Figure 5C**, P=0.034). On the same line, T-bet expressing CD8$^+$ T cells were mildly elevated in the spleen of the CD1d$^{-/-}$ group (**Figure 5D**, P=0.077). The CD1d$^{-/-}$ mast cell carrying mice showed higher T_{H1} levels in their spleen, in relation

to the control group (**Figure 5E**, P=0.010). No difference was detected between the groups in the levels of T-bet expression within the entire CD4$^+$ T cell population of the spleen (**Figure 5F**). Of note, both groups showed a similar percentage of total CD8$^+$ and CD4$^+$ T cells in the blood (**Supplemental Figure 3C**,**D**) and spleen (**Supplemental Figure 3E**,**F**).

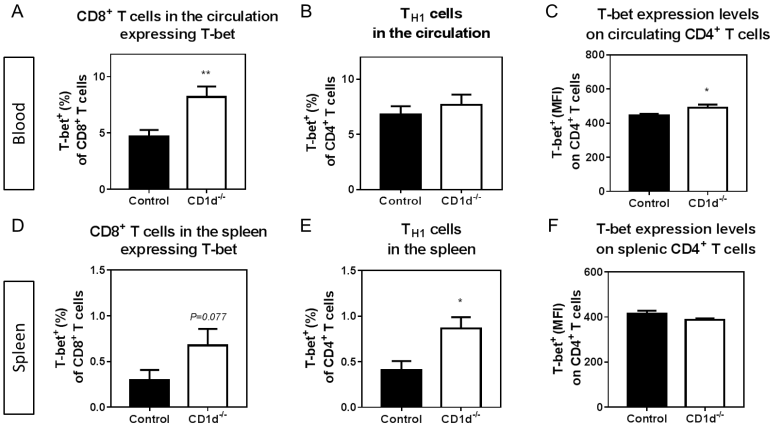

Figure 5: T-bet expression is increased in the circulating and splenic T cells of CD1d$^{-/-}$ mast cell reconstituted mice. (**A**) The percentage of T-bet expressing CD8$^+$ T cells in the blood was elevated upon CD1d$^{-/-}$ mast cell reconstitution as compared to control mast cell reconstitution (n=9/grp). (**B**) No difference was observed in the circulating CD4$^+$ T_{H1} cells between the groups; however (**C**) T-bet expressing CD4$^+$ T cells were elevated in the blood of CD1d$^{-/-}$ reconstituted mice as compared to control reconstituted mice. (**D**) Splenic T-bet expressing CD8$^+$ T cells (n=9/grp) as well as (**E**) CD4$^+$ T_{H1} cells are elevated in the spleen of CD1d$^{-/-}$ reconstituted mice in comparison to control reconstitution. (**F**) No difference was detected in the T-bet expression levels of the total CD4$^+$ T cell population between the two groups. All values are depicted as mean±SEM. **P<0.05, **P<0.01*

4. Discussion

In this study, we examined the CD1d-mediated effect of mast cells on NKT cells during atherosclerosis progression. We discovered that disruption of this axis aggravates atherosclerotic plaque development and destabilization through increased infiltration of CD4$^+$ T cells in the plaque intima. The T cell population in the plaques of mice which lacked CD1d-expressing mast cells showed high pro-inflammatory cytokine production, in the form of IFNγ, TNFα and IL-17. This may have caused the increased macrophage accumulation in the plaque. Furthermore, after 3 weeks of WTD, these animals showed a slight increase in serum cholesterol levels, and although temporary, it might have contributed to the observed plaque progression. The above results suggest that mast cell CD1d expression, influences the function of NKT cells and the subsequent control of CD4$^+$ T cell infiltration in a protective manner, which is previously unreported.

Early through WTD, following the adoptive transfer of CD1d$^{-/-}$ mast cells, we

detected an increase in the circulating levels of NKT cells. This increase was also apparent in the thymus of CD1d$^{-/-}$ mast cell reconstituted mice and could be explained by the elevated stage 2 NKT cells. As mentioned, thymic NKT cells of this stage migrate into peripheral tissues[33], which may thus explain their enhanced abundance in the circulation. However, while stage 2 are migrators and potent cytokine secretors, transition to stage 3 requires glycolipid presentation through CD1d[34]. Interestingly, we observed a reduction in the stage 3 thymic population of NKT cells, indicating that signal through CD1d is partially absent in the CD1d$^{-/-}$ mast cell reconstituted mice, as it was expected. This is implied also by the reduced activation and co-stimulation of the NKT population in the liver. These data suggest that the proatherogenic phenotype observed does not arise from a possible positive feedback signaling of other CD1d-expressing cells on the NKT population. It rather seems that NKT cells, generated in the thymus progress to stage 2 and migrate to the periphery at higher frequencies, perhaps to counteract for the absence in the CD1d signaling cascade initiated by the mast cells.

As stated before, NKT cells can potently activate T cells[35] and, in atherosclerosis, NKT cells are suggested to act prior to and pave the way for T cell infiltration in the plaque. In contrast to the existing concept on the proatherogenic role of NKT cells in atherosclerosis, and despite the reduced NKT cell activation, in this study we observed increased CD4$^+$ T cells within the plaque intima of the aortic root and an elevated cytokine production mainly by CD4$^+$ T cells of the aortic arch. While this seems surprising, particularly since CD1d signaling is known to induce a proatherogenic response on the NKT cells[18], a previously published study demonstrated that NKT cells in mice fed a high-fat diet, control T_{H1} cell responses specifically *via* a reduced IFNγ secretion in the serum upon α-GalCer injection[36]. Other reports have also stated that CD1d-mediated effects on the NKT cells can subsequently control T_{H1} cells. For example, in arthritis an autoimmune condition characterized by the presence of mast cells, loss of CD1d-mediated NKT activation exacerbated the T_{H1} responses, again in an IFNγ mediated manner[37]. In our study the pro-inflammatory IFNγ production of both the CD4$^+$ and CD8$^+$ T cell subsets seemed to be under the control of transcription factor T-bet, which showed elevated expression in T cells of both the spleen and the circulation. T-bet directly induces the production of IFNγ[38], whereas T_{H1} cells can also secrete TNFα[39]. In addition, NKT cells have been previously reported to inhibit T_{H17} cell differentiation[40], the main CD4$^+$ T cell subset secreting cytokine IL-17[41] that increases in proatherogenic conditions[42]. Therefore, it seems that loss of CD1d expression by the mast cells, and subsequent partial loss of NKT cell activation, led to an increase in T_{H1} cells, which in turn augmented atherosclerotic plaque progression. Hence, there is a tight balance between NKT and T_{H1} cells with mast cells playing a fundamental role in it.

The increase in pro-inflammatory CD4$^+$ T cells observed within the atherosclerotic

plaques of CD1d$^{-/-}$ mast cell reconstituted mice may have influenced the collagen deposition in the area. Specifically, the atherosclerotic plaques of these mice contained less collagen, signifying reduced plaque stability[43]. Cytokine IFNγ, has been accounted for its destabilizing manner[43,44]. Pro-inflammatory cytokine secretion by T cells could also explain the enhanced macrophage infiltration observed in the plaque, particularly since these cells inflict damage on the endothelium[45] and induce macrophage accumulation in the area[46]. Macrophages in turn produce a vast array of matrix metalloproteinases and are greatly implicated in plaque destabilization[47]. Therefore the increased macrophage content may have contributed to the destabilizing phenotype observed. It is also important to mention that the slight increase detected in circulating cholesterol levels may have contributed to the enhanced plaque development.

Up to date there are limited reports on the CD1d mediated action of mast cells on NKT cells. Mast cells on their own are potent cytokine secretors in organs where NKT cells reside, such as the thymus[48], the atherosclerotic plaque[29] and the liver[49]. Mast cell activation results in the release of proteases and cytokines, with IFNγ and TNFα considered among the typical cytokines excreted in their microenvironment. Furthermore, CD1d expression on the mast cell surface has been associated with mast cell activation *via* their classical receptor, FcεR[26]. Therefore, CD1d on the mast cell surface could also partly affect the activation of mast cells and, through that, the local response inside the plaque. The above study examining the function of mast cells and NKT cells, indicated that mast cells express also the costimulatory protein CD40; albeit not in a manner that influenced the NKT cell response in their experimental setup. Our results however showed that CD1d$^{-/-}$ mast cells seem to negatively affect the CD40L expression on liver NKT cells, thus supporting this mode of action. Additional co-stimulatory pathways may also participate in the observed mast cell-NKT cell interaction since ox40L and CD48 expression on the mast cells are reported to regulate cytokine secretion by NKT cells[27] .

To conclude, here we describe for the first time an *in vivo* interaction through mast cell-CD1d and NKT cells in atherosclerosis. This relationship seems to be protective, since its interruption increases atherosclerotic plaques, showing a highly pro-inflammatory CD4^{+} T cell content. A reduction in the mast cell-mediated CD1d activation of the NKT cells may therefore result in a compensatory operation of their close relatives, the T cells. While we have no further evidence of the exact lipid antigen presented by mast cell-CD1d to NKT cells, or why disruption of a classical proatherogenic pathway would enhance instead of hamper atherosclerosis, it is important to remember that NKT cells are also involved in many tolerogenic responses with presentation of endogenous lipids as to control autoimmune reactions[50]. Additionally, in the past it was shown that activation of NKT cells protects against autoimmune conditions, such as

diabetes[51] or arthritis[52]. In atherosclerosis, mast cells *via* the expression of CD1d and subsequent lipid presentation, appear to be fine-tuning the NKT cell actions, leading thus to a newly described mechanism of action in this disease.

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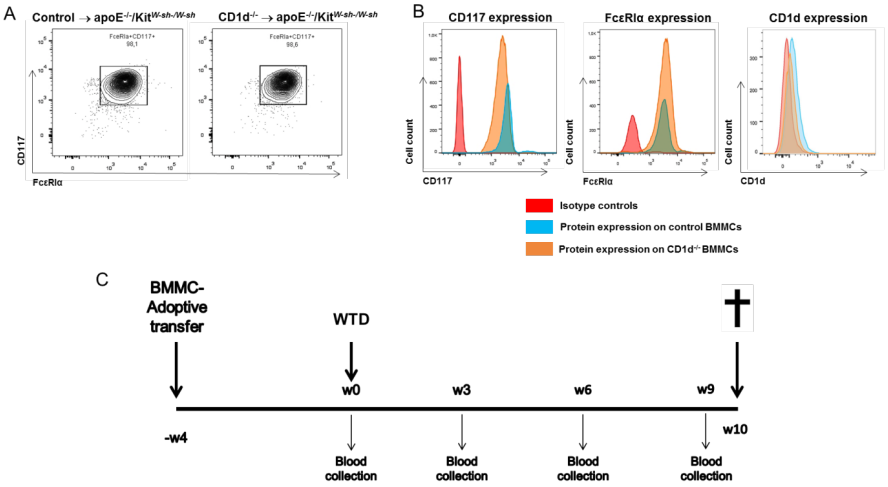
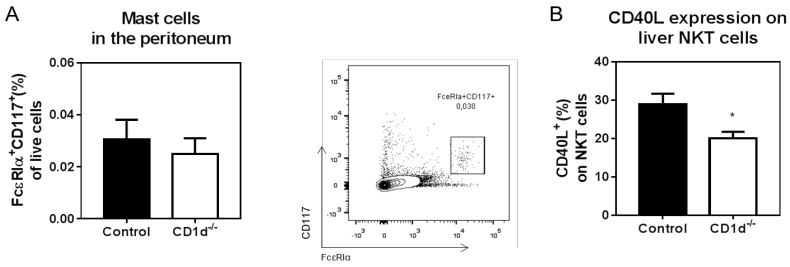
Supplementary Information



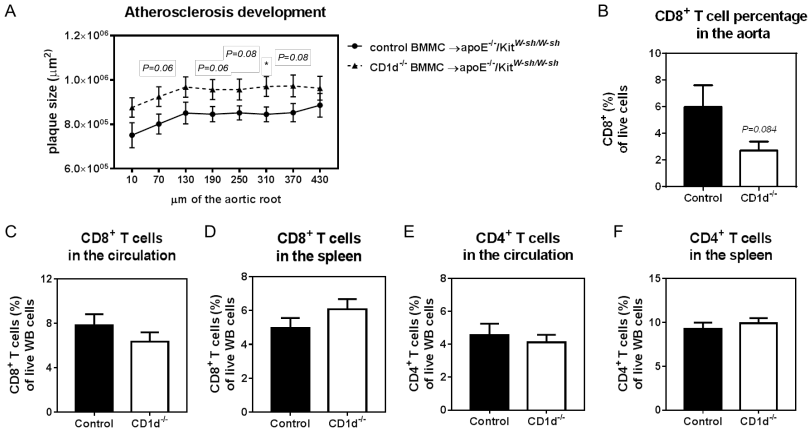
Supplemental Figure 1: Experimental set-up and controls. (**A**) Flow cytometry purity check, 1 day prior to mast cell reconstitution. (**B**) Isotype controls for CD117, FcεRIα and CD1d expression on the transferred BMMCs. (**C**) Mast cell deficient apoE/Kit$^{W\text{-}sh/W\text{-}sh}$ mice were adoptively transferred through i.v. injections with 10*10^6 mature bone marrow derived mast cells of either CD1d$^{-/-}$ mice or control control mice. After a 4 week-recovery period, the mice were placed on a WTD for an additional 10 weeks.

Antibody	Clone	Concentration	Fluorochrome	Company
Fixable Viability Dye	-	0.05 µg/sample	eFluor 780	eBioscience
FcεRIα	MAR-1	0.1 µg/sample	PercP Cy5.5	Biolegend
FcεRIα	MAR-1	0.1 µg/sample	APC	eBioscience
CD117	2B8	0.1 µg/sample	APC/PE Cy7	eBioscience
CD1d	1B1	0.1 µg/sample	PE	eBioscience
CD1d-tetramer	-	0.125 µg/sample	BV 420	-
CD69	H1.2F3	0.1 µg/sample	PE	eBioscience
CD24	M1/69	0.1 µg/sample	PercP Cy5.5	BD Biosciences
CD44	IM7	0.1 µg/sample	PE Cy7	eBioscience
NK1.1	PK136	0.1 µg/sample	FITC	eBioscience
CD40L	MR1	0.1 µg/sample	APC	eBioscience
CD4	GK1.5	0.1 µg/sample	FITC/eFluor 450	eBioscience
CD8a	53-6.7	0.1 µg/sample	PercP	BD Biosciences
CD8a	53-6.7	0.1 µg/sample	APC	eBioscience
Thy1.2	53-2.1	0.1 µg/sample	PE Cy7	eBioscience
IFNγ	XMG1.2	*0.3 µg/sample*	BV650	BD Biosciences
IL-17	eBio17B7	*0.3 µg/sample*	Alexa Fluor 488	eBioscience
TNFα	MP6-XT22	*0.3 µg/sample*	PE	eBioscience
T-bet	eBio4B10	*0.3 µg/sample*	Alexa Fluor 660	eBioscience

Supplementary Table 1. Flow cytometry antibodies: List of extracellular and *intracellular* antibodies



Supplemental Figure 2: Mast cells are detected in the peritoneum of mast cell deficient apoE$^{-/-}$/Kit$^{W\text{-}sh/W\text{-}sh}$ mice in both groups at the experimental endpoint. (A) Mast cell levels in the peritoneum of apoE$^{-/-}$/Kit$^{W\text{-}sh/W\text{-}sh}$ mice reconstituted with either control or CD1d$^{-/-}$ BMMCs show no difference between the two groups. (B) The percentage of liver NKT cells that express the costimulatory molecule CD40L is decreased upon CD1d$^{-/-}$ BMMC reconstitution, as compared to control BMMC reconstitution. All values are depicted as mean±SEM. **P<0.05*



Supplemental Figure 3: Atherosclerotic plaques in the heart of the CD1d$^{-/-}$ reconstituted mice develop at a higher rate across the aortic root area. (**A**) CD1d$^{-/-}$ mast cell reconstitution results in increased atherosclerosis development through the aortic root, as compared to reconstitution of mice with control mast cells (n=9/grp). (**B**) The CD8^{+} T cell content in the aortas of CD1d$^{-/-}$ reconstituted mice is slightly reduced in comparison to control mice. No difference was detected in the total (**C**) circulating CD8^{+} (**D**) splenic CD8^{+} or (**E**) circulating CD4^{+} T cell levels and (**F**) spleen CD4^{+} T cells between the two groups of mice. All values are depicted as mean±SEM. **P<0.05*