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

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HIGHLIGHTS

- CD1d-deficiency on mast cells increases atherosclerotic plaque development.
- *CD1d*$^{-/-}$-mast cell transferred animals show less stable plaques with increased CD4^{+} T cell content.
- Proinflammatory cytokine production increases upon *CD1d*$^{-/-}$-mast cell deficiency.
- Disruption of the CD1d-mediated mast cell-NKT cell axis augments atherosclerosis by altering T cell responses.

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ABSTRACT

Background and aims: The development of atherosclerosis is tightly regulated by the innate and adaptive immune system. Communication between these two compartments occurs, among others, upon presentation of lipid antigens to the NKT cell population by CD1d-expressing antigen-presenting cells. Recent evidence states that also mast cells express CD1d and can directly communicate with NKT cells. However, no such relationship has been reported in atherosclerosis. Here, we aimed to elucidate *in vivo* the CD1d-mediated interaction between mast cells and NKT cells upon atherosclerosis progression.

Methods: We adoptively transferred *CD1d*$^{-/-}$ or control mast cells to mast cell-deficient *apoE*$^{-/-}$*Kit*$^{W\text{-}sh/W-sh}$ mice and subsequently placed the animals on a Western-type diet for 10 weeks.

Results: At the end of the Western-type diet period, the aortic root of *CD1d*$^{-/-}$ mast cell-reconstituted mice displayed increased plaque size, with less collagen deposition and higher intraplaque CD4^{+} T cells, as compared to control mice. In addition, T cells inside the aortic arch showed higher pro-inflammatory cytokine production in the form of IFNγ, TNFα and IL-17. Finally, T-bet expression was found elevated in both CD4^{+} and CD8^{+} circulating T cells.

Conclusions: 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 pro-inflammatory T cell responses. This 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 medium and large-sized 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 disease development, with macrophage foam cells taking up modified lipids, thus initiating atherosclerotic plaques [2]. Along with foam cell formation, macrophages process and present digested lipoprotein-derived antigens to various T cell populations, through their MHC-machinery [3]. 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]. Upon activation, NKT cells secrete vast amounts of T_{H1} or T_{H2} cytokines, such as IFNγ and TNFα or IL-4 and IL-13 respectively, depending on the quality and prevalence of the activating ligand [6]. NKT cell activation can be differentially shaped by co-stimulatory pathways, such as through

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CD40^{-}CD40L [7], but can also be cytokine-induced, independently of CD1d ligation [8]. NKT cells reside at high numbers in the liver [9], but are also reported within atherosclerotic plaques of *LDLr*$^{-/-}$ and *apoE*$^{-/-}$ mice [10] and human atheromata [11], where they show a proatherogenic phenotype [12]. Furthermore, NKT cells appear to act mainly in the initial phase of atherosclerosis and exacerbate plaque progression [10], until the adaptive response develops. Thereupon, primarily IFNγ-producing CD4^{+} T_{H1} cells [13] take over. However, NKT cells have also been described to influence advanced atherosclerosis, mainly by inducing plaque destabilization and necrotic core formation [14]. Previous studies established that NKT cell activation through α-galactosylceramide (α-GalCer), their most potent known activator, increases atherosclerotic plaque progression in *apoE*$^{-/-}$ mice [14,15], whereas CD1d deficiency impairs neointima formation [16]. In addition, CD1d-protein expression is elevated inside human atherosclerotic tissues compared to normal intima [17] and *CD1d*$^{-/-}$*LDLr*$^{-/-}$ mice upon high fat diet develop smaller plaques [14]. In fact, *CD1d*$^{-/-}$ mice completely lack NKT cells, indicating how essential CD1d molecules are for the thymic development of the NKT population [18]. NKT cells arise from CD4^{+}CD8^{+} NKT precursors in the thymus and undergo 4 stages of maturation, defined by the expression of markers CD24, CD44 and NK1.1 [19]. After reaching stage 2, (CD24^{-}CD44^{+}NK1.1^{-}) NKT cells may migrate to the periphery [20]. Subsequently, in the periphery or the thymus, these cells advance to stage 3 (CD24^{-}CD44^{+}NK1.1^{+}) upon glycolipid presentation through CD1d [19].

CD1d is expressed by macrophages, dendritic cells and B cells, all of which can activate NKT cells [21–23]. However, CD1d has also been reported on the membrane of non-antigen presenting populations, such as mast cells [24]. A recent study suggests that CD1d expression on peritoneal mast cells can lead to NKT cell proliferation and cytokine release *in vitro* [25]. This crosstalk may shape the allergic asthma immune response *in vivo*, as the CD1d-mediated interaction of mast cells with NKT cells aggravated airway inflammation by prompting B cells to produce more IgE [24], and thereby triggering mast cell activation. Mast cells are classical pro-inflammatory intermediaries in atherosclerosis [26], detected at an activated state within atherosclerotic plaques [27]. Under the influence of modified lipids, mast cells secrete cytokines exacerbating disease progression [28], a characteristic they share with NKT cells.

However, no direct interaction between these two cell types has been described in atherosclerosis. 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*$^{W\text{-}sh/W-sh}$ mice and analyzed the progression of atherosclerosis in hyperlipidemic conditions.

2. Materials and methods

2.1. Cell culture

Bone marrow isolated from female control *LDLr*$^{-/-}$ or *CD1d*$^{-/-}$*LDLr*$^{-/-}$ mice was cultured for 4 weeks at 37 °C and 5% CO_2, in RPMI 1640 medium containing 25 mM HEPES (Lonza) supplemented with 10% fetal calf serum (FCS), 60 μM β-mercaptoethanol (Sigma), 100 U/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 [29]. CD1d expression and mast cell purity, based on the expression of FcεRIα and CD117, were assessed at the adoptive transfer point (Supplementary Fig. 1A and 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*$^{W\text{-}sh/W-sh}$ mice with an average age of 15 weeks (n = 10/grp) underwent an i.v. adoptive transfer with 10*10^{6} fully maturated bone marrow derived mast cells, isolated from either *LDLr*$^{-/-}$ or *CD1d*$^{-/-}$*LDLr*$^{-/-}$ mice and henceforth referred to as control or *CD1d*$^{-/-}$ mast cells, respectively. After 4 weeks, mice were placed on a Western-Type diet (WTD) [0.25% cholesterol, 15% cocoa butter (SDS, Essex, UK)] for a period of 10 weeks. The experimental set-up is described in Supplementary Fig.1C. During the study, blood was collected from the tailvein, and serum was obtained upon centrifugation at 8.000 rpm for 10 min. Total cholesterol levels were assessed for weeks 0, 3, 6 and 9 of WTD, 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 analyzed for cholesterol levels. At the experimental endpoint all animals were subcutaneously anesthetized with a mix of ketamine (40 mg/mL), sedazine (6.25 mg/mL) and atropine (0.05 mg/mL).

2.4. Immunohistochemistry

The hearts of all mice were dissected below the atria and frozen in OCT compound (Sakura). Hearts were sectioned horizontally and towards the aortic arch. Upon identification of the tri-valve leaflets of the aortic root, 10 μm 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 fashion on a Leica DM-RE microscope using a Leica QWin software (Leica Imaging Systems, UK).

2.5. Aortic cell cytokine stimulation

Aortic arches were digested in a mix of collagenase I (450 U/mL; Sigma) and XI (250 U/mL Sigma), DNase (120 U/mL; Sigma) and hyaluronidase (120 U/mL; Sigma) [30]. Single aortic cells were obtained with a 70 μm cell strainer. Subsequently, cells were stimulated with a mix of PMA/Ionomycin (50/500 ng/ml; Sigma) in the presence of Brefeldin A (eBioscience) for 2 h. Cytokine production was measured using flow cytometry, after cell fixation and permeabilization (BD Bioscience).

2.6. Flow cytometry

Blood and spleen samples were lysed using erythrocyte lysis buffer (0.1 mM EDTA, 10 mM $NaHCO_3$, 1 mM NH_4Cl, pH = 7.2) to obtain white blood cells. Spleen and thymus samples were processed through a 70 μm 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 2). Measurements were performed on a FACS Canto II (Becton Dickinson, Mountain View, CA) and analyzed using

FlowJo software.

2.7. qPCR

Messenger-RNA isolation on the liver of 6 mice/group was performed according to the guanidium isothiocyanate method [31]. Reverse transcription was achieved by the M-MuLV reverse transcriptase (RevertAid, Leon-Roth). Gene expression was measured on a 7500 Fast-real time PCR (Applied Biosystems, Foster City, CA) using SYBR Green Technology. Relative gene expression was determined using two housekeeping genes. The complete primer list can be found in Supplementary Table 2.

2.8. Statistics

Data are depicted as mean ± SEM and analyzed 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 the context of atherosclerosis. We adoptively transferred $CD1d^{-/-}$ or control mast cells into mast cell deficient $apoE^{-/-}Kit^{W\text{-}sh/W-sh}$ mice and subsequently fed both groups 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 control, and showed no difference after (Fig. 1A., w3: $CD1d^{-/-}$: 3.04 ± 0.5% *vs*. control: 1.77 ± 0.2%, $p = 0.0006$). However, no difference was detected in the percentage of activated (CD69$^+$) NKT cells in the blood (Fig. 1B). At the experimental endpoint, the overall NKT cell population in the thymus was increased upon $CD1d^{-/-}$ mast cell reconstitution, as compared to control mast cell reconstitution (Fig. 1C, $CD1d^{-/-}$: 0.65 ± 0.05% *vs*. control: 0.48 ± 0.04%, $p = 0.014$). Within the thymic NKT cell population of the $CD1d^{-/-}$-mast cell mice, the stage 2 NKT cells ($CD24^-CD44^+NK1.1^-$) which migrate to the periphery [20], did not show any differences in terms of absolute cell counts (Fig. 1D). The same was observed for the absolute cell counts of CD1d-restricted [19] stage 3 ($CD24^-CD44^+NK1.1^+$) NKT cells between the two groups (Fig. 1E). The liver NKT cell population did not differ between the groups (Fig. 1F). However, the liver NKT cells of the $CD1d^{-/-}$ mast cell group were significantly less activated as compared to the control group (Fig. 1G, $CD1d^{-/-}$: 55.9 ± 1.8% *vs*. control: 63.5 ± 2.3%, $p = 0.018$). It is worth noting that at the endpoint, we were able to detect the adoptively transferred mast cells on a similar level inside the peritoneal cavities of both the control and $CD1d^{-/-}$ group (Supplementary Fig.2A), whereas *Cd1d1* expression in the liver, as well as CD40L levels on the NKT population of $CD1d^{-/-}$ reconstituted mice, was found reduced as compared to the control (Supplementary Fig. 2B and C).

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

In the course of the study, we analyzed total serum cholesterol levels in both groups of mice. We observed that 3 weeks after the start of WTD, total cholesterol levels were significantly elevated in $CD1d^{-/-}$ mast cell reconstituted mice, as compared to control mice (Fig. 2A, w3: $CD1d^{-/-}$: 1258 ± 74 mg/dL *vs*. control: 1066 ± 46 mg/dL, p = 0.02). Thereafter, we did not observe a significant difference in cholesterol levels between the two groups. At the end of the experiment, we examined the cholesterol distribution over the various lipoprotein fractions. We did not detect any difference among the (V) LDL and HDL fractions between the groups (Fig. 2B).

3.3. CD1d-disruption on mast cells increases atherosclerotic plaque size

After 10 weeks of WTD, the aortic root was analyzed to determine atherosclerotic plaque size and morphology. The atherosclerotic plaque size was determined using an ORO staining (Fig. 3A), the macrophage content in the area was detected by a MOMA-2 staining (Fig. 3B) while the collagen composition of the plaque was determined using a Masson's trichrome staining (Fig. 3C). The atherosclerotic plaque in the aortic root of $CD1d^{-/-}$ mast cell reconstituted mice was significantly increased by 15% as compared to the plaque size of control mast cell reconstituted mice (Fig. 3D, $CD1d^{-/-}$: $95*10^4 \pm 3.7*10^4\ \mu m^2$ *vs*. control: $83*10^4 \pm 3.8*10^4\ \mu m^2$, $p = 0.033$). Plaque development was also elevated along the entire aortic root of the heart, as measured from the start of the three-valve area up to the receding of the valves (Supplementary Fig.3A). The macrophage content in the plaque area of $CD1d^{-/-}$ mast cell reconstituted mice was slightly, but not significantly, elevated (Fig. 3E, $CD1d^{-/-}$: $37*10^4 \pm 1.4*10^4\ \mu m^2$ *vs*. control: $32*10^4 \pm 2.5*10^4\ \mu m^2$, $p = 0.066$). In contrast, the plaques of the $CD1d^{-/-}$ mast cell reconstituted group showed a significant reduction in collagen content, as compared to control (Fig. 3F, $CD1d^{-/-}$: 55.7 ± 2% *vs*. control: 61.1 ± 1%, $p = 0.038$). However, no mast cells were detected in the aortic root of both control and $CD1d^{-/-}$ group (Supplementary Fig.3B).

3.4. T cells in the atherosclerotic site show increased pro-inflammatory cytokine production upon CD1d-disruption

Upon further characterization of the aortic root, we quantified the CD4$^+$ and CD8$^+$ T cell content. The number of CD4$^+$ T cells within the plaque of $CD1d^{-/-}$ mast cell reconstituted mice tended to increase as compared to control mice (Fig. 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 intimal CD8$^+$ T cells between the groups (Fig. 4B). In addition, we analyzed the proportion of CD4$^+$ and CD8$^+$ T cells (as part of the total T cell [Thy1.2$^+$] population) in the aortic arch of these mice, using flow cytometry. The $CD1d^{-/-}$ mast cell bearing mice showed a significantly higher percentage of CD4$^+$ T cells in the plaque, in relation to control mice (Fig. 4C, $CD1d^{-/-}$: 32.2 ± 2.8% *vs*. control: 24.6 ± 2.1%, $p = 0.041$). No significant difference was detected in the percentage of CD8$^+$ T cells between the two groups (Fig. 4D). 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 $CD1d^{-/-}$ group presented a significant increase in the production of cytokines IFNγ (Fig. 4E, $p = 0.012$), IL-17 (Fig. 4F, $p = 0.025$) and TNFα ($p = 0.0016$) as opposed to the control group. On that note, we observed that the percentages of NKT cells within the aortic arch were similar in both groups (Supplementary Fig.3C). NKT cells appeared at a low percentage, and although they showed an active cytokine production profile for IFNγ, TNFα and IL-17, there was no difference between control and $CD1d^{-/-}$ group (Supplementary Fig. 3D-F).

3.5. T-bet expression is higher in circulating $CD4^+$ and $CD8^+$ T cells

Having in mind the close relationship between NKT and T_{H1}/T_{H2} cells and upon observing the T cell differences in the atherosclerotic tissue, we aimed to analyze the systemic T cell response. We examined circulating T cells for the expression of transcription factor T-bet, which controls pro-inflammatory cytokine IFNγ secretion and generation of CD4 T_{H1} cells [32]. We detected a substantial increase on T-bet expression levels of the CD4$^+$ T cell population in the blood of $CD1d^{-/-}$ mast cell reconstituted mice, as compared to control mast cell reconstituted mice (Fig. 5A, $p = 0.034$). On the contrary, GATA-3 expression,

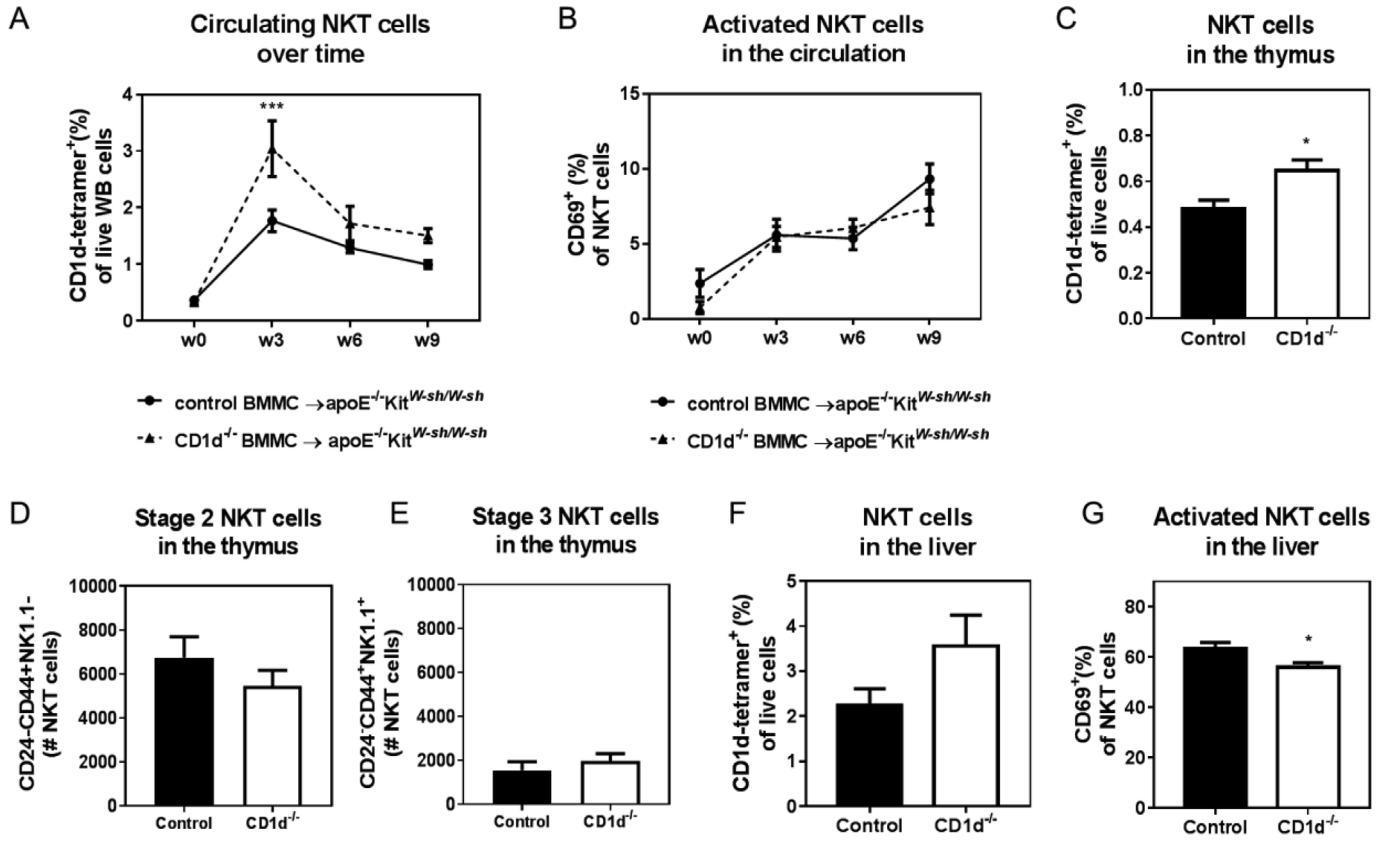

Fig. 1. NKT cell levels are elevated but cells appear less activated upon reconstitution of mice with $CD1d^{-/-}$ mast cells.
(A) Circulating levels of NKT cells increased at 3 weeks of WTD in the $CD1d^{-/-}$ reconstituted mice as compared to control. (B) No difference was detected on NKT cell activation in the blood over time, between the groups. (C) Total thymic NKT cells were elevated, in comparison to mice reconstituted with control mast cells. (D) Stage 2 as well as (E) stage 3 NKT cell counts in the thymus of mice reconstituted with $CD1d^{-/-}$ mast cells did not show any difference, as compared to control mice. (F) No difference was detected in the total NKT cell populations of the liver between the two groups. (G) The NKT cells in the liver of $CD1d^{-/-}$ mast cell reconstituted mice showed decreased activation as compared to control mast cell reconstituted mice. All values are depicted as mean ± SEM. $^{*}p < 0.05$, $^{***}p < 0.001$.

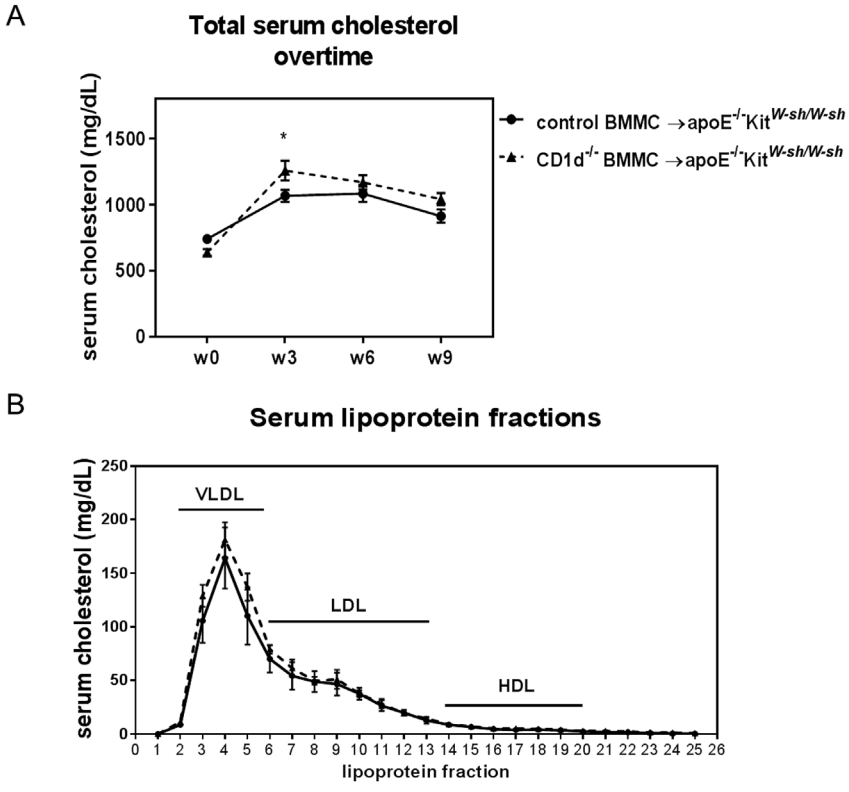

Fig. 2. Cholesterol levels in the circulation show a mild increase in $CD1d^{-/-}$ mice at week 3.
(A) Total cholesterol levels in the serum of $CD1d^{-/-}$ mast cell reconstituted mice showed a slight increase over time of WTD, as compared to control mast cell reconstituted mice. (B) No difference was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

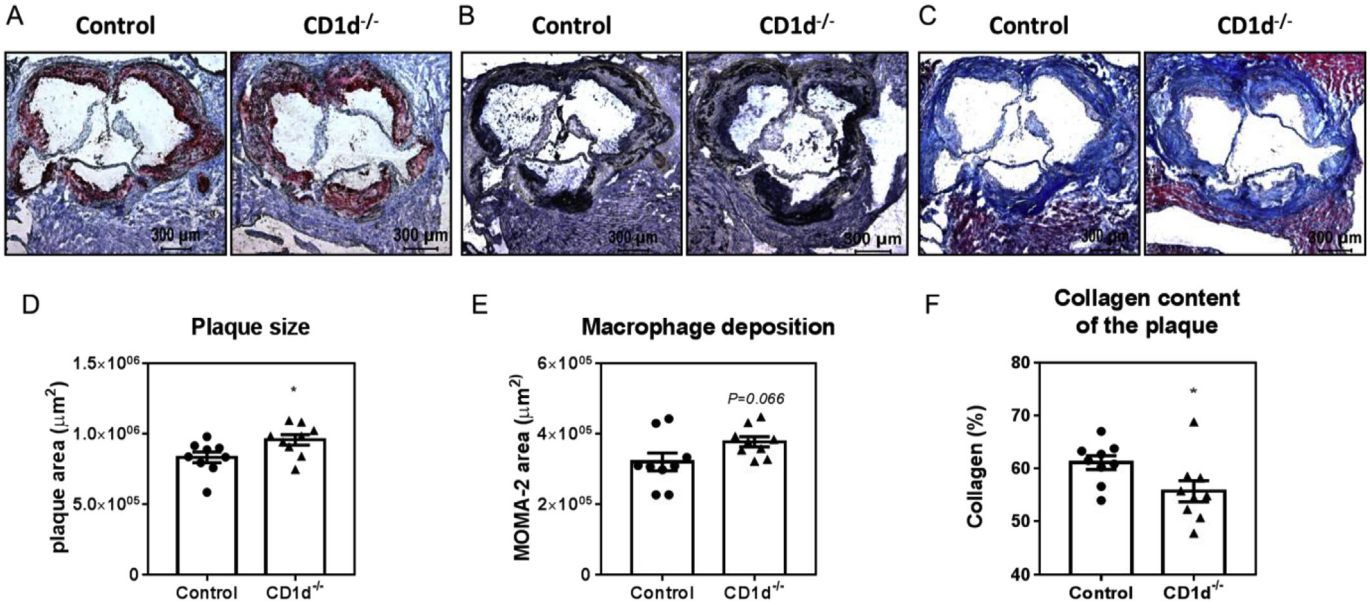

Fig. 3. Mice reconstituted with *CD1d*$^{-/-}$ mast cells show increased atherosclerosis in the aortic root, as compared to mice reconstituted with control mast cells. Representative pictures per group of mice for (A) ORO staining, (B) MOMA-2 staining and (C) Masson's trichrome staining (n = 9/grp). The aortic root of mice reconstituted with *CD1d*$^{-/-}$ mast cells shows (D) elevated atherosclerotic plaque size, (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. *$p < 0.05$.

which characterizes T_{H2} cells [33], was significantly decreased in circulating CD4$^+$ T cells upon *CD1d*$^{-/-}$ mast cell reconstitution (Fig. 5B, $p = 0.025$). Additionally, T-bet expressing CD8$^+$ T cells were found elevated in the circulation of the *CD1d*$^{-/-}$ group as compared to control (Fig. 5C, *CD1d*$^{-/-}$: 8.2 ± 0.9% *vs.* control: 4.7 ± 0.5%, $p = 0.004$). Both groups showed no difference in the absolute cell count of CD8$^+$ and CD4$^+$ T cells in the blood (Fig. 5D and E). Interestingly, while activated T cells were enhanced over time of WTD (statistics) for both groups, the *CD1d*$^{-/-}$ group showed a slight increase over week 9 as compared to control (Fig. 5F).

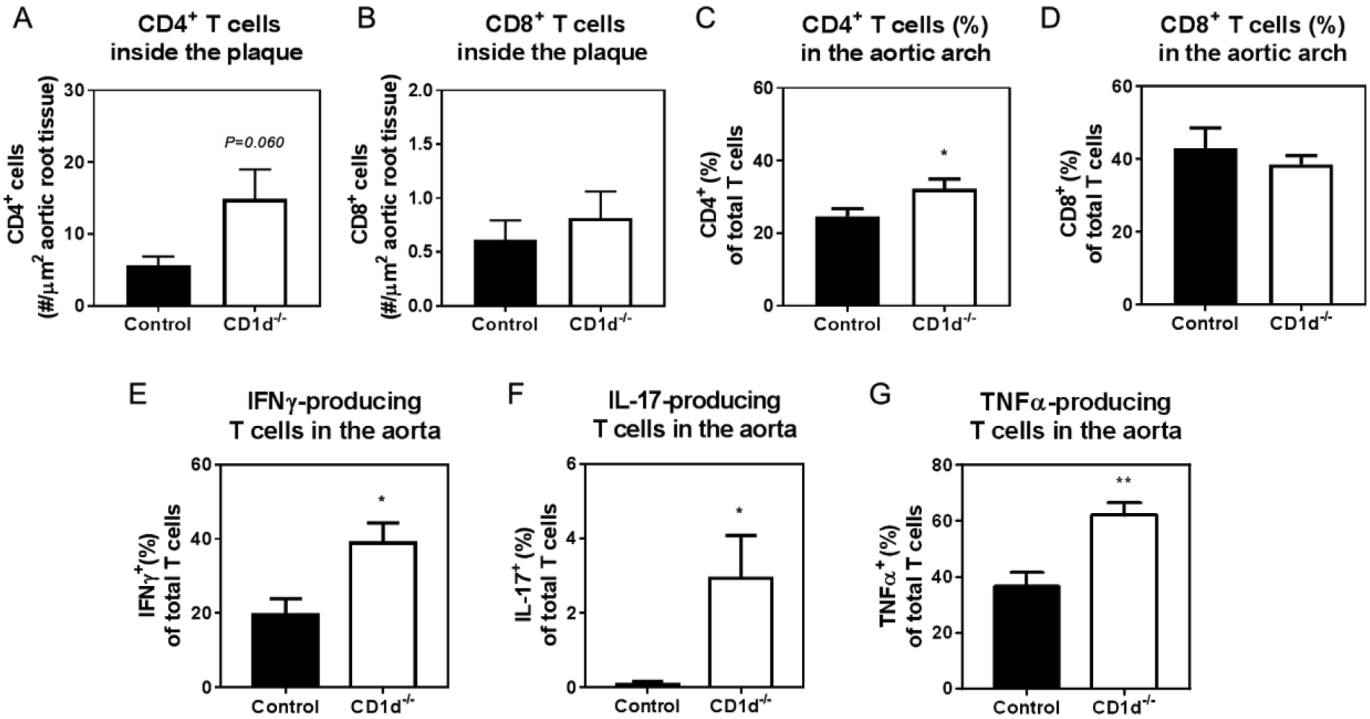

Fig. 4. CD4$^+$ T cells are increased in the atherosclerotic sites of *CD1d*$^{-/-}$ mast cell reconstituted mice and show increased pro-inflammatory cytokine production. (A) CD4$^+$ T cells in the plaque intima of the aortic root increased upon *CD1d*$^{-/-}$ mast cell reconstitution, as compared to control. (B) No difference was observed in the intimal CD8$^+$ T cell numbers between the groups. (C) The CD4$^+$ T cell levels of the total T cell population were elevated in the *CD1d*$^{-/-}$ mast cell group as compared to control. (D) The percentages of CD8$^+$ T cells in the aorta showed no difference between the *CD1d*$^{-/-}$ mast cell reconstituted group and control mice. (E) IFNγ-producing, (F) IL-17-producing and (G) TNFα-producing T cells, in the aorta of *CD1d*$^{-/-}$ reconstituted mice were increased in comparison to control mice. All values are depicted as mean ± SEM. *$p < 0.05$, **$p < 0.01$.

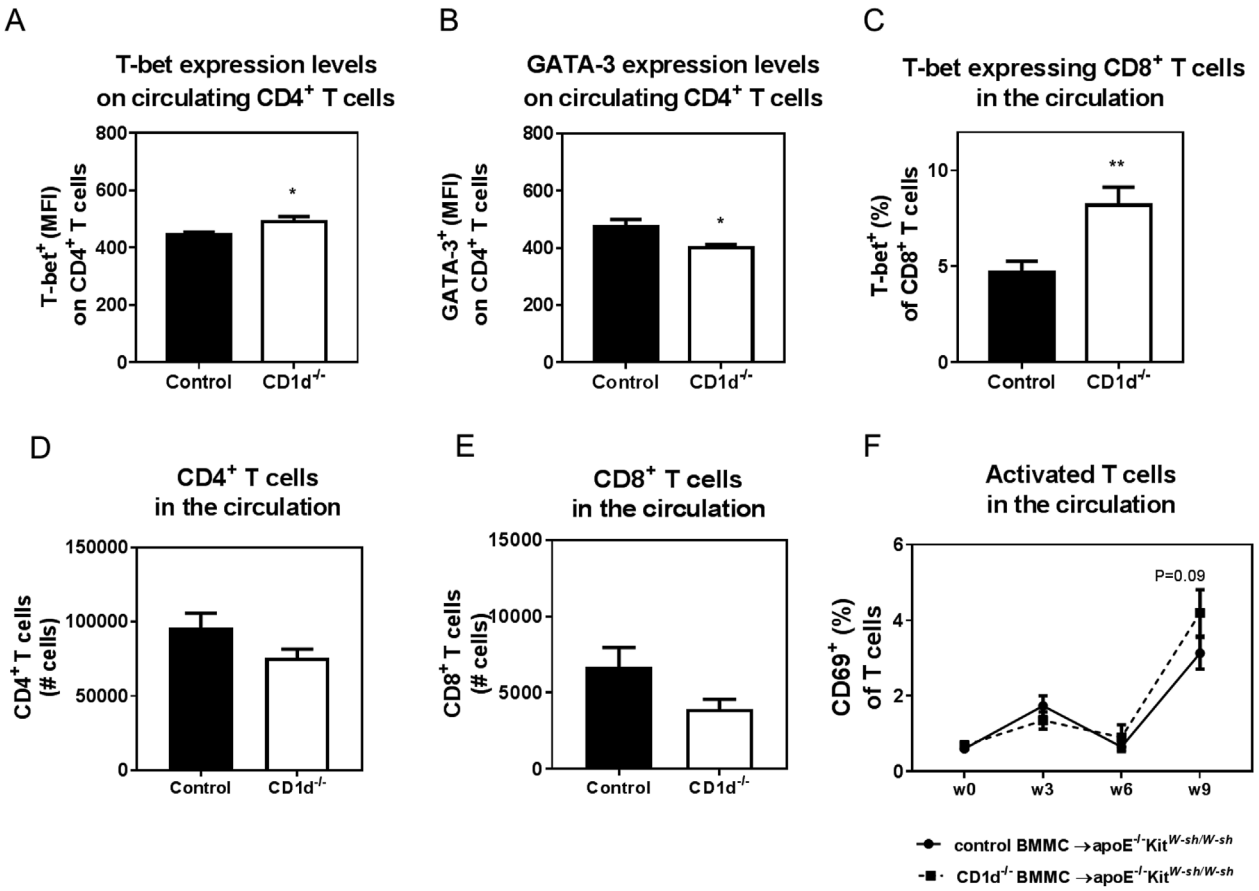

Fig. 5. T-bet expression is increased in circulating T cells of *CD1d*$^{-/-}$ mast cell reconstituted mice.
(A) T-bet expressing CD4^{+} T cells were elevated in the blood of *CD1d*$^{-/-}$ mast cell reconstituted mice as compared to control mice. (B) GATA-3 expression on CD4^{+} T cells was reduced in the *CD1d*$^{-/-}$ mast cell reconstituted mice as compared to control mice. (C) The percentage of T-bet expressing CD8^{+} T cells in the blood was elevated upon *CD1d*$^{-/-}$ mast cell reconstitution as compared to control. No difference was detected in the total cell count of (D) circulating CD4^{+} or (E) circulating CD8^{+} T cell numbers between the two groups at the experimental endpoint. (F) Activated (CD69^{+}) T cells of the *CD1d*$^{-/-}$ group showed a slight increase at week 9 of WTD, as compared to control. 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 upon atherosclerosis development. We discovered that the adoptive transfer of mast cells lacking CD1d expression increases atherosclerotic plaque size and destabilization through enhanced infiltration of CD4^{+} T cells into the plaque. The T cell population in the plaques of mice repopulated with *CD1d*$^{-/-}$ mast cells showed increased pro-inflammatory cytokine production, in the form of IFNγ, TNFα and IL-17. This effect may have caused the observed reduction in collagen deposition within the plaque. After 3 weeks of WTD, these animals showed a modest increase in serum cholesterol levels; however, the overall total cholesterol was not different between the groups, indicating no major cholesterol contribution to the observed plaque increase. The above results suggest that mast cell-CD1d expression has a protective influence on the function of NKT cells and probably controls T cell responses in atherosclerosis.

Through the course of WTD, we detected increased levels of circulating NKT cells in the *CD1d*$^{-/-}$ mast cell reconstituted mice. This increase was also apparent in the thymus of the *CD1d*$^{-/-}$ group. As mentioned, thymic NKT cells of stage 2 and 3 migrate into peripheral tissues [34], which may thus explain their abundance in the circulation. However, while stage 2 are migrators and potent cytokine secretors, transition to stage 3 requires glycolipid presentation through CD1d [35]. In this study we did not observe a reduction in the absolute amount of stage 2 and 3 NKT cells. However, a reduction in the expression of CD1d as well as in the activation and co-stimulation of the NKT population in the liver was observed. These data suggest that NKT cells, generated in the thymus, migrate to the periphery of *CD1d*$^{-/-}$ mast cell reconstituted mice at higher frequencies, perhaps to counteract for the absence of mast cell-initiated CD1d signaling.

As stated before, NKT cells can potently activate T cells and, in atherosclerosis, NKT cells are suggested to act prior to and pave the way for intraplaque T cell infiltration. In contrast to the existing concept of proatherogenic NKT cells, and despite the reduced NKT cell activation, in this study we observed that lack of CD1d expression by mast cells led to increased intraplaque CD4^{+} T cells within the aortic root and elevated proatherogenic cytokine production, mainly by CD4^{+} T cells, in the aortic arch. This seems surprising since CD1d signaling is known to induce proatherogenic responses through NKT cells [15]. However, a previously published study demonstrated that α-GalCer-activated NKT cells in mice fed a high-fat diet, control T_{H1} cell responses via reduced IFNγ secretion in the serum [36]. Other reports have also stated that CD1d-mediated effects on NKT cells can subsequently control T_{H1} cells. For example, in autoimmune arthritis, characterized by the vast presence of mast cells, disruption of CD1d-mediated NKT cell activation exacerbated T_{H1} responses, in an IFNγ-mediated manner [37]. In our study, pro-inflammatory IFNγ production was found elevated in aortic T cells of the *CD1d*$^{-/-}$ mast cell reconstituted mice and coincided with increased T-bet expression by circulating CD4^{+} T cells. Transcription factor T-bet directly induces T_{H1} cell skewing [32] and IFNγ production [38], and T_{H1} cells are also characterized by TNFα secretion [39]. In addition, NKT cells have been previously reported to inhibit T_{H17} cell differentiation [40], the main CD4^{+} T cells secreting cytokine IL-17

[41] which is increased in proatherogenic conditions [42]. Interestingly, in our setup, increased T-bet expression on the circulating $CD4^+$ population was accompanied by decreased expression levels of GATA-3, the transcription factor regulating T_{H2} cell skewing [33]. CD1d-mediated presentation is known to act differentially upon the NKT cells, depending on the glycolipid antigen that is presented. Subsequently, NKT cells can alter their response as to secrete either T_{H1} or T_{H2} cytokines [6] and the respective NKT cytokine secretion can in turn affect T_{H1} or T_{H2} subsets [40]. Provided that despite the presence of NKT cells within the plaque area, their cytokine secretion profile showed no difference between the control and *CD1d*$^{-/-}$ groups, this may suggest how disruption of the *CD1d*$^{-/-}$-mediated mast cell-NKT cell interaction indirectly modified the cytokine production of $CD4^+$ T cells. We also observed that circulating $CD8^+$ T cells of *CD1d*$^{-/-}$ mast cell reconstituted mice show increased T-bet expression. It has been previously reported that T-bet expression on $CD8^+$ T cells leads to IFNγ production [43]. NKT cells have been found to negatively regulate $CD8^+$ T cell mediated responses. Specifically, the absence of NKT cells enhanced $CD8^+$ T cell specific-IFNγ production and $CD8^+$ T cell infiltration in allergic dermatitis [44]. 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 IFNγ production by T cells, which in turn augmented atherosclerotic plaque progression, as also indicated by the slight increase in T cell activation of the *CD1d*$^{-/-}$ group upon 9 weeks of WTD.

The increase in pro-inflammatory $CD4^+$ T cells observed within the atherosclerotic plaques of *CD1d*$^{-/-}$ mast cell reconstituted mice may have influenced collagen deposition. Specifically, the atherosclerotic plaques of these mice contained less collagen, signifying reduced plaque stability [45]. IFNγ has been accounted for its plaque destabilizing effect [46].

Up to date there are limited reports on the CD1d-mediated action of mast cells on NKT cells. In this study, we did not detect any mast cells in the atherosclerotic plaque area of the heart. However, this is not surprising since adoptively transferred mast cells are known to show difficulties in repopulating the heart tissue [47]. Furthermore, mast cells are attracted inside tissues according to the local chemokine signals released. The fact that at the point of adoptive transfer in this study setup there was no ongoing inflammatory stimulus within the heart area, as the mice were not on Western-type diet, is a possible reason why mast cells did not appear in the plaque area at the experimental endpoint. Mast cells are potent cytokine secretors and populate organs where NKT cells reside, such as the thymus [48], the atherosclerotic plaque [26] and the liver [49]. Therefore, the multitude of tissues that mast cells can populate suggests that the effects observed in this study are more a systemic effect of *CD1d*$^{-/-}$ mast cell reconstitution rather than a local effect on NKT cells of the atherosclerotic plaque.

The expression of CD1d on the mast cell surface was previously associated with mast cell activation via their classical receptor FcεR [24]. Mast cell activation results in cytokine release, with IFNγ [50] and TNFα [51] considered among the typical ones excreted. However, there is also an anti-inflammatory role exerted by this cell type [52], e.g. through the release of the atheroprotective cytokine IL-10. The microenvironment can have a strong influence on the downstream effects of mast cells and determine their pro- or anti-inflammatory character, as recently indicated through the effect of cytokine IL-37 [53], or vitamin E [54] on the mast cell actions. Therefore, CD1d expression on the mast cell surface could also partly affect the activation of mast cells.

Mast cells have been implicated in a direct interaction with the adaptive immune system [55], and the above-mentioned study which examined the expression of CD1d on mast cells indicated that mast cells express also the costimulatory protein CD40; albeit not in a manner that influenced the NKT cell response [24]. Our results however showed that $CD1d^{-/-}$-mast cells seem to negatively affect CD40L expression on liver NKT cells. Additional co-stimulatory pathways may also participate in the observed mast cell-NKT cell interaction since ox40L and CD48 expression on mast cells are reported to influence NKT cell cytokine secretion [25].

To conclude, here we describe 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. Therefore, in the absence of mast cell-mediated CD1d activation, NKT cells may aggravate the local T cell immune responses. In this study setup, we were unable to fully elucidate the molecular mechanism through which NKT cells can act upon the $CD4^+$ T cell population. In addition, the exact lipid antigen presented by mast cell-CD1d to NKT cells remains currently unknown. Additional *in vivo* experiments may shed more light on these mechanistic questions in the future. Of note, NKT cells are also involved in tolerogenic responses with presentation of endogenous lipids as to control autoimmune reactions [56] and can thus exert also protective functions. In atherosclerosis, mast cells appear to be fine-tuning the NKT cell actions via CD1d and subsequent glycolipid presentation, leading thus to a novel mechanism of action in this disease.

Conflicts of interest

The authors declared they do not have anything to disclose regarding conflict of interest with respect to this manuscript.

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Author contributions

E.K., and I.B. designed the present study, executed the experiments and wrote the manuscript. G.H.M.v.P., J.v.D., J.E.N., T.vd.H., M.B., C.G., F.H.S. and M.J.K. performed experiments. J.K., I.B. and G.H.M.v.P. provided intellectual input and revised the manuscript. All authors reviewed the final manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.atherosclerosis.2018.11.027.

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