Single-cell immune profiling of atherosclerosis: from omics to therapeutics

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Chapter 8

Inhibition of Interleukin-4 Induced Gene 1 (IL4I1) stimulates a pro-inflammatory immune environment without affecting early atherosclerotic lesion development in LDL receptor knockout mice.

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Abstract

Interleukin-4 Induced Gene 1 (IL4I1) is produced by antigen presenting cells upon T cell activation. It functions as a suppressor of cytotoxic T cell activation and inhibition of IL4I1 has been proposed as a promising therapeutic strategy for cancer. However, immune therapy in cancer has been associated with cardiovascular health problems. In this study, the role of IL4I1 in atherosclerosis was investigated.

Single-cell RNA sequencing data from human atherosclerotic plaques revealed IL4I1 expression on foamy TREM2^{hi} macrophages. Female low-density lipoprotein receptor deficient (LDLr$^{-/-}$) mice were fed a Western-type diet for 6 weeks to induce atherosclerosis development and treated either with the IL4I1 inhibitor CB-668 (25 mg/kg) or vehicle control twice a day by oral gavage. Treatment with CB-668 had no effect on the main immune cell populations in the circulation. However, CB-668 administration did lead to a shift from naïve to effector and/or central memory CD4^{+} and CD8^{+} T cells and an increase in cytotoxic CD8^{+} T cells in the spleen and the atherosclerotic aorta. Histological analysis of the three-valve area in the heart showed that CB-688 did not affect atherosclerotic lesion size nor composition compared to the controls.

Our data show that inhibition of IL4I1 using the small molecule inhibitor CB-668 could result in a more pro-inflammatory immune environment but does not affect early atherosclerotic lesion development. This is the first indication that application of CB-668 in anti-cancer therapy likely does not lead to an increased risk of atherosclerosis.

Keywords: inflammation, the immune system, atherosclerosis, macrophage, IL4I1

Introduction

Interleukin 4 induced gene 1 (IL4I1) is a secreted amino-acid catabolizing enzyme which converts its substrate L-phenyl alanine into phenyl pyruvate, ammonia, and hydrogen peroxide.[1] IL4I1 was initially discovered in a rare type of B-cell lymphoma[2-4], and is currently known to be overexpressed in different types of cancer, including breast cancer, renal cancer, and melanoma. In experimental studies in murine models, overexpression of IL4I1 is associated with a poor diagnosis due to negative regulation of the antitumor effects of $CD8^+$ T cells and thereby stimulating tumor immune evasion.[5,6] Recently, a selective small molecule inhibitor of IL4I1, named CB-668, has been described as a potent novel anti-cancer therapy.[7] Blockade of IL4I1 using CB-668 in murine tumor models resulted in increased expression of pro-inflammatory immune genes in the tumor and led to reduced tumor growth, particularly in combination with anti-programmed cell death 1 (PD1) ligand therapy. This implies a key role for IL4I1 in suppression of the immune response in cancer. Indeed, recent studies have shown that IL4I1 limited T cell proliferation and differentiation as well as B cell proliferation.[1,8]

In immune cells, IL4I1 is primarily produced by antigen presenting cells such as macrophages and dendritic cells and secreted in the immune synapse at the interface between the antigen presenting cell and the T cell. It is crucial for cell-cell communication and functions as a negative regulator of T cell activation. Aubatin *et al.* have demonstrated that IL4I1 modulates T cell activation by interfering with signaling pathways downstream of the T cell receptor independently of its enzymatic activity.[9] Besides antigen presenting cells, certain T cell subtypes are also able to produce IL4I1, including T helper 17 cells (T_{h17}) and regulatory T cells (T_{regs}).[10,11] Production of IL4I1 by T_{h17} cells is induced in an autocrine manner and blocks T_{h17} cell cycle progression through the inhibitory effects of Tob1, a member of the TOB/BTG 1 family of anti-proliferative proteins and thus functions as a cell cycle inhibitor.[12] In addition, Cousin *et al.* demonstrated that IL4I1 promotes the differentiation of naïve T cells into regulatory $FOXP3^+$ T cells *in vitro*. Notably, increased numbers of $FOXP3^+$ T cells have been associated with augmented tumorigenesis, an effect related to their T cell suppression activity.[13,14]

In summary, IL4I1 is an important immunoregulator and has shown to be a promising therapeutic target in cancer to limit immune evasion in the tumor microenvironment. However, important to note is that blockade of immune checkpoints inhibits the natural "brake" on the immune response. Although this is beneficial in cancer therapy in which an augmented immune response is essential for effective removal of the aberrant cancer cells, it does increase the risk for an autoimmune response. Recent studies

showed substantial accumulation of T cells in human atherosclerotic plaques[15,16], making it likely that checkpoint inhibitors will affect T cell immune responses in atherosclerosis. In line, checkpoint inhibitors that are successful in cancer therapy, such as anti-PD-L1 and anti-T lymphocyte-associated protein 4 (CTLA4) have been associated with an increased risk for atherosclerosis.[17-20] Yet, it remains unknown whether blockade of the immunoregulator IL4I1 stimulates atherosclerosis risk.

In the current study we determined if IL4I1 is expressed in atherosclerotic plaques and established the effects of IL4I1 inhibition on atherosclerosis development in hypercholesterolemic low-density lipoprotein receptor (LDLr) knockout mice *in vivo*.

Material and methods

Single-cell RNA sequencing of human atherosclerotic plaques

Human carotid atherosclerotic plaques were obtained from 18 patients (14 male, 4 female) during carotid endarterectomy surgery and included in the AtheroExpress biobank.[18] Briefly, single cells were isolated, live cells were sorted into 384-wells plates and processed for single-cell RNA sequencing, as previously described.[15] All studies were performed in accordance with the Declaration of Helsinki. Informed consent was obtained from all patients involved in the study.

Mice

Female 8-10 weeks old Low-Density Lipoprotein receptor knockout (LDLr-/-) mice were bred in-house and provided with food and water *ad libitum*. Mice were housed in a standard laboratory setting and fed a Western-type diet (0.25% cholesterol, 15% cocoa butter, Special Diet Services, Essex, UK) for six weeks to induce atherosclerosis development. The mice were treated with either the IL4I1 inhibitor CB-668 (25 mg/kg, Calithera Biosciences) or a vehicle control (Ctrl) (n = 14-15 per group) twice daily through oral gavage. Blood was drawn from by tail vein bleeding in week 2 and 4. At week 6, mice were sacrificed after subcutaneous injection of anaesthetics (ketamine (40 mg/mL), atropine (0.1 mg/mL), and xylazine (8 mg/mL)). Blood was collected by orbital bleeding in regular tubes (Eppendorf), for serum analysis) or in K2-EDTA blood tubes (Sarstedt AG & Co. KG) for flow cytometry) and mice were perfused with PBS through the left cardiac ventricle. Subsequently, organs were collected for analysis. All animal experiments were approved by the Ethics Committee for Animal Experiments and the Animal Welfare Body of Leiden University, and performed in compliance with the European Parliament's Directive 2010/63/EU on the protection of animals used for research purposes.

Serum cholesterol levels

Serum was collected by centrifugation of whole blood at 8000 rpm for 10 minutes at 4°C and stored at -80°C until further use. Serum concentrations of total cholesterol were determined using enzymatic colorimetric assays.[21] Precipath (Roche Diagnostics) was used as internal standard.

Cell isolation

To obtain a white blood cell suspension, whole blood was lysed with ACK lysis buffer (0.15 M NH_4Cl, 1 mM $KHCO_3$, 0.1 mM Na_2EDTA, pH 7.3). Spleens were passed through a 70 µm cell strainer and subsequently lysed with ACK lysis buffer to obtain a splenocyte suspension. Heart lymph nodes were passed through a 70 µm cell strainer to obtain a single cell suspension. Aortic arches were cut into small pieces and digested using an enzyme mix containing collagenase I (450 U/mL), collagenase XI (250 U/mL), DNAse (120 U/mL), and hyaluronidase (120 U/mL; all Sigma-Aldrich) for 30 minutes at 37°C while shaking. Next, digested samples were passed through a 70 µm cell strainer. All single cell suspensions were subsequently used for flow cytometry.

Flow cytometry

Single cell suspensions were stained with a mixture of fluorescent antibodies directed extracellular markers for 30 minutes at 4°C. Intracellular staining was performed using the FoxP3 Transcription Factor Staining Buffer Set (ThermoFisher) following the manufacturer's instructions. Briefly, cells were fixed for 45 minutes at 4oC and washed twice at 1500 rpm for 5 minutes. Fixed cells were stained with a mixture of intracellular antibodies for 30 minutes at 4°C, washed, and subsequently measured with flow cytometry. The antibodies used for flow cytometry are listed in **Table 2**. All measurements have been performed on a Cytoflex S (Beckman Coulter) and analysed with FlowJo V10.7 (Treestar).

Histological analysis

Hearts were isolated, dissected, embedded, and frozen in Tissue-Tek OCT compound (Sakura). Prior to histological analyses, cryosections of 10 µm were made of the aortic root. Neutral lipids were stained with Oil-Red-O (Sigma Aldrich) to assess mean plaque size and vessel occlusion. Aortic root sections were stained with Masson's Trichrome to measure collagen content and necrotic core size. The monocyte/macrophage area was stained using a MOMA-2 primary antibody (1:1000; rat IgG2b; Bio-Rad) and visualized using a secondary biotinylated rabbit α-rat IgG antibody (1:100, Vector) and the Vectastain ABC HRP kit and Vector ImmPACT NovaRed (Vector). Sections were digitalized with a Panoramic 250 Flash III slide scanner (3DHistech) and quantified using ImageJ software.

Table 2. List of extracellular and intracellular antibodies used.

Marker	Fluorochrome	Clone	Supplier
CD3e	BV650	145-2C11	BD biosciences
CD4	PercP	RM4-5	BD biosciences
CD8	BV510	53-6.7	Biolegend
CD11b	BV605	M1/70	Biolegend
CD11c	APC	N418	eBioscience
CD25	PE-texas red	PC61.5	Biolegend
CD44	APC	IM7	eBioscience
CD45	AF700	30-F11	Biolegend
CD45	PE	30-F11	Biolegend
CD62L	BV605	MEL14	Biolegend
F4/80	FITC	BM8	Biolegend
Foxp3	eFluor 450	FJK-16s	eBioscience
Ly6C	BV510	HK1.4	Biolegend
Ly6G	PerCP-Cy5.5	1A8	Biolegend
MHCII	BV650	M5/114.15.2	Biolegend
ROR γT	PE	AFJKS-9	eBioscience
T-bet	PE-Cy7	eBio4B10	eBioscience

Statistical analysis

Data are depicted as mean ± SEM and analysed using GraphPad Prism 9 software. Normal distribution was tested using a Shapiro Wilk test. Outliers were identified by a Grubb's outlier test. A two-tailed Student's t-test was performed with normal distributed data, otherwise, the Mann–Whitney test was used. Probability values of $p<0.05$ were considered statistically significant.

Results

IL4I1 expression on human plaque TREM2⁺ macrophages detected by single-cell RNA sequencing

IL4I1 is known to be expressed by antigen presenting cells in multiple diseases. Apart from cancer, IL4I1⁺ macrophages accumulate in both inflammatory and infectious diseases, such as rheumatoid arthritis and COVID19.[22,23] Furthermore, in sarcoidosis and tuberculosis IL4I1 is highly expressed by macrophages and dendritic cells in Th1 granulomas.[24] However, whether IL4I1 is also expressed in antigen presenting cells in human atherosclerotic plaques remains unknown. Recently, we applied single-

cell RNA sequencing (scRNAseq) on human carotid atherosclerotic plaques of 18 patients.[15] Fourteen distinct cell clusters (0-13) were identified amongst which four myeloid cell clusters consisting of both macrophages and dendritic cells (cluster 5, 6, 7, 12) (**Fig. 1A**). Indeed, *IL4I1* expression was detected in this data set, primarily in the *TREM2* (Triggering receptor expressed on myeloid cells 2) expressing macrophages (**Fig. 1B**).[15,16,25] To confirm IL4I1 expression in murine plaque antigen presenting cells, we used PlaqView (www.plaqview.com) to look up *Il4i1* in an integrated data set of 12 data sets of murine atherosclerotic aorta's and, as described earlier by Zernecke *et al*., this gene was mainly expressed by cells of the mature dendritic cell cluster.[26,27] Together, these data show that IL4I1 is expressed in the atherosclerotic plaque and this expression is restricted to antigen presenting cells. We next aimed to determine whether inhibition of IL4I1 affected T cell polarization in the atherosclerotic plaque.

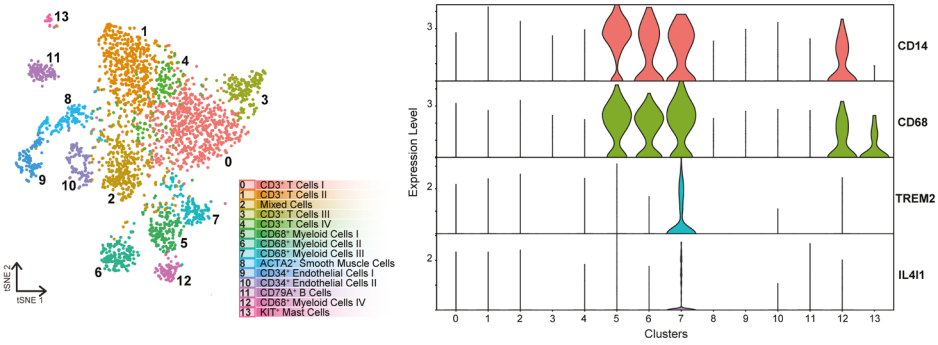

Figure 1. Single cell RNA sequencing reveals expression of IL4I1 on TREM2⁺ macrophages in human atherosclerotic lesions. (A) Single-cell RNA sequencing of human carotid atherosclerotic plaques reveals tSNE with 14 distinct clusters. **(B)** Violin plots of expression of CD14, CD68, TREM2 and IL4I1.

Inhibition of IL4I1 does not affect immune cell populations in the circulation and spleen

Because scRNAseq has shown that IL4I1 is expressed locally in the atherosclerotic lesion by myeloid cells, we have examined whether inhibition of IL4I1 could affect immune activation and atherosclerotic plaque development. Female LDLr$^{-/-}$ mice (8-10 weeks old) were fed a Western-type diet for 6 weeks to induce atherosclerosis. During these 6 weeks, mice were treated with CB-668, a small molecule inhibitor of IL4I1, or with vehicle control via oral gavage twice a day (25mg/kg)(**Fig. 2A**). Inhibition of IL4I1 using CB-668 did not affect the body weight throughout the experiment (t=6 weeks, Ctrl: 21.1±0.4 g vs. CB-668: 20.6±0.4 g; **Fig. 2B**). As expected, Western-type diet feeding led to an increase of serum total cholesterol levels in both groups

(**Fig. 2C**). IL4I1 inhibition did not significantly affect the serum cholesterol levels during the 6 weeks of treatment (t=6 weeks, Ctrl 1733.8±91.3 mg/dL vs. CB-668 1907.2±84 mg/dL; **Fig. 2C**). To study the systemic immune effects of IL4I1 inhibition using CB-668, general immune cell populations were assessed in the blood and spleen. In the circulation, no differences were found in either lymphocytes or myeloid cell subsets upon CD-668 treatment (**Fig. 2D**). In spleen, however, CB-668 did induce a significant decrease in $CD4^+$ (Ctrl: 15.70±0.84%, CB-668: 14.50±0.61%, $p<0.05$) and $CD8^+$ (Ctrl: 11.70±0.49%, CB-668: 10.80±0.44%, $p<0.05$) T cells. Furthermore, inflammatory monocytes were slightly increased ($CD11b^+Ly6C^{hi}$; Ctrl: 0.58±0.055%, CB-668: 0.81±0.091%, $p<0.05$). No differences were observed in splenic B cells, $CD11b^+$ cells, $Ly6G^+$ Neutrophils, or $CD11b^+$ $CD11c^+$ dendritic cells.

Treatment with CB-668 leads to a shift from naïve to effector or central memory $CD4^+$ and $CD8^+$ T cells

Because IL4I1 has been described as an immune regulator that may be involved in T cell proliferation and function[28], we have examined whether inhibition of IL4I1 could affect the T cell activation state in different immune organs such as the spleen, heart lymph nodes (HLN), and in the atherosclerotic aortic arch (AA). The percentages of naïve $CD4^+$ and $CD8^+$ T cells (T_{naive})($CD44^-CD62L^+$) were decreased in the spleen as well as in the HLN (spleen: $CD4^+$ T_{naive} -14.0%, $CD8^+$ T_{naive} -14,0%, HLN: $CD4^+$ T_{naive} -17.0%, $CD8^+$ T_{naive} -7.0%; $p<0.05$)(**Fig. 3A-B**). Moreover, this decrease was accompanied by an increase of effector T cells (T_{eff}) ($CD44^+CD62L^-$) and/or central memory T cells (T_{cm}) ($CD44^+CD62L^+$). Splenic $CD8^+$ T_{eff} cells were increased upon IL4I1 inhibition ($CD8^+$ T_{eff} +66.0%, $p<0.05$)(**Fig. 3A**), whereas there was no difference of $CD8^+$ T_{eff} cells in the HLN (**Fig. 3B**). Central memory $CD8^+$ T cells in the spleen as well as central memory $CD4^+$ and $CD8^+$ cells in the HLN were increased upon treatment with CB-668 (spleen: $CD8^+$ T_{cm} +15.0%, HLN: $CD4^+$ T_{cm} +39.0%, $CD8^+$ T_{cm} +11%, $p<0.05$)(**Fig. 3A-B**). In the atherosclerotic aorta, no differences were found in the activation state of $CD4^+$ T cells. However, we did observe a trend towards lower naïve $CD8^+$ T cells and an increase in effector $CD8^+$ T cells was observed ($CD8^+$ T_{eff} +45.8%, $p<0.01$)(**Fig. 3C**).

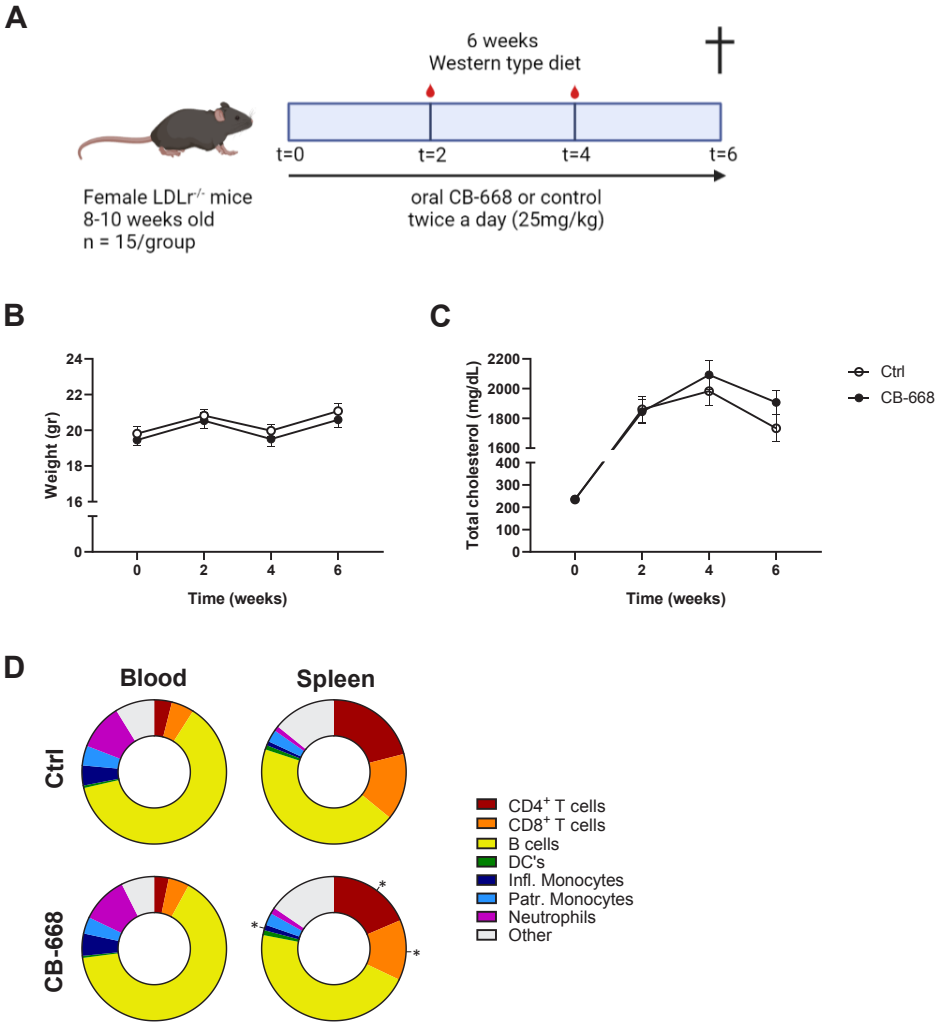

Figure 2. IL4I1 inhibition does not affect the bodyweight, serum cholesterol levels and general immune cells in blood and spleen. (A) Schematic overview of the experimental set-up. Female LDLr$^{-/-}$ were put on a western type diet for 6 weeks and were treated with 0.25 mg/kg CB-668 or vehicle control twice a day by oral. Blood was drawn via tail vein bleeding at week 2 and week 4. At week 6, all mice were sacrificed, after which organs were collected and processed for analysis. **(B)** Body weight of mice was measured every two weeks during treatment and was not affected by CB-668. **(C)** Plasma cholesterol levels were obtained at t=0, t=2, t=4 and t=6. **(D)** The frequency of CD4^{+} T cells, CD8^{+} T cells, CD19^{+} B cells, CD11b^{+} myeloid cells, CD11b^{+}CD11c^{+} Dendritic cells (DCs), CD11b^{+}Ly6c^{Hi} inflammatory monocytes (Inf. monocytes), CD11b^{+}Ly6c^{lo} patrolling monocytes (Patr. monocytes) and CD11b^{+}Ly6G^{Hi} Neutrophils was assessed and displayed as parts-of-whole of the live cell population. Statistical significance was determined using an unpaired Students t-test ($p<0.05$*). Data represent mean ± SEM (n = 14-15 per group).

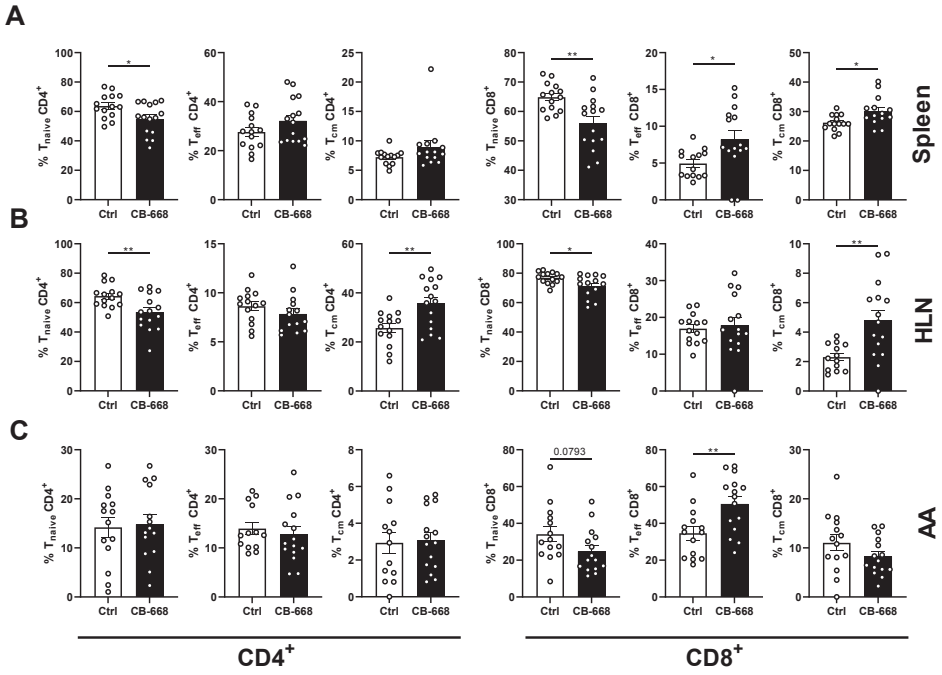

Figure 3. IL4I1 inhibition leads to a shift from naïve to effector or central memory CD4⁺ and CD8⁺ T cells (A) Naïve T cell (T_{naive}; $CD44^{-}CD62L^{+}$), effector T cells (T_{eff}; $CD44^{+}CD62L^{-}$) and central memory T cell (T_{cm}; $CD44^{+}CD62L^{+}$)(of live CD4 or CD8 cells) populations in the spleen, **(B)** HLN and **(C)** aortic arch (AA). Statistical significance was determined using an unpaired Students t-test ($p<0.05$*, $p<0.01$**). Data represent mean ± SEM (n = 14-15 per group).

Subsequently, we assessed whether CB-668 treatment also affected specific T helper (T_h) cell subsets. Whereas CB-668 treatment did not alter the percentages of T_h17 ($ROR\gamma T^{+}$ $CD4^{+}$) and regulatory T cells (T_{reg}; $Foxp3^{+}CD4^{+}$), it did lead to an increase of T_h1 cells ($Tbet^{+}CD4$ +98.0%) in the HLN and an increase in splenic cytotoxic $CD8^{+}$ T cells ($Tbet^{+}CD8^{+}$; T_c1 +33.0%)(**Fig. 4A-B**). A trend towards increased cytotoxic $CD8^{+}$ T cells was observed in the aortic arch as well (+37%; p=0.07) (**Fig. 4C**). Together, this suggests that IL4I1 inhibition induced a shift towards a central or effector T cell phenotype, mainly by an increase of T_c1 cells in the spleen and trend towards increase in the aortic arch and an increase in T_h1 cells in the HLN.

Inhibition of IL4I1 does not affect lesion development

Innate as well as the adaptive inflammatory immune responses are known to drive the development and progression of atherosclerosis. Because IL4I1 inhibition has been reported to affect these immune responses, we have examined whether IL4I1 inhibition could affect initial atherosclerosis development. Oil-red-O staining of aortic

root sections did not reveal any differences in both plaque size (Ctrl 169788±22661 μm^2 vs CB-668 184507±24990 μm^2) and vessel occlusion (Ctrl 16.9±2.0% vs CB-668 19.2±2.5%)(**Fig. 5A**). Further analysis of the plaque composition revealed that CB-668 treatment also did not affect the monocyte/macrophage positive area (Ctrl 26975±4382 μm^2 vs CB-668 24055±4289 μm^2) or macrophage content (Ctrl 56.4±3.2% vs CB-668 55.1±3.0%)(**Fig. 5B**). In addition, inhibition of IL4I1 did not have an effect on the collagen area (Ctrl 7596±1665 μm^2 vs CB-668 7695±1662 μm^2) or relative collagen content (Ctrl 21.8±2.2% vs CB-668 22.0±1.9%)(**Fig. 5C**). These data indicate that CB-668 treatment did not affect plaque development and composition and therefore does not lead to an increased risk of atherosclerosis in LDLr-/- mice.

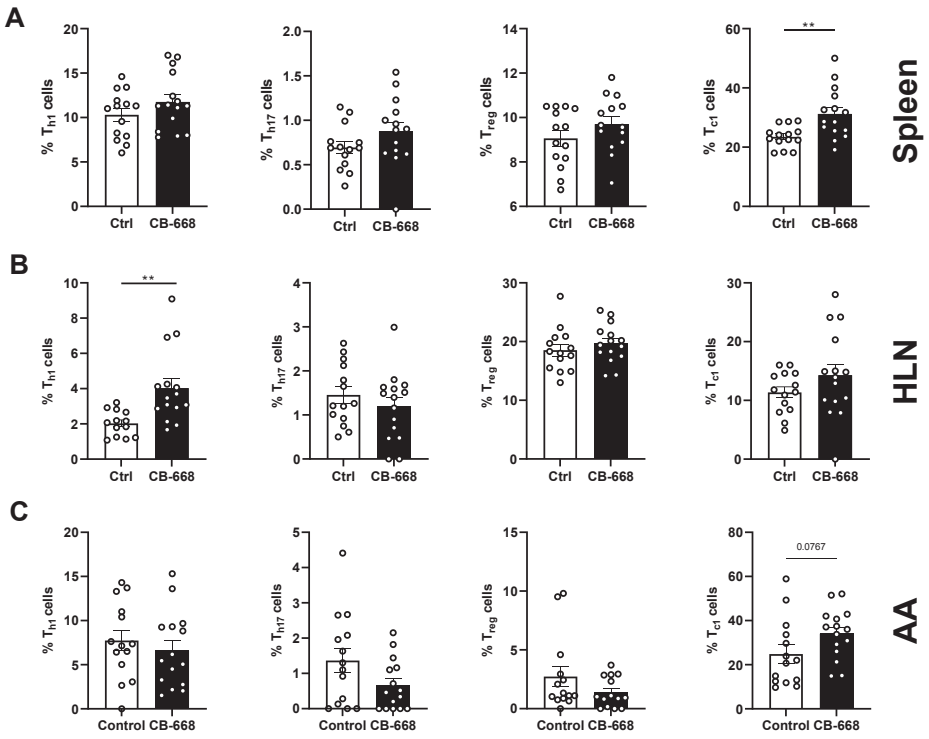

Figure 4. Blockage of IL4i1 does not have major effects on the CD4$^+$ and CD8$^+$ T cell subsets. **(A)** The percentages of T_h1 (CD4$^+$Tbet$^+$), T_h17 (CD4$^+$RORγT$^+$), regulatory T cells (T_{regs}; CD4$^+$FoxP3$^+$) and cytotoxic T cells (T_c1; CD8$^+$Tbet$^+$)(of live CD4 or CD8 cells) were determined in **(A)** the spleen, **(B)** HLN and **(C)** aortic arch (AA). Statistical significance was determined using an unpaired Students t-test ($p<0.05$*, $p<0.01$**). Data represent mean ± SEM (n = 14-15 per group).

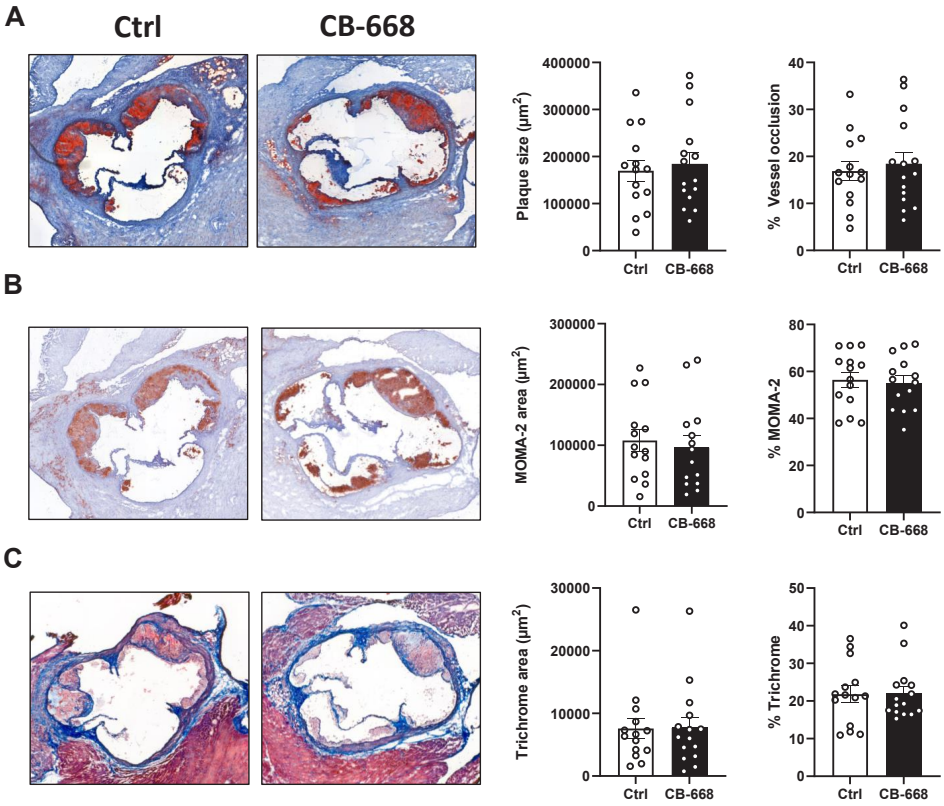

Figure 5. Inhibition of IL4I1 does not affect initial lesion development Representative pictures of aortic root atherosclerotic lesions stained with **(A)** Oil-red-O to visualize neutral lipids and quantification of the lesion size and vessel occlusion. **(B)** MOMA-2 antibodies for quantification of the absolute and relative macrophage content, and **(C)** Masson trichrome for quantification of the absolute and relative collagen content. Significance was determined using a Student's T-test. Individual data points and mean ± SEM (n = 14-15 per group).

Discussion

IL4I1 has been shown to be a promising immunological target for cancer treatment, as blockade induces cytotoxic $CD8^+$ T cell responses.[7] Cancer and atherosclerosis share multiple immunological pathways contributing to both diseases in different manners. Specific T cell responses are for example beneficial in cancer, but not in atherosclerosis. Moreover, scRNAseq data from human and murine atherosclerosis has shown that IL4I1 is highly expressed in myeloid cell subsets.[29] Intervening in the IL4I1 pathway could thus also affect this disease.

Our data demonstrate that IL4I1 inhibition by CB-668 treatment did not induce major effects on general immune cells on a systemic level. However, IL4I1 inhibition did lead to a shift from naïve to effector or central memory T cells, mainly by an increase in cytotoxic $CD8^+$ T cells in the spleen and a trend towards increased cytotoxic $CD8^+$ T cells the aortic arch. These results are in line with the effects observed in cancer, in which IL4I1 inhibition induced an accumulation of IFNγ-producing T_{c1} $CD8^+$ T cells in the tumor, which normally are activated through this pathway and impede tumor escape.[5,7] This suggests that IL4I1 secretion might be used as an immune evasion strategy by the tumor. Whereas cytotoxic $CD8^+$ T cells are beneficial in cancer, the role of these T cells in atherosclerosis is ambiguous. Both the phenotype induced by local stimuli present in the atherosclerotic lesion as well as the stage of atherosclerosis determines whether $CD8^+$ T cells exert pro-atherogenic or athero-protective functions.[30] Although the shift of naïve to effector or central memory T cells was abundant in the $CD8^+$ T cells, we did also observe this shift in $CD4^+$ T cells in the HLN. Olsen *et al*. has shown that decreased naïve and increased memory $CD4^+$ T cells are linked to subclinical atherosclerosis.[31] Although we observed increased effector and central memory $CD4^+$ T cells, we could not confirm the previously described *in vitro* effects of IL4I1 on T_{h17} and T_{regs} in our *in vivo* atherosclerosis model.[12,13] Nevertheless, we did see an increase in T_{h1} $CD4^+$ T cells in the HLN which, which corresponds with a previous *in vitro* study highlighting IL4I1 as inhibitor of T_{h1} proliferation upon interaction with IL4I1 secreting antigen presenting cells.[24]

IL4I1 inhibition results in a more systemic pro-inflammatory environment and a slight increase in T cell activation that might contribute to atherosclerosis development and progression. However, we did not find any differences in atherosclerotic plaque development or composition. The findings in our murine model suggest that treatment with the IL4I1 inhibitor to repress cancer might not induce the risk of atherosclerosis initiation. It, however, remains to be established if CD-668 treatment affects the progression or stability of advanced lesions, as human atherosclerosis is often asymptomatic and diagnosed at an advanced stage.[32] T cells predominantly play a role in the chronic inflammatory response leading to the formation of advanced atherosclerotic lesions.[33] Furthermore, in human atherosclerotic lesions, T cells make up the biggest and most diverse leukocyte population.[34] T cells constitute more than half of all leukocytes in human plaques, whereas in initial murine plaques T cells are less common and account for only between 6% to 25% of total leukocytes.[34–37] In advanced murine atherosclerotic lesions, the percentage T cells of total leukocytes is reported to increase.[34]

While murine models do not perfectly mimic human disease pathophysiology, they remain useful in the portrayal of immune cell-mediated immune responses in atherosclerosis.[39] In addition, comparison of coronary artery disease associated pathways from human genome wide associated studies with mouse studies has shown that the pathways involved in the immune system are highly overlapping.[40]

It is therefore likely that the increased pro-inflammatory $CD4^+$ and $CD8^+$ T cell subsets upon treatment with CB-668 in our study, could potentially affect advanced stages of atherosclerosis to a bigger extent. Furthermore, it would be of interest to examine if treatment with CB-668 in cancer patients with cardiovascular disease could lead to changes in the atherosclerotic plaque microenvironment to confirm in human subjects that this anticancer therapy is not correlated with an increased risk of cardiovascular disease development.[41]

In conclusion, our study demonstrated that inhibition of IL4I1 results in a more pro-inflammatory immune environment but does not affect initial atherosclerotic lesion development in $LDLr^{-/-}$ mice. Therefore, this study highlights that the use of CB-668 as an anti-cancer treatment does not lead to increased risk of atherosclerosis initiation. Still, further research will have to confirm whether inhibition of IL4I1 using CB-668 does not add to the risk of atherosclerosis progression and plaque destabilization, for example by including carotid intima-media thickness tests and the occurrence of major adverse cardiovascular events in clinical trial design.

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