Mast cells as immune regulators in atherosclerosis

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Chapter 7

Mast cell depletion in advanced atherosclerosis does not induce plaque regression

Manuscript in preparation

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Abstract

Regression of atherosclerotic plaques is a continuous challenge in the prevention of acute cardiovascular syndromes. While many pharmacological approaches have proven successful in arresting progressing atherosclerosis, it is still complicated to reverse the process of plaque formation and reduce pre-formed stenotic tissue in arteries. Mast cells are plaque components, known for their pro-inflammatory role during atherosclerosis progression. Numerous studies have shown that this cell type accumulates inside atherosclerotic plaques and their activation, and subsequent degranulation, can enhance plaque development, while their stabilization hampers plaque progression. However, mast cells have also been implicated in atherosclerosis regression and in this project, we aimed to determine whether mast cell depletion can induce plaque regression. We made use of the DTR-conditional depletion model, to abolish the presence of mast cells in RMB-LDLr$^{-/-}$ mice while simultaneously altering the food content from high-fat to lipid-low diet. We did not observe any differences in atherosclerotic plaque size, as well as macrophage or smooth muscle cell content, indicating that mast cell depletion in this setup did not induce plaque regression. The absence of mast cells however, did markedly reduce the amount of infiltrating neutrophils and collagen content in the plaque area. In conclusion, depletion of mast cells is not an optimal therapeutic target for the reversion of pre-established atherosclerosis.

1. Introduction

Acute cardiovascular events, generally caused by atherosclerotic plaque progression in large arteries, are identified as the major cause of death in Western societies[1]. Atherosclerosis progresses at a chronic, yet silent, fashion that can remain undetected for years; until the plaque reaches an advanced stage, where it may either destabilize, or rupture, leading to a myocardial infarction or a stroke[2]. Up to date, the only therapeutic method available to eradicate an atherosclerotic plaque, is surgical intervention, for instance through coronary angioplasty with or without stent-placement, which can however lead to secondary (re)stenosis[3]. A possible reduction of the atherosclerotic plaque size, upon induction of plaque regression *via* non-invasive therapy, would help in the struggle against unwanted side-effects. From a pharmacological perspective, statin use can decrease the (very) low density-lipoprotein content, providing plaque stability. Still, lipoprotein fraction modulation alone, without taking the inflammatory context into account, is not able to induce plaque regression[4,5]. Previously, it has been shown in mice, that lowering of the lipid diet content in combination with anti-inflammatory treatment, can induce regression of atherosclerotic plaques[6,7]. However, until now no such method has been successfully applied on human subjects[8]. In a recent experimental model study, successful plaque regression was achieved in LDL-receptor$^{-/-}$ mice by targeting the adaptive immune response, through the inhibition of the co-stimulatory pathway triggered by OX40 and OX40-ligand[7]. Interestingly, this intervention affected also the mast cell content of the atherosclerotic plaque, which appeared reduced in number and activation status. This event was of particular importance, considering the role that mast cells play in atherosclerosis.

Mast cells accumulate within the plaque tissue in the intima and adventitia[9] and promote disease progression and plaque destabilization[10]. The adverse effect of mast cells is mainly attributed to their degranulating nature, which leads to the release of pro-inflammatory mediators[11] such as cytokines and proteases. Our group has previously found that apoE$^{-/-}$ mice develop increased plaques upon systemic mast cell activation, an effect which was reversed by using a mast cell stabilizer[12]. Furthermore, mast cell deficient LDLr$^{-/-}$/Kit$^{W-sh/W-sh}$ mice show lower plaque size and lipid deposition, as compared to control LDLr$^{-/-}$ mice[13]. Importantly, in a clinical study where human plaque specimens from carotid arteries were analysed, mast cells were found to be the only inflammatory cell type that positively correlated with increased intra-plaque haemorrhage and future cardiovascular events[14]. For these reasons, mast cell targeting appears to be significantly interesting as a possible method to control atherosclerosis.

Therefore, in this study we aimed to assess the therapeutic potential that mast

cell deficiency may exert, in combination with a lipid lowering diet.

2. Materials & Methods

2.1 Animals

All animal work was performed according to the guidelines of the European Parliament Directive 2010/63EU and all experimental work was approved by the Animal Ethics committee of Leiden University. The mice were bred and housed in the facility under *ad libitum* water and food supplies. The red mast cell and basophil mouse strain (RMB mice, official name: B6.Ms4a2^{tm1Mal}) was provided by the laboratory of dr. P. Launay INSERM U1149, Paris, France[15]. These mice express the simian diphtheria toxin receptor (DTR)[16] under control of the FcεR1β gene promoter, a receptor which is specifically expressed by mast cells[17]. Injection of diphtheria toxin (DT) will therefore induce apoptosis of all FcεR1β expressing cells. RMB mice were subsequently crossed with LDLr$^{-/-}$ mice (Jackson Laboratories, USA) to obtain atherosclerosis-prone RMB-LDLr$^{-/-}$ mice. LDLr$^{-/-}$ mice were included in the study as a control group (control-LDLr$^{-/-}$) for DT-mediated side effects[18].

2.2 Atherosclerosis

Male RMB-LDLr$^{-/-}$ (n=12/group), and control-LDLr$^{-/-}$ (n=6) of average age 19-20 weeks were fed a Western type diet (WTD) [0.25% cholesterol, 15% cocoa butter; Special Diet Services, Essex, UK] for 10 weeks, to develop atherosclerosis. After the 10-week WTD period, a baseline group of mice was sacrificed for assessment of the high-lipid diet effects. The remaining groups of mice were placed on a low-lipid (chow) diet for a subsequent period of 6 weeks. Simultaneously the DT/PBS injection scheme was initiated. Diphtheria toxin was injected intraperitoneally (0.5mg/mL) in order to achieve specific depletion of all FcεR1β-expressing cells. DT was injected 3 times within the first week of the depletion period, for optimal cell clearance (boost period). After the first week, DT was injected at a maintenance fashion every 13 days. Before the baseline sacrifice and DT-administration period, all mice were randomized according to their age, weight and total serum cholesterol levels. All remaining mice were monitored for body weight changes throughout the compound administration period and until the endpoint of the experiment. After the 6-week chow period all mice were anesthetized by subcutaneous injections using ketamine (100mg/mL), sedazine (25mg/mL) and atropine (0.5mg/mL) and their vascular system was perfused by PBS administration at a continuous low flow *via* heart puncture in the left ventricle.

2.3 Total cholesterol analysis

Serum was obtained upon separation of blood, collected through the tail-vein. At the experimental endpoint, serum was collected from blood collected *via* the eye vein. Serum separation was achieved by centrifugation at 8.000 rpm for 10 minutes. Total cholesterol levels in the serum were assessed using an enzymatic colorimetric assay with Precipath as an internal control (Roche Diagnostics).

2.4 Immunohistochemistry

The hearts of all mice were fixed in formalin for 24 hours and subsequently dissected below the atria. All hearts were embedded in O.C.T. compound (Sakura) and sectioned horizontally to the aortic axis and towards the aortic arch. Upon identification of the aortic root, defined by the

trivalve leaflets, 10μm sections were collected. Mean plaque size (in μm^2) was calculated for three sequential sections, displaying the highest plaque content, using an Oil-red-O staining (Sigma Aldrich), which is specific for neutral lipid marking. Mast cell and neutrophil content was manually quantified following a Naphthol AS-D chloro-acetate esterase kit (Sigma Aldrich). The distinction of neutrophils from mast cells was performed according to their cellular characteristics, specifically the pink granular cytoplasm and lobular nucleus. Plaque macrophages were stained with a MOMA-2 antibody at a 1:1000 concentration (rat IgG2b, Serotec Ltd.). Smooth muscle cell quantification was performed following a staining with α-smooth muscle actin (αSMA) at a concentration of 1:1000 (Abcam). Intraplaque collagen was assessed using a Masson's trichrome staining kit (Sigma Aldrich). T cell content in the aortic root was determined after a CD3 staining at a 1:150 concentration (clone SP7, ThermoScientific). All analyses were performed on a Leica DM-RE microscope and staining quantification levels were measured using a Leica QWin software (Leica, Imaging Systems, UK) and through blinded independent analysis.

2.5 Flow cytometry

Blood, spleen and heart lymph node tissues were harvested for further analysis of their immune cell populations. To obtain blood leukocytes, red blood cell lysis was performed with a solution of 0.1mM EDTA, 10mM $NaHCO_3$, 1mM NH_4Cl, pH=7.2. Splenocytes as well as cells from the draining heart lymph nodes were obtained upon organ grinding through a 70μm filter and subsequent erythrocyte lysis. All cells were stained with fluorescently labeled antibodies against the markers of interest (**Table 1**). All antibodies were used at a concentration of 0.1μg/sample. The flow cytometry experiments were performed with a FACS Canto II and data were analyzed using a FlowJo software.

Antibody	Fluorochrome	Clone	Company
CD11b	eFluor 450	M1/70	Ebioscience
Ly6G	PE	1A8	BD Biosciences
NK1.1	FITC	PK136	Ebioscience
FcεR1α	PercP Cy5.5	MAR-1	Biolegend
CD117	APC	2B8	Ebioscience
CD4	eFluor 450	GK1.5	Ebioscience
CD8a	PercP	53-6.7	BD Biosciences

Table 1: Flow cytometry antibodies

2.6 Statistics

Data collected were analyzed for normal distribution and are presented as mean±SEM. For the analysis of one variable between more than two groups a one-way ANOVA was performed, with a Bonferroni post-test for multiple comparisons. For the analysis of one variable between two individual groups, a 2-tailed Student's *t*-test was used, with the Bonferroni post-test for multiple comparisons. The probability for all tests was set to 0.05 with lower values considered significant ($P<0.05$).

3. Results

3.1 Conditional depletion of mast cells using the Diphtheria toxin mouse model

In this study, we aimed to evaluate effects of mast cell depletion on atherosclerosis, in combination with a lipid-lowering diet. After a 10-week period of WTD, a baseline group of RMB-LDLr$^{-/-}$ mice was sacrificed, to determine baseline plaque characteristics. The remaining groups of mice were switched to a chow diet while injected with DT or PBS for a period of 6 weeks. An additional group of LDLr$^{-/-}$ mice served as controls to estimate possible toxic effects of diphtheria toxin. Through the injection period we monitored all three groups of mice (PBS, DT & control) for possible changes in body weight. However, we did not observe any substantial differences in body weight development between the control group and the PBS or DT groups (**Figure 1A**). This indicated that administration of DT in such fashion does not elicit any toxic side effects and hence we proceeded with comparing the baseline, PBS and DT groups. To ensure that the diet-reversal indeed reduced the circulating lipid content, we measured the total cholesterol levels in the serum at the end of the study. While the baseline group showed high total serum cholesterol, the PBS and DT groups displayed a significant reduction in their circulating cholesterol levels, as expected upon diet switch (**Figure 1B**, $P<0.0001$). We next aimed to assess the efficiency of DT to deplete mast cells, by specifically examining the presence of mast cells in the perivascular tissue of the aortic root in the mouse hearts; an area prone to develop atherosclerosis. Upon manual quantification of cell numbers, we observed that mast cells were absent in the DT group, as compared to the baseline and PBS groups of mice (**Figure 1C**, DT: 0.77±0.29 cells per section vs PBS: 12.83±1.94 cells per section, $P<0.0001$; vs baseline: 9.52±1.65 cells per section, $P=0.0005$). Representative pictures of the Naphthol AS D-chloroacetate esterase staining performed in the aortic root of the hearts demonstrated that in the DT group all mast cells were virtually absent (**Figure 1D**).

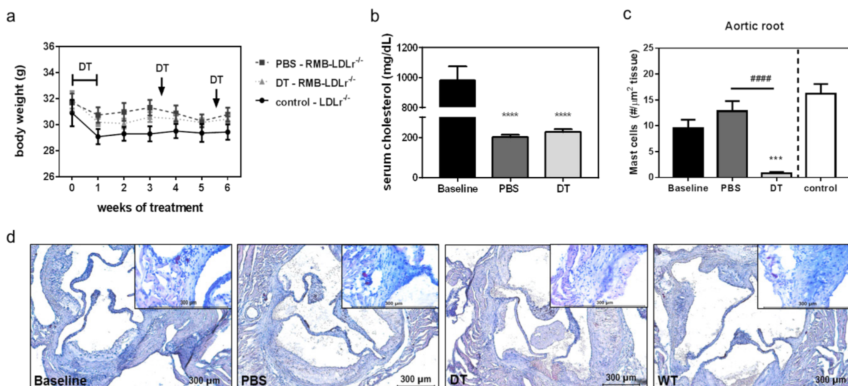

Figure 1: RMB-LDLr$^{-/-}$ conditional depletion using diphtheria toxin resulted in the abolishment of mast cells in the aortic root of the hearts. (A) Diphtheria toxin (DT) i/p injections (0.5mg/mL) did not evoke unspecific effects on the body weight of the DT or control group of mice, as compared to the PBS group. **(B)** Reversal from WTD to chow diet significantly reduced total cholesterol levels in the serum in both groups of mice (PBS & DT) as compared to the baseline group. **(C)** The number of mast cells in the aortic root of the heart after 6 weeks of DT-specific mast cell depletion was diminished, in comparison to the PBS group. **(D)** The number of mast cells was quantified with the use of a Naphthol AS-D chloro-acetate esterase staining; representative pictures of 10μm sections in the aortic root of the heart are shown. All values (n=12/grp) are depicted as mean±SEM. ****P<0.001, ****P<0.0001* compared to the baseline group; *####P<0.0001* compared to the PBS-treated group.

3.2 Mast cell depletion does not alter plaque size upon chow diet

Upon establishing the successful depletion of mast cells inside the atherosclerotic tissue, we moved on to characterize the impact of their absence in atherosclerotic plaque size. We used an ORO staining to visualize the lipid content of the plaque (**Figure 2A**) in the aortic root. However, quantification of the plaque size did not display any significant differences among the three groups of mice (**Figure 2B**, DT: $26*10^4 \pm 3*10^4$ μm^2, PBS: $30*10^4 \pm 2*10^4$ μm^2 baseline: $29*10^4 \pm 5*10^4$ μm^2, *P>0.05***)**. Subsequently, we assessed the macrophage content of the plaque with a MOMA-2 staining (**Figure 2C**). Diet reversal from WTD to chow revealed a sharp reduction in the intraplaque macrophage content of both the PBS and DT groups **(Figure 2D**, P<0.0001**)**. Additionally, analysis of the plaque T cell content in the hearts was performed by an immunohistochemical staining against membrane protein CD3 (**Figure 2E**). We observed a significant decrease in T cell numbers in the plaques of the DT group as compared to the baseline; yet no difference was observed among DT and PBS mice (**Figure 2F**, DT: 12.2±1.4 cells vs baseline: 39.9±10.3 cells, P=0.012).

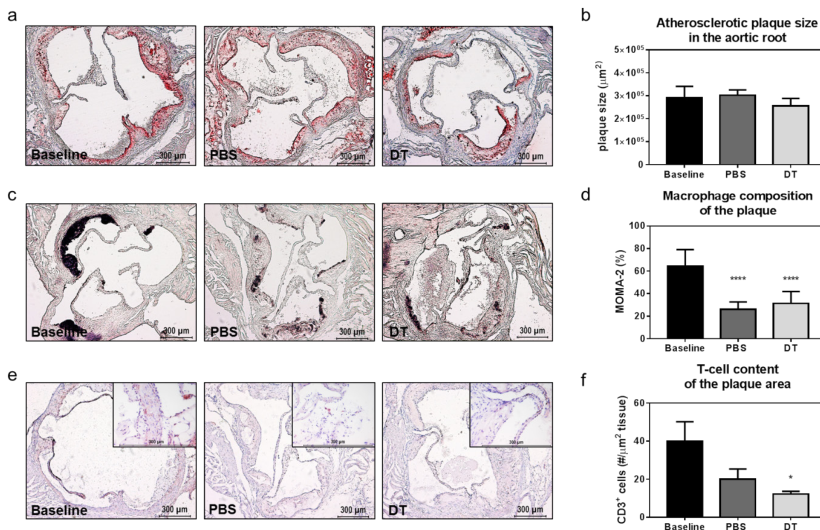

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Figure 2: Conditional depletion of mast cells upon chow diet did not affect atherosclerotic plaque size, macrophage content or T cell number in the aortic root of the heart. (A) An Oil-Red-O staining in the aortic root was performed to determine the atherosclerotic plaque size among the groups; representative pictures are shown. **(B)** No significant difference was observed between the Baseline, PBS and DT groups. **(C)** The macrophage content of the atherosclerotic plaques was quantified using a MOMA-2 staining; example pictures of the staining are depicted for each group. **(D)** Diet reversal from WTD to chow, resulted in a substantial reduction in the macrophage plaque content, with no difference observed between the PBS and DT groups. **(E)** The number of T cells was assessed using a staining against CD3; representative pictures are shown. **(F)** The number of T cells present in the aortic root of DT mice was significantly reduced as compared to the baseline group, however no significant changes were observed between the DT and PBS groups. All values (n=12/grp) are depicted as mean±SEM. **P<0.05, ****P<0.0001* compared to the baseline group.

3.3 Atherosclerotic plaques appear less stable upon mast cell depletion

The collagen deposition, as measured by a Masson's Trichrome staining, in the plaques of the DT mice was found significantly reduced in comparison to the PBS group of mice (**Figure 3A**,**3B**, DT: $88*10^4 \pm 13*10^4$ µm^2 vs PBS: $136*10^4 \pm 17*10^4$ µm^2, P=0.044). No significant changes were detected when comparing the baseline and DT mice. Of note, no difference was observed in the necrotic core area of the plaques between the different groups of mice (DT:$53*10^4 \pm 11*10^4$ µm^2, PBS: $68*10^4 \pm 9*10^4$ µm^2, baseline: $58*10^4 \pm 15*10^4$ µm^2). We also determined the smooth muscle cell (SMC) content of all groups (**Figure 3C**), however, this did not differ between the three groups of mice (**Figure 3D**).

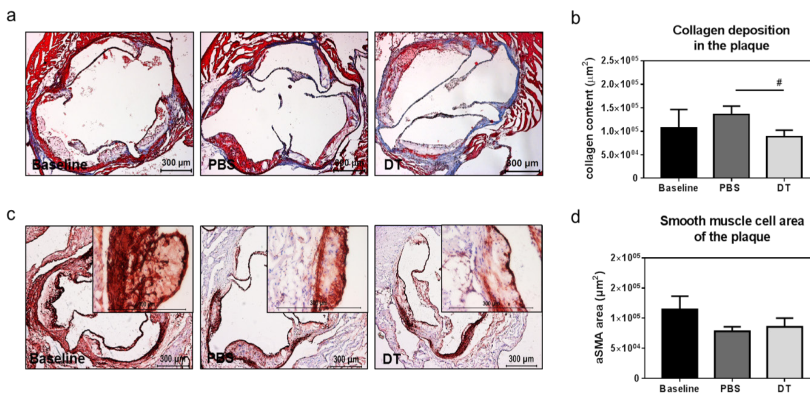

Figure 3: Collagen deposition in the aortic root of the hearts was reduced upon mast cell depletion. (A) The collagen content of the aortic root was quantified using a Masson's Trichrome staining; representative pictures are presented. **(B)** The deposition of collagen inside the atherosclerotic plaques of DT mice was found significantly reduced as compared to the PBS group of mice. **(C)** Quantification of the smooth muscle cell content in the plaque area of the heart was performed using an αSMA staining; representative pictures per group are shown. **(D)** No difference was observed between the PBS and DT groups in the total smooth muscle cell area of the aortic root. All values (n=12/grp) are depicted as mean±SEM. $^{\#}$*P<0.05* compared to the PBS treated group.

3.4 The neutrophil content is negatively affected by the absence of mast cells in the plaque

As mast cells were previously established to induce neutrophil chemotaxis[19], we analysed the neutrophil content in the circulation and the atherosclerotic plaque of all mice. We did not observe any differences in the percentage of circulating neutrophils, defined as $Ly6C^+/CD11b^+Ly6G^-/NK1.1^-$ cells (**Figure 4A**). However, in line with previous data from our group[19], we observed a significant reduction in the intraplaque neutrophil numbers upon mast cell depletion, as compared to both the PBS and baseline groups (**Figure 4B**, DT: 5.4±1.1 cells vs PBS: 17.7±2.3 cells, P<0.0001; vs baseline: 16.3±1.9 cells, P=0.0005).

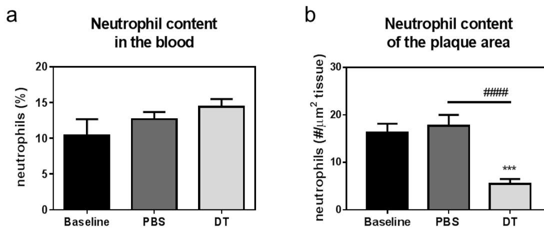

Figure 4: The neutrophil content of the aortic root was significantly diminished following the depletion of mast cells upon chow diet. (A) The proportion of neutrophils present in the circulation was not altered among the PBS and DT groups. **(B)** The number of neutrophils accumulating in the aortic root of DT treated mice was highly reduced during mast cell depletion, as compared to the PBS and baseline groups. All values (n=12/grp) are depicted as mean±SEM. ****P<0.001* compared to the baseline group; *####P<0.0001* compared to the PBS-treated group.

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3.5 Mast cell absence reduces the levels of $CD4^+$ and $CD8^+$ T cells in the heart lymph nodes

Since the inflammatory cell content of the atherosclerotic plaque is in direct communication with the proximal heart lymph nodes (hLN)[20], an area rich in T cells, we aimed to analyze the local $CD4^+$ and $CD8^+$ T cell content using flow cytometry. The $CD4^+$ percentage in the draining hLNs was significantly reduced in the DT as compared to the PBS group (**Figure 5A**, DT: 17.75±0.59 % as compared to PBS: 20.69±0.63 %, P=0.003). However, no difference was observed among the percentages of $CD4^+$ T cells circulating in the blood (**Figure 5B**). Along the same line, $CD8^+$ T cells appeared at a substantially lower percentage in the hLNs of the DT mice as compared to the PBS mice (**Figure 5C**, DT: 15.6±0.9 % compared to PBS: 18.9±0.9 %, P=0.020). Finally, no differences were observed among the three groups in the levels of circulating $CD8^+$ T cells (**Figure 5D**).

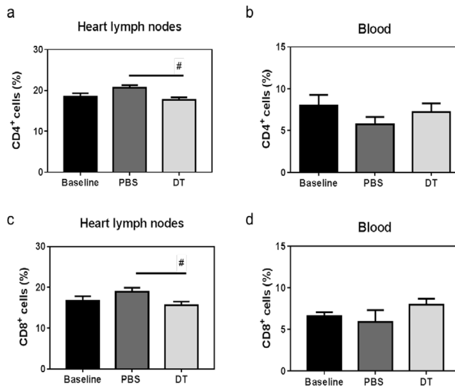

Figure 5: CD4⁺ and CD8⁺ T cell levels were reduced in the heart lymph nodes of mast cell depleted mice. (A) The percentage of CD4⁺ T cells in the heart lymph nodes was significantly reduced in the DT group as compared to the PBS. **(B)** The amount of circulating CD4⁺ T cells did not differ between the groups of mice. **(C)** The CD8⁺ T cell percentage in the heart lymph nodes was decreased in the DT group as compared to the PBS. **(D)** Blood CD8⁺ T cells were not altered upon DT treatment, in comparison to the other two groups of mice. All values (n=12/grp) are depicted as mean±SEM. $^{\#}$*P<0.05* compared to the PBS-treated group.

4. Discussion

In the present study, we aimed to assess the possible therapeutic effect of mast cell depletion on pre-existing atherosclerotic plaques, in the presence of a lipid lowering diet. We confirmed that the switch from WTD to chow diet reduced the total cholesterol levels in the circulation and furthermore, that DT treatment depleted the mast cell content from the tissues, including the aortic root of the heart. Despite the successful depletion of mast cells, we did not detect regression in the atherosclerotic plaque size of the aortic root; indicating that mast cell depletion in this context is not relevant as therapeutic means for the regression of atherosclerosis.

Macrophages were markedly affected by the diet reversal in both chow-fed groups of mice, similarly as described previously[7]; but the absence of mast cells did not have any additional effect on the plaque macrophage content.

In contrast, collagen deposition in the plaques was found reduced in the mast cell depleted mice. At first glance this seems surprising, considering that mast cell chymase inhibition has been reported to increase the intraplaque collagen content in apoE$^{-/-}$ mice[21]. In addition, mast cell degranulation products have been implicated in the degradation of extracellular matrix components[11], with mast cell deficient LDLr$^{-/-}$/Kit$^{W-sh/W-sh}$ mice showing higher collagen levels as compared to their wild-type counterparts[13]. In line with the above, mast cell stabilization with tranilast was reported to increase collagen deposition in a diabetes study[22]. However, mast cell tryptase has been noted to increase collagen synthesis in renal fibrosis[23]. This suggests that the level and quality of degranulating mast cell products can have a substantially different effect on the collagen deposition process. Furthermore, mast cells have been proven to have a regenerative capacity under tissue repair conditions. For instance, mast cell infiltration and degranulation mediators in the myocardium has been proven crucial in extracellular

matrix remodelling[24], during the healing phase following an infarct episode. In contrast to the observed collagen reduction, the smooth muscle cell content of the plaques was not altered upon mast cell depletion. While this result may seem inexplicable, smooth muscle cell α-actin is a cytoskeletal protein expressed in terminally differentiated arterial smooth muscle cells, and not in the SMCs undergoing proliferation[25]. However, it is the proliferative state of SMCs that is characterized by collagen production inside the atherosclerotic plaques[26]. The collagen reduction observed here may, thus, be linked primarily to proliferative smooth muscle cells and, therefore, not directly determined by α-SMA.

The neutrophil intraplaque content was also found highly reduced upon mast cell depletion. Mast cell tryptase has been previously reported to attract neutrophils in the surrounding tissue, through endothelial mediated cytokine release[27]. Furthermore, recently our group has observed that neutrophil infiltration within atherosclerotic plaques is greatly affected by mast cells and particularly through their release of chemokine CXCL1 that can direct neutrophils inside the plaques by acting on their CXCR2 receptor[19]. Moreover, a close relationship between mast cells and neutrophils was also reported in rheumatoid arthritis[28].

In addition, the total T cell population appeared significantly reduced in the hearts of chow-fed mice that did not contain mast cells, as compared to WTD-fed mice with mast cells. Lately it is being proposed that mast cells can interact with T cells in a direct fashion[29]. In the case of atherosclerosis, stabilization of mast cells in $LDLr^{-/-}$ mice has been reported to reduce $CD4^+$ T cell numbers in the plaques[30], whereas the intraplaque amount of mast cells was positively associated with the number of $CD8^+$ T cells in human atherosclerotic specimens[31]. Therefore, it could be that the physical presence of mast cells is needed, to a certain extend, for T cells to infiltrate or survive within the atherosclerotic plaques. Interestingly, in the draining heart lymph nodes, both $CD4^+$ and $CD8^+$ T cell populations appeared markedly reduced in the absence of mast cells, suggesting that mast cells may shape the immune response of secondary lymphoid organs. However, no effect was observed in the circulating rate of both $CD4^+$ and $CD8^+$ T cells. The fact that the absence of mast cells seems to affect mainly the lymph node population that is adjacent to the atherosclerotic site, and less the systemic T cell response, indicates that the observed effects are local and atherosclerosis specific.

Overall, the atherosclerotic plaques of mice upon lipid lowering appeared to contain similar amounts of macrophages, T cells and smooth muscle cells, regardless of whether mast cells were present or not. However, the absence of mast cells significantly affected the amount of collagen deposited in the atherosclerotic area as well as the local neutrophil infiltration. In this experimental setup, mast cell depletion did not induce atherosclerosis regression upon a lipid-lowering diet. It is worth mentioning

that intervention on the $CD3^+$ axis has been previously shown to induce atherosclerosis regression[6]. Be that as it may, in regressing plaques it is equally crucial for foam cells to emigrate in the draining lymph nodes[32], where they can act on the local T cell response. In this study, we saw that while mast cells were able to shape the $CD4^+$/$CD8^+$ lymph node content they did not regulate macrophage emigration from the plaques, and therefore plaque regression. Ultimately, even though mast cells seem to firmly assist atherosclerosis regression[7] they do not seem able to trigger it. Nevertheless, the above data indicate that although mast cells are regarded as classical pro-inflammatory effectors, they may also have protective effects that favour plaque stabilization. Therefore, in atherosclerosis it may be optimal to refine individual pathways of mast cell action and selectively intervene on the adverse ones in order to successfully interfere in disease progression.

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