Mast cells as immune regulators in atherosclerosis

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Chapter 4

Hypercholesterolemia induces a mast cell - CD4⁺ T cell interaction in atherosclerosis

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Abstract

Mast cells are potent innate immune cells that aggravate atherosclerosis through the release of pro-inflammatory mediators inside atherosclerotic plaques. Similarly, $CD4^+$ T cells are constituents of the adaptive immune response and accumulate within the plaques following lipid-specific activation by antigen presenting cells. Lately it is being proposed that these two cell types can interact, upon request, in a direct manner. However, no indication of such an interaction has been previously investigated in atherosclerosis. In our study, we aimed to examine whether mast cells can act as non-classical antigen presenting cells in atherosclerosis, thereby shaping the $CD4^+$ T cell response. We observed that mast cells can increase their MHC-II expression under hyperlipidemic conditions both *in vivo* and *in vitro*. Furthermore, we showed that mast cells can present antigens *in vivo via* their MHC-II molecules. High-fat diet serum enhanced the expression of the co-stimulatory molecule CD86 on the mast cell surface *in vitro,* while co-culture of OVA-peptide loaded mast cells shifted the OT-II $CD4^+$ T cell response towards a T_{H1} cell subset. In accordance, the aortic T_{H1} cell content of atherosclerotic mice that lack mast cells was found reduced as compared to their wild-type counterparts. Importantly, we here established that mast cells in advanced human atherosclerotic plaques express HLA-DR, indicating that they are capable of antigen presentation inside human atherosclerotic tissues. Therefore, we are the first to show that mast cells may function as non-classical antigen presenting cells and directly modulate adaptive immunity in atherosclerosis.

1. Introduction

Atherosclerosis is a chronic, lipid-mediated, autoimmune disease of the medium and large arteries, which involves both innate and adaptive immune reactions[1]. The communication between the innate and adaptive immune response is achieved through professional antigen presenting cells, such as dendritic cells, that can specifically activate T lymphocytes with antigens taken up from the atherosclerotic plaque[2]. It has previously been established that during the progression of atherosclerosis, antigens from the oxidized low-density lipoprotein (oxLDL) molecules can elicit immune responses by activating an array of T cell subtypes, like the T_{H1} and T_{H2} cell types[3,4]. This happens inside secondary lymphoid tissues, such as the spleen and the local draining lymph nodes and requires three key-signals that involve presentation of the antigenic fragments. Antigen presentation is triggered by major histocompatibility complex I or II (MHC-I/II) and followed by a strong co-stimulatory response, in the form of proteins like CD86, along with secreted cytokines priming the T cell activation process[5]. In response, activated T cells subsets translocate to the atherosclerotic plaque and influence the disease outcome locally[6]. The differential T cell subset effect is partially attributed to the different cytokine milieu secreted by the two cell types. Specifically, T_{H1} cells, which are the most abundant type inside progressing atherosclerotic plaques, secrete large amounts of interferon-γ (IFNγ) while T_{H2} cells are found at lower numbers and release mainly interleukin-4 (IL-4), interleukin-5 (IL-5) and interleukin-13 (IL-13)[7,8]. $CD4^+$ T_{H1} cells have been proven to be proatherogenic[9], while T_{H2} cells appear to counteract for the T_{H1}-mediated effects[10] and through secretion of IL-5 show an atheroprotective function. On the other hand, IL-4 cytokine is reported to have a pro-atherogenic function with lower plaques developing upon its absence[11] making thus the exact role of T_{H2} cells in atherosclerosis hard to define[12]. Therefore, the diversity, in terms of quality and quantity of the T cell subsets, can exert differential effects and inversely affect the progression of atherosclerosis. Still, although activated T cells have in principle a pre-established fate upon entering the vessel wall, local costimulation in the plaques[13,14] may alter their behaviour.

Mast cells are tissue resident innate immune cells that have long been established to contribute to atherosclerosis progression[15]. These cells accumulate in the atherosclerotic tissue upon plaque development and, according to their classical mode of action, release pro-inflammatory mediators such as proteases and cytokines[16,17]. Yet, increasing evidence suggests that mast cells can also adopt an inducible antigen presenting phenotype that may influence the immune response inside tissues[18]. While mast cells are not classical antigen presenting cells like dendritic cells, peritoneal mast cells have been found to upregulate MHC-II in the presence of cytokines IL-4 and IFNγ[19], or inside the lymph nodes of mice that have been injected with lipopolysaccharide (LPS)[20].

Importantly, mouse bone marrow derived mast cells have been reported to present peptides of the model-antigen ovalbumin (OVA) to $CD4^+$ T cells[21]. Likewise, human cultured mast cells can directly present antigens to $CD4^+$ T cells through the human MHC-II equivalent protein HLA-DR[22,23]. However, up to date it is not known whether mast cells can directly influence the adaptive immune response during atherosclerotic plaque progression. Therefore, we aimed to study the antigen presentation capacity of mast cells to $CD4^+$ T cells in atherosclerosis.

2. Materials & Methods

2.1 Animals

All animal work was performed in compliance with the guidelines directed by the European Union Directive 2010/63EU and the Dutch Government. The experimental work was approved by the Animal Ethics committee of Leiden University. The animals were originally obtained by the Jackson Laboratories and subsequently bred in the local animal facility, while provided with food and water *ad libitum*. At experimental endpoint, mice were subcutaneously anesthetized with an injection mix of ketamine (100mg/mL), sedazine (25mg/mL) and atropine (0.5mg/mL) and their vascular system was perfused with PBS upon heart puncture in the left ventricle.

2.2 WTD-timecourse experiment

In order to examine the effect of WTD on the antigen presentation capacity of mast cells we performed a timecourse experiment (**Supplemental Figure 1A**). Male $LDLr^{-/-}$ mice (n=10/grp) of an average age 8-10 weeks old were placed on a Western-type diet (WTD) (0.25% cholesterol, 15% cocoa butter; Special Diet Services, Essex, UK) for either 4 or 8 weeks, or kept on a chow diet until the experimental endpoint. Mouse peritoneal cells were collected by peritoneal lavage, using 10mL PBS, to study the mast cell peritoneal population according to their characteristic expression of markers FcεRIα and CD117. Notably, the peritoneal mast cell population was also examined for the expression of the myeloid cell marker CD11c, to ensure that the observed population was not $MHC\text{-}II^+$ dendritic cells or macrophage foam cells, as these cells have been previously reported to express inducible Fcε-receptors in atopic dermatitis, allergy and inside atherosclerotic plaques[24,25]. The para-aortic lymph nodes of these mice were isolated at the experimental endpoint and fixated in formalin for 24 hours. Subsequently, all lymph nodes were embedded in OCT medium (Sakura) and 6μm cryosections were collected. Mast cell detection was performed using a Naphthol AS-D chloro-acetate esterase kit (Sigma Aldrich). The T cell population in the para-aortic lymph nodes was detected through antibody staining against CD3 at a 1:150 concentration (clone SP7, Thermoscientific). Determination of all mast cell and $CD3^+$ T cell numbers was performed by blinded and independent manual quantification of 2 collected sections per mouse. Representative pictures were obtained using a Leica DM-RE microscope (Leica Systems, UK).

2.3 Eα-presentation experiment

In order to study the ability of mast cells to present antigens through their MHC-II molecule we made use of the Eα-peptide presentation system whereupon an Eα-GFP peptide, after internalization and degradation, can be presented by the MHC-II molecules on the surface of APCs and is detected by specifically designed antibodies against Eα-fragments[26] we performed an Eα-peptide presentation experiment (**Supplemental Figure 1B**). Male $LDLr^{-/-}$ mice (n=14/

grp, average age:13 weeks) were placed on a WTD for 4 weeks. 24 hours prior to the end of the experiment one group was injected intraperitoneally with Eα-GFP[27], (100μg/mouse) while an additional group was administered sterile PBS as a control. Peritoneal cells isolated from Eα-treated mice were further stimulated *ex vivo* with Eα-GFP (200μg/mL) for either 3 or 24 hours and compared with peritoneal cells isolated from the PBS injected mice. The expression levels of an Y-Ae-Streptavidin antibody against Eα were determined by flow cytometry in comparison to the control-PBS expression.

2.4 Bone marrow derived mast cell stimulation

To investigate the antigen presentation function of mast cells *in vitro*, bone marrow derived mast cells (BMMCs) were cultured from isolated bone marrow cells in RPMI 1640 medium containing 25mM HEPES (Lonza) supplemented with 10% fetal calf serum (FCS), (1x) minimal essential medium non-essential amino-acids (MEM NEAA; Gibco), 60μM β-mercaptoethanol (Sigma), 1% mix of penicillin/streptomycin (PAA), 1% sodium pyruvate (Sigma) and 2% L-glutamine (Lonza) for 4 weeks with cytokine IL-3 (5 ng/mL; Immunotools). Mast cell purity was assessed by the expression of FcεRIα and CD117 and routinely found to be above 98%. Mature mast cells kept in culture for weeks 4 - 6 were used for all experiments. MHC-II induced mast cells (iMCs) were obtained by a mix of cytokines IL-4 (20ng/mL) and IFNγ (50ng/mL). Subsequently, to study the effect of hyperlipidemic serum, iMCs were stimulated for 24h with 10% serum isolated from either chow fed or 4 weeks WTD fed $LDLr^{-/-}$ mice and all conditions were renewed for an additional period of 24h. iMCs were additionally stimulated with 40% peritoneal fluid which was obtained by peritoneal lavage with 10ml PBS and after centrifugation at 1.600 RPM for 5 minutes. After a period of 24h all conditions were renewed accordingly. Cytokines IL-12p70, IFN-γ and IL-4 secretion was measured by ELISA (BD Biosciences) according to the manufacturer's protocol.

2.5 Mast cell-OT-II CD4⁺ T cell co-culture

To study the direct presentation of antigens by mast cells to CD4⁺ T cells *in vitro* we made use of the OVA presentation system. In a co-culture experiment, iMCs were repeatedly treated with 10% chow/WTD serum, as described above, in the presence of an OVA-peptide (10μg/mL) that can directly bind the MHC-II surface molecules and is directly presented to the T cell receptor (TCR) without requiring internalization. CD4⁺ T cells were isolated from genetically modified OT-II mice[28] using a magnetic-bead isolation kit (Miltenyi) and activated with αCD3 (1μg/mL) and αCD28 (0.5μg/mL) for 24h. Subsequently, after washing off the stimulants, mast cells and T cells were placed together in a co-culture system. Cells were seeded in quintuplicate per condition at a 1:1 ratio for 72 hours. At the end of the experiment, all samples were prepared for flow cytometry. For the proliferation assay radioactive ^{3}H-Thymidine (0.5μCi/well; PerkinElmer) was added to the samples after 48h and the incorporation rate was measured 15h later using a liquid scintillation analyzer (Tri-Carb 2900R). The levels of cytokines IL-4 and IFNγ were determined by ELISA (BD Biosciences) according to the manufacturer's protocol.

2.6 Mast cell deficiency experiment

To investigate the effect of mast cells on the T cell population in atherosclerosis, male mast cell deficient $apoE^{-/-}/Kit^{W\text{-}sh/W\text{-}sh}$ mice as well as control $apoE^{-/-}$ mice (**Supplemental Figure 1C**) of (n=16/grp, average age 16 weeks), were fed a WTD for 6 weeks. The intraplaque cell content was obtained through enzymatic digestion of the aortic tissue for 30 minutes at 37°C, with a mix of collagenase I (450u/mL; Sigma) and XI (250U/mL Sigma) and DNase (120U/mL; Sigma), hyaluronidase (120 U/mL; Sigma) and samples were further processed using flow cytometry.

2.7 Human specimens

To explore the ability of human intraplaque mast cells to present antigens, 20 anonymous atherosclerotic plaques were collected post-operatively from carotid or femoral artery endarterectomy surgeries, performed between July 2016 and December 2016 at the Haaglanden Medical Center (HMC), Westeinde, The Hague, The Netherlands. The handling of all human samples was performed in accordance with the "Code for Proper Secondary Use of Human Tissue". All atherosclerotic samples were processed in single cell suspensions, as described previously[29]. In short, cell suspensions from human atherosclerotic plaques were obtained upon digestion with collagenase IV (Gibco) and DNase (Sigma) for 2 hours at 37°C prior to single-cell separation through a 70μm cell strainer. All human white blood cell populations were characterized by flow cytometry, based on the expression of the pan-leukocyte marker CD45. The mast cells were further defined by the FcεRIα and CD117 antibodies.

2.8 Flow cytometry

All cell populations are depicted as the percentage of viable cells, determined according to a fixable viability dye solution (eBioscience). Gating strategies and controls are shown in **Supplemental Figure 2**. Cells were stained with antibodies against extracellular proteins or fixated and permeabilized using a transcription factor kit (eBioscience) for intracellular stainings (**Table 1**). Flow cytometry measurements were performed on a FACS Canto II and data were analysed using FlowJo software.

Targeted Species	Antibody	Fluorochrome	Clone	Concentration	Company
Mouse/ Human	Fixable Viability Dye	eFluor 780	-	0.1 μg/sample	eBioscience
Mouse	FcεRIα	PercP Cy5.5	MAR-1	0.1 μg/sample	Biolegend
Mouse	CD117	APC/PE Cy7/ PE	2B8	0.1 μg/sample	eBioscience
Mouse	MHC-II	FITC/PE	M5/ 114.15.2	0.1 μg/sample	eBioscience
Mouse	CD11c	PE	N418	0.1 μg/sample	eBioscience
Mouse	CD86	PE/APC	GL1	0.1 μg/sample	eBioscience
Mouse	Ea52-68	Biotin	EBioY-Ae	0.5 μg/sample	eBioscience
Mouse	Streptavidin	APC	-	0.3 μg/sample	eBioscience
Mouse	Thy1.2	PE Cy7	53-2.1	0.1 μg/sample	eBioscience
Mouse	CD4	PercP	RM4-5	0.1 μg/sample	BD Biosciences
Mouse	CD8	eFluor 450	53-6.7	0.1 μg/sample	eBioscience
Mouse	*T-bet*	*Alexa-Fluor 660*	*eBio4B10*	*0.3 μg/sample*	eBioscience
Mouse	*Ki-67*	*FITC*	SolA15	*0.3 μg/sample*	eBioscience
Human	CD45	PE Cy7	2D1	0.3μg/sample	eBioscience
Human	FcεRIα	APC	AER-37	0.1μg/sample	eBioscience
Human	CD117	PercP Cy5.5	104D2	0.1μg/sample	BD Biosciences
Human	HLA-DR	PE	L243	0.3μg/sample	eBioscience
Human	CD63	PE	H5C6	0.1μg/sample	eBioscience

Table 1: List of extracellular and *intracellular* antibodies used in all flow cytometry experiments

2.9 Statistics

All data are presented as mean±SEM. Values within groups were tested for normal distribution and corrected with a Bonferroni post-test for multiple comparisons. In the event of one-variable analysis among two groups a 2-tailed Student's *t*-test was used, while among more than two groups a one-way ANOVA was performed. For the analysis of two variables between groups, a two-way ANOVA test was used. Non-Gaussian distributed data were analyzed with a Mann-Whitney *U* test. Pearson's correlation was used to estimate the association between two variables in human mast cells. The probability (alpha) value for all tests was set to 0.05, with differences lower than this considered significant ($P<0.05$).

3. Results

3.1 Western-type diet increases the MHC-II expression on peritoneal mast cells

To determine the effects of high lipid diet on the mast cells, we fed LDLr$^{-/-}$ mice a WTD for a period of 4 to 8 weeks and compared the peritoneal mast cell population with LDLr$^{-/-}$ mice fed a chow diet. Upon increasing periods of WTD, the relative amount of peritoneal mast cells increased significantly (**Figure 1A**, chow: 0.39±0.1% vs WTD 4wk: 1.97±0.4%, P=0.014; chow vs WTD 8wk: 2.02±0.3%, P=0.011). Likewise, the absolute number of peritoneal mast cells increased after 4 weeks of WTD (**Figure 1B**, P=0.060), however this number returned to chow-levels upon 8 weeks of WTD. Interestingly, we observed that during 4 weeks of WTD the peritoneal mast cell population showed an enhancement in expression of the antigen presenting protein MHC-II (**Figure 1C**, chow-MCs:30.38±1.7% vs WTD 4wk-MCs:52.48±5.9%, P=0.014). The absolute number of mast cells that express MHC-II upon 4 weeks of WTD showed a 4-fold increase as compared to the number of MHC-II^{+} MCs in chow diet (**Figure 1D**, chow: 3677±367 mast cells vs WTD 4wk: 21561±5071 mast cells, P=0.009). Also here, 8 weeks of WTD restored the amount of MHC-II^{+} expressing mast cells to chow-levels. Furthermore, the para-aortic lymph nodes of these mice showed a slight increase in the number of mast cells after 4 weeks of WTD, as compared to the chow-fed mice. The mast cells accumulated particularly in the subcapsular sinuses of the lymph nodes (**Supplemental Figures 3A**,**B**), an area where soluble antigens meet professional antigen presenting cells[30]. However, no difference was observed between the groups in the number of T cells in the area (**Supplemental Figure 3C**).

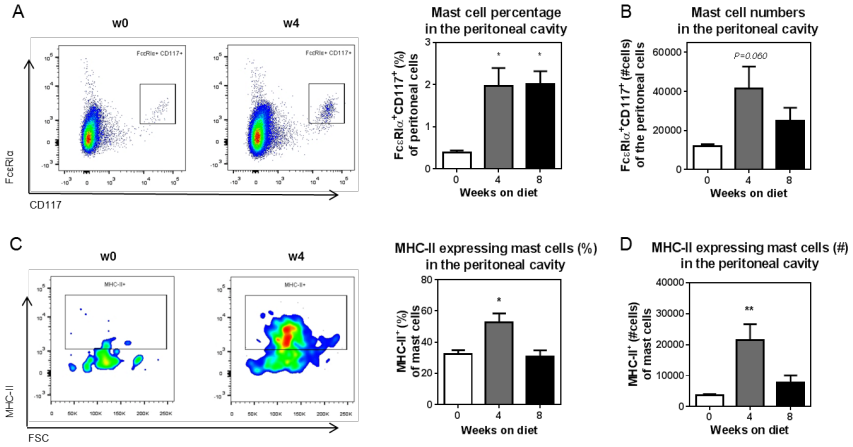

Figure 1: Peritoneal mast cells of LDLr$^{-/-}$ mice increase in amount and MHC-II expression after 4 weeks of WTD. Representative flow cytometry plots are shown, with the mast cell population defined by the expression of receptors FcεRIα and CD117. The mast cell percentage in the peritoneal cavity of LDLr$^{-/-}$ mice increased after 4 and 8 weeks of WTD (**A**). The mast cell numbers peaked after 4 weeks of WTD and were restored to normal levels after 8 weeks of WTD (**B**). Characteristic dot plots of the MHC-II expression on the mast cell population show the peritoneal mast cells that express protein MHC-II (**C**). The MHC-II-expressing mast cells increased significantly in percentage and numbers after 4 weeks of WTD, while returning to normal after 8 weeks (**D**). All values (n=5/gr) are depicted as mean±SEM; *$p<0.05$, **$p=0.01$

3.2 The MHC-II molecules on the peritoneal mast cells can present antigens *in vivo*

We next sought to examine whether the MHC-II molecules on the peritoneal mast cell surface are functional and capable of presenting antigens *in vivo* by using the Eα-peptide system[26]. We therefore injected the Eα-peptide or PBS-control in the peritoneum of WTD-fed LDLr$^{-/-}$ mice. After a period of 24 hours we observed that the MHC-II expression was enhanced in the Eα-injected mast cells as compared to control (**Figure 2A**, PBS:16.3±4.9% Eα:29.75±6.1% P=0.052). Importantly, Eα-peptide fragments were detected on the mast cell surface of the Eα-injected group (**Figure 2B**, PBS:2.73±0.3% vs Eα:7.07±1.5%, P=0.009). We further examined the capacity of peritoneal mast cells to present the Eα-peptide *ex vivo*. Upon 3h of peptide addition, Eα-GFP^{+} signal was detected in the peritoneal mast cells, indicating that the peptide is internalized but not yet processed by the lysosomal compartment (**Figure 2C**, PBS: 3.41±1.9% vs Eα(3h): 27.7±6.2%, P=0.008). No Eα-GFP^{+} cells were observed after 24 hours. However, at this timepoint Eα-peptide fragments were detected on the peritoneal mast cell surface, indicating that during this period the Eα-peptide was fully processed by the lysosomes and its fragments were presented on the mast cell surface through the MHC-II molecule (**Figure 2D**, PBS: 4.46±1.2% vs Eα(24h): 22.08±9.1%, P=0.062).

As expected, no fragment presentation by mast cells was detected in the first 3h of Eα-addition.

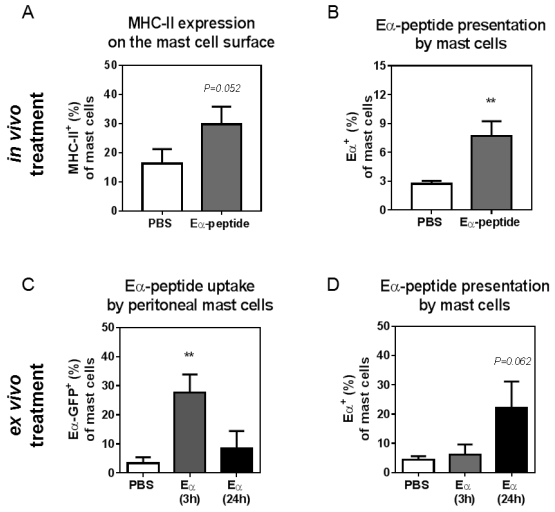

Figure 2: The MHC-II protein on the peritoneal mast cell surface is able to present antigenic fragments of the Eα-peptide, upon WTD. LDLr$^{-/-}$ mice, fed a WTD for 4 weeks, increase their MHC-II expression levels 24h after i/p injection of Eα-GFP, as compared to the PBS mice (n=10/grp) (**A**). The Eα-peptide fragments are detected *in vivo* on the surface of peritoneal mast cells, 24h after administration (n=11/grp) (**B**). Peritoneal cells isolated from the PBS and Eα-administered mice (n=6/grp) were further treated *ex vivo* with Eα-GFP peptide. Eα-GFP uptake by peritoneal mast cells was detected 3h after addition, while 24h later there was no detectable Eα-GFP signal (**C**). Eα-peptide fragments were observed 24h later, on the peritoneal mast cell surface (**D**). All values are depicted as mean±SEM (n=10/grp); ***p<0.01*.

3.3 WTD serum enhances the presentation capacity of bone marrow derived mast cells *in vitro*

Since WTD-induced hypercholesterolemia seemed to enhance the presentation machinery of mast cells, we aimed to further investigate the direct effect of hyperlipidemic serum on the non-classical MHC-II expression of mast cells. We therefore used bone-marrow derived mast cells (BMMCs) to examine the effects of atherogenic serum isolated from mice fed a WTD for 4 weeks or chow diet serum. It is worth mentioning here that, unlike peritoneal mast cells[31], bone marrow derived mast cells are not able to constitutively express MHC-II[32] unless induced with specific factors[19,20]. For that reason, in our BMMC experiments we induced MHC-II expression on the mast cells (iMCs) using IL-4 and IFNγ (**Supplemental Figure 4A**). This does not seem to be necessary *in vivo* because hyperlipidemic serum already contains these cytokines (**Supplemental Figure 4B**,**C**). We observed that upon repeated treatment of iMCs, for 48h with WTD (4wk) serum, mast cells increased their MHC-II expression levels by 2-fold as compared to chow serum (**Figure 3A**, chow-iMC: 3.12±0.3% vs WTD-iMC: 10.25±0.1%; P<0.0001). Furthermore, the costimulatory protein CD86, which is necessary for the strengthening of the antigen presentation process[33], was also found to increase in a WTD specific manner on the surface of the WTD-iMCs, as compared to chow-iMCs. (**Figure 3B**, chow-iMC: 1.73±0.1% vs WTD-iMC: 2.53±0.1%, P=0.0012). Finally, for antigen presentation to be efficient, cytokine signals are also needed. Therefore, we measured the levels of

cytokine IL-12, which is one of the most potent cytokines for T_{H1} subset skewing[34]. We did not observe any differences between the WTD and chow treated iMCs (**Figure 3C**), however it was certain that mast cells, in the presence of serum are able to provide the third signal needed for $CD4^+$ T_{H1} cell skewing. Interestingly, when iMCs were treated with fluid collected from the peritoneal cavity of $LDL^{-/-}$ mice fed either chow or WTD (4 weeks) they showed a marked increase in their MHC-II expression (**Supplemental Figure 4D**). We therefore measured the IL-4 and IFNγ levels in the peritoneal cell fluid and observed that both cytokines are present (**Supplemental Figure 4E**,**F**), indicating that peritoneal mast cells seem to have the necessary signals needed for MHC-II induction *in vivo*.

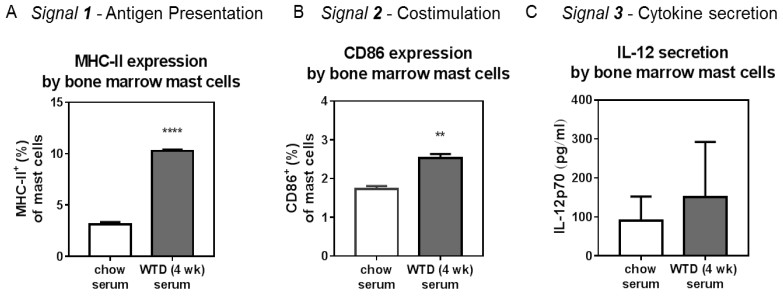

Figure 3: Inducible bone marrow derived mast cells repeatedly treated with WTD serum possess all three signals required for antigen presentation. The expression of MHC-II on the surface of bone marrow mast cells increased upon repeated (48h) *in vitro* treatment with 10% serum isolated from $LDLr^{-/-}$ mice that were fed a WTD for 4 weeks, as compared to chow serum (**A**). Co-stimulatory molecule CD86 was enhanced on the surface of mast cells after repeated treatment with 10% WTD (4wk) serum from $LDLr^{-/-}$ in comparison to chow-serum (**B**). Bone marrow mast cells were capable of secreting cytokine IL-12 after repeated serum treatment, with no significant difference detected between chow or WTD samples (**C**). All values are shown as mean±SEM; *$p<0.05$, **$p<0.01$.

3.4 OVA-peptide specific $CD4^+$ T cell proliferation is enhanced upon co-culture with WTD-treated mast cells

Thereafter, we aimed to unravel the direct effect of mast cell mediated antigen presentation to $CD4^+$ T cells. We isolated $CD4^+$ T cells from genetically modified OT-II mice, that specifically recognize ovalbumin antigens[28], and after 24h of pre-activation with αCD3/CD28 we placed them in a coculture with iMCs loaded with an OVA-peptide (OVAp) in the presence of either chow or WTD diet. OVAp-loaded iMCs showed a significant increase on their MHC-II expression in the presence of $CD4^+$ T cells, regardless of the type of serum (**Figure 4A**, $P<0.001$). Furthermore, the OVAp-iMC population pretreated with WTD-serum showed an increase in their CD86 expression when co-cultured with $CD4^+$ T cells (**Figure 4B**). $CD4^+$ T cells increased their proliferation rate in an OVAp specific manner with significant enhancement in the proliferation rate when co-cultured with WTD-pretreated iMCs, as detected by

the incorporation rate of ^{3}H-Thymidine (**Figure 4C**, chow-iMCs:9033±2558 dpm vs WTD-iMCs: 25331±2889 dpm, P<0.0001). The WTD effect of OVAp-carrying iMCs on the proliferation rate of CD4⁺ T cells was further confirmed by flow cytometry after detection of the intracellular proliferation marker Ki-67 on the CD4⁺ T cell population (**Figure 4D**, chow-iMCs:17.76±0.09% vs WTD-iMCs:25.56±1.7%, P=0.004). Finally, we measured the secretion of IFNγ and IL-4 in the co-culture since, aside from inducing MHC-II on the mast cells, they are the most important cytokine "fingerprints" of the CD4⁺ T_{H1} or T_{H2} subsets respectively. The secretion of IFNγ was slightly increased in the WTD-iMC conditions upon the presence of CD4⁺ T cells; however, no difference was observed between the OVAp-induced IFNγ secretion by CD4⁺ T cells (**Figure 4E**). In contrast, IL-4 secretion was significantly increased in the chow iMC conditions, effect which was not observed in the WTD iMC-CD4⁺ samples, resulting thus in a significant difference in the IL-4 levels between chow and WTD conditions (**Figure 4F**, chow-iMC:14.16±2.3 pg/ml vs WTD-iMC:5.85±1.9 pg/ml, P=0.002) suggesting that mast cell induced antigen presentation in hyperlipidemic conditions favors the secretion of IFNγ over IL-4 and therefore T_{H1} cell skewing over T_{H2}.

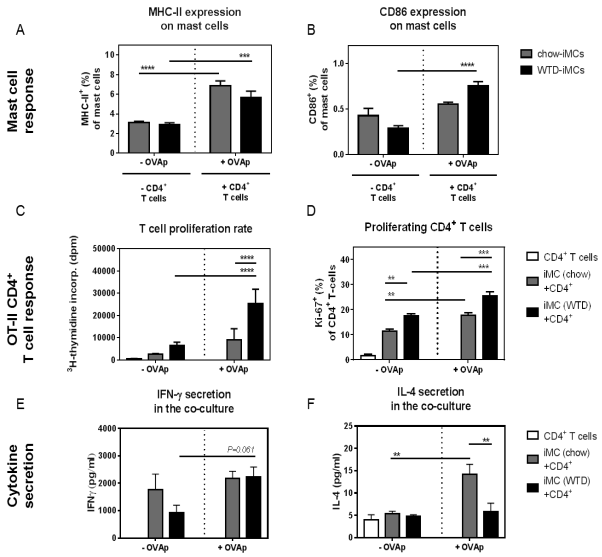

Figure 4: Bone marrow iMCs, repeatedly treated with WTD-serum and loaded with an OVA-peptide, enhance the proliferation rate of pre-activated OT-II CD4⁺ T cells. The expression of MHC-II on the mast cell surface was increased in OVAp-loaded mast cells at the presence of CD4⁺ T cells (**A**). The percentage of costimulatory molecule CD86 was found increased only WTD-treated mast cells that were loaded with OVAp and in the presence of CD4⁺ T cells (**B**). The proliferation rate of pre-activated OT-II CD4⁺ T cells increases upon presentation of OVAp by mast cells treated with WTD as compared to chow-treated MCs, as measured by both radioactive thymidine incorporation and expression of proliferation marker ki-67⁺ with flow cytometry (**C** and **D**). The IFNγ secretion by CD4⁺ T cells was increased upon presentation of the OVAp by WTD-treated mast cells as compared (**E**). The secretion of IL-4 by CD4⁺ T cells was increased upon presentation of OVAp by the chow-treated mast cells (**F**). All values (n=5/grp) are shown as mean±SEM; *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$.

3.5 Aortic CD4$^+$ T cells show reduced proliferation in mast cell deficient mice

To verify whether mast cells affect the CD4$^+$ T cell population in the atherosclerotic plaque we placed mast cell deficient apoE$^{-/-}$/Kit$^{W\text{-}sh/W\text{-}sh}$ mice on a WTD and compared the intraplaque CD4$^+$ T cell content in their aortas with that from WTD-fed control apoE$^{-/-}$ mice. Using flow cytometry, we observed a reduction in the percentage of Ki-67$^+$ CD4$^+$ T cells in the aortas of atherosclerotic mast cell deficient mice as compared to controls (**Figure 5A**, apoE$^{-/-}$: 30.91±5.2 % vs apoE$^{-/-}$/Kit$^{W\text{-}sh/W\text{-}sh}$:13.7±1.2, P=0.013). This reduction was also apparent when comparing the absolute number or proliferating CD4$^+$ T cells (**Figure 5B**, apoE$^{-/-}$: 1725±567 #cells vs apoE$^{-/-}$/Kit$^{W\text{-}sh/W\text{-}sh}$: 389±59 #cells, P=0.037). Interestingly, a decline in the number of T_{H1} cells was also detected in the aortas of mast cell deficient apoE$^{-/-}$ mice as compared to the control apoE$^{-/-}$ mice (**Figure 5C**, apoE$^{-/-}$: 148±25 #cells vs apoE$^{-/-}$/Kit$^{W\text{-}sh/W\text{-}sh}$: 45±16 #cells, P=0.056). Of note, the total T cell percentage is found slightly reduced in the aortas of the mast cell deficient apoE$^{-/-}$ (**Figure 5D**). This reduction is not due to the CD8$^+$ T cell population which remains unchanged (**Figure 5E**) but rather due to the reduction of the CD4$^+$ T cells (**Figure 5F**, apoE$^{-/-}$: 4.72±0.8 % vs apoE$^{-/-}$/Kit$^{W\text{-}sh/W\text{-}sh}$: 2.37±0.7 #cells, P=0.028).

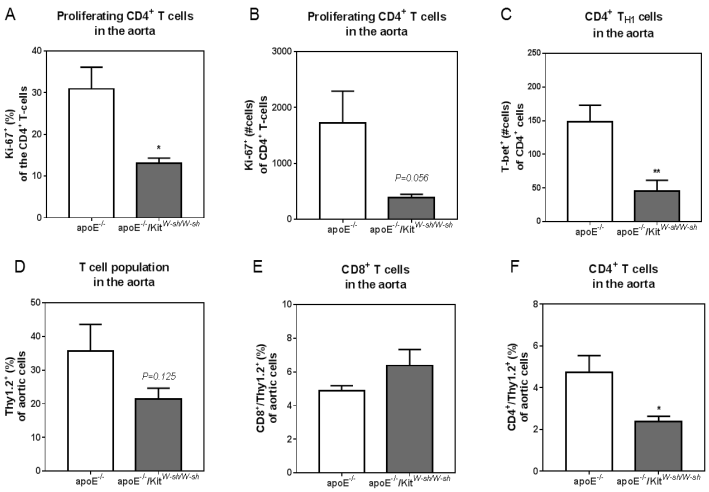

Figure 5: Mast cell deficient apoE$^{-/-}$/Kit$^{W\text{-}sh/W\text{-}sh}$ mice show a reduction in the proliferation of CD4$^+$ T_{H1} cells in the aortic arch. The percentage and absolute numbers of proliferating ki-67$^+$/CD4$^+$ T cells were reduced in the aortic arch of apoE$^{-/-}$/Kit$^{W\text{-}sh/W\text{-}sh}$ mice, fed a WTD for 6 weeks, as compared to apoE$^{-/-}$ mice (**A** and **B**). The numbers of T_{H1} cells in the aorta of mast cell deficient apoE$^{-/-}$ mice were decreased, as measured by the expression of transcription factor T-bet on the aortic CD4$^+$ T cell population (**C**). Mast cell deficient apoE$^{-/-}$ mice fed a WTD show a slight reduction in their aortic T cell percentage (**D**). The aortic CD8$^+$ T cell content of apoE$^{-/-}$ mice was not affected by the absence of mast cells (**E**). The aortic CD4$^+$ T cell content was significantly decreased in of apoE$^{-/-}$ mice fed a WTD upon the absence of mast cells (**F**). All values (n=8/group) are depicted as mean±SEM; *$p<0.05$, **$p<0.01$.

3.6 Human intraplaque mast cells express HLA-DR

Finally, to establish whether our data can be translated to the human disease setting, we analyzed the mast cell content of 20 endarterectomy plaques collected from carotid or femoral arteries. We are the first to use flow cytometry for the detection of human intraplaque mast cells, in femoral as well as carotid arteries as measured by the expression of receptors FcεRIα and CD117(**Figure 6A**, carotid hMCs: 0.81±0.1%, femoral hMCs: 1.26±0.2%). Focusing on the antigen presentation capacity of mast cells inside the atherosclerotic plaques, we further stained them for the expression of protein HLA-DR. We observed that mast cells inside human atherosclerotic plaques can express HLA-DR in both the femoral and the carotid arteries (**Figure 6B**, carotid hMCs: 9.30±4.3% vs femoral hMCs: 23.8±6.9%). Interestingly, the HLA-DR expressing cells showed a negative association with the activated mast cell population, as detected by marker CD63[35], which comprises the majority of mast cells within the atherosclerotic plaque (**Figure 6C**, P=0.024).

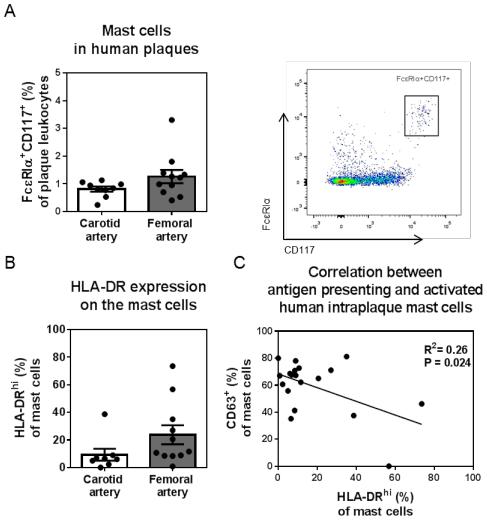

Figure 6: Human mast cells from carotid and femoral atherosclerotic plaques express HLA-DR. The mast cell population in the plaques of human subjects (n=20), obtained from endarterectomy surgery, are detected based on the high expression of FcεRIα and CD117. Representative flow cytometry plots of human mast cells, gated according to the expression of receptors FcεRIα and CD117 (**A**). Human intraplaque mast cells express HLA-DR, in the femoral artery and carotid artery samples (**B**). The HLA-DR^{hi} mast cells show a negative correlation with the activated CD63^{+} mast cell population in the atherosclerotic arteries (**C**).

4. Discussion

In this project, we sought to examine whether mast cells can act as non-professional antigen presenting cells in atherosclerosis. We observed that hyperlipidemic conditions induce the expression of MHC-II on the peritoneal mast cell surface and furthermore, that mast cells are able to process and present antigens *in vivo*. Within the atherosclerotic tissue, mast cells appear to augment the T_{H1} response in a manner that seems to partially involve a direct interaction between these two cell types. Importantly, we show

that antigen presentation by mast cells may even take place within the atherosclerotic plaques of human subjects, a process that appears to associate in a negative manner with the classical mast cell activation taking place within the plaque.

The capacity of mast cells to directly shape T cell responses, upon request by certain signals in their microenvironment, is a process that has recently gained attention in the field[36]. However, the question was posed long ago in a study reporting that rat mast cells incubated with IFNγ showed increased MHC-II expression; thus arguing that mast cells, aside from their classical degranulating nature may also be capable of presenting antigens[37]. The regulation of the MHC-II molecule expression by IFNγ has previously been established in professional antigen presenting cells[38], but also in human mast cells[39]. Additionally, peritoneal mast cells, in the presence of IFNγ/IL-4[19], have been shown to increase their MHC-II expression levels. This effect may explain why mast cells increase their MHC-II levels in an atherosclerotic environment, since it is known that patients with coronary heart disease show increased levels of IFNγ in their serum[40]. Moreover, human plaque tissue contains high amounts of IFNγ[41]. Here we observed that mast cells increase their MHC-II levels both *in vivo* and *in vitro* in a WTD-specific manner in accordance to detecting high levels of IFNγ, in the serum and peritoneal fluid of hyperlipidemic mice. We also saw that the absence of mast cells significantly reduced the number of T_{H1} cells, the main producers of IFNγ, inside the atherosclerotic plaques. Furthermore, antigen presentation through WTD-treated mast cells increased the IFNγ secretion by T cells, which, when combined with the reduced IL-4 secretion, supports the generation of T_{H1} cells. Mast cells have also been found to enhance plaque progression in an IFNγ dependent manner[16], with mast cell deficient mice developing smaller atherosclerotic plaques[42] which suggests that IFNγ may also function as a regulator of the direct interaction between mast cells and T_{H1} cells within the atherosclerotic plaque. It is worth noting that while WTD-treated mast cells seem to support a T_{H1} response, chow-treated mast cells tend to favor the T_{H2} phenotype. Interestingly, allergic asthma is a classic T_{H2} mediated condition where mast cells are famously known for their adverse effects[43]. In contrast, atherosclerosis is shaped by the action of T_{H1} cells, as mentioned before. Therefore, it seems that mast cells can act on the adaptive immune system *in situ* and according to the local inflammatory status.

Our study also shows that mast cells can express, upon request, all three signals required for antigen presentation. The presence of multiple costimulatory molecules on the mast cell surface, such as OX40L, CD80 and CD86, has been previously stated to be a main assistant in their direct communication with $CD4^+$ T cells[20,44]. Here we observed that WTD conditions increase CD86, however we cannot exclude that other costimulatory molecules may participate in the described interaction with $CD4^+$ T cells. Furthermore, mast cells are known for their potent cytokine secretion, and TNFα

release has also been reported to enhance local antigen presentation[32]. In the case of atherosclerosis, signal 3 could be provided by the mast cells, for instance in the form of IL-12 which skews T cells towards a T_{H1} subset, but also by the inflammatory cytokine content of the hypercholesterolemic serum.

In addition, we here establish that the MHC-II protein on the surface of mast cells is a fully functional molecule that can bind antigens processed by the lysosomal machinery of the cell and successfully transport them to the cell surface where they can be introduced to CD4⁺ T cells. However, while the Eα and OVA peptides are model antigens that serve in exploring the presentation capacity of a cell, the WTD-specific induction of MHC-II implies that lipid specific antigens may be presented by mast cells. Although it is still unclear which peptide fragments elicit an adaptive immune response in atherosclerosis it is worth mentioning that oxLDL has been previously reported to regulate the expression of MHC-II on macrophages in an autophagy mediated pathway[45]. It would therefore be intriguing to see if such a pathway is shaping also the lipid-specific mast cell presentation capacity that we observe here. Overall, it is interesting to investigate the means through which mast cells take up antigens from their surrounding atherosclerotic environment since, to our knowledge, mast cells are not capable of ingesting lipid proteins through the classic scavenger receptors such as those that mediate macrophage foam cell formation.

The way by which mast cells are activated within the atherosclerotic tissue has not yet been fully deciphered. While the classical FcεR-pathway is the most plausible manner, it is known that oxLDL can activate human mast cells in a Toll-like receptor (TLR)4-dependent mode[46]. This is of importance considering that TLR4 signaling on APCs enhances antigen presentation[47]. In line with the above, CD63 is an activation marker linked to the FcεR-mediated degranulation[35]. Notably, this study is the first to use flow cytometry as a method to detect human mast cells within atherosclerotic plaques. While we saw that intraplaque mast cells express high levels of CD63, their expression was negatively correlated to the HLA-DR expressing population. Even though this does not indicate a causal relationship, it seems that mast cells that are engaged with the presentation of antigens are not at that current moment activated. Furthermore, HLA-DR expression by human mast cells has also been previously reported in human tonsils[22], a common centre of inflammation in the body. Importantly, a very recent study investigating the presentation capacity of human skin mast cells provides additional evidence that these cells express HLA-DM in an IFNγ-specific manner and directly present antigens to CD4⁺ T cells further shaping thus not only the T_{H1} response[48], but also prompting T cells to act back on the mast cells. Likewise, we saw that CD4⁺ T cells act on the mast cell MHC-II expression in a positive way. It therefore seems that T cells can in turn affect the mast cells in a manner that favors the induced antigen presentation

ability of the latter.

Lastly, it is worth to mention that *in vitro* examination of the mast cell antigen presentation capacity in different studies shows slight discrepancies. This diversity probably lies on the *in vitro* generation of mast cells from bone marrow progenitors and the stimuli used. It is however important to remember that mast cells are found only within tissues, whereupon they undergo the last step of differentiation according to local stimuli.

In conclusion, this study showed that mast cells are capable of presenting antigens in a hypercholesterolemic manner and this effect is potently intensifying the proatherogenic T_{H1} cell type within atherosclerotic plaques. Importantly, mast cells seem capable of antigen presentation within human plaques, indicating that these cells can also shape the adaptive immune response apart from exacerbating the innate inflammatory pathways. Further research on how to modulate this interaction may induce novel ways to limit atherosclerosis development.

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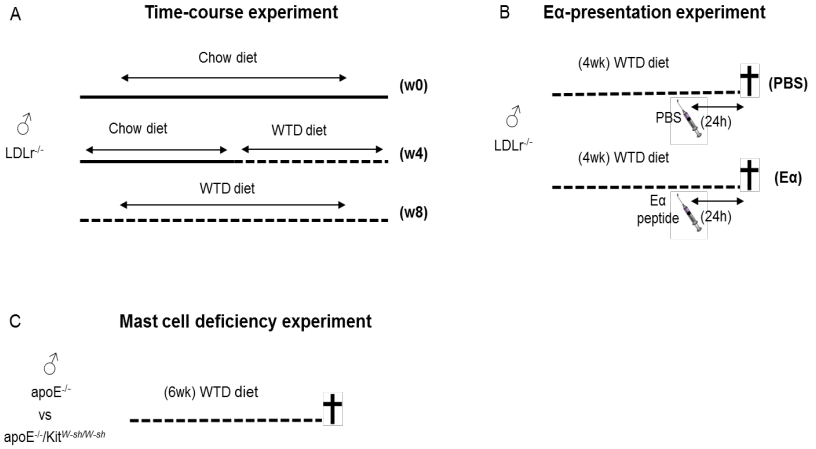
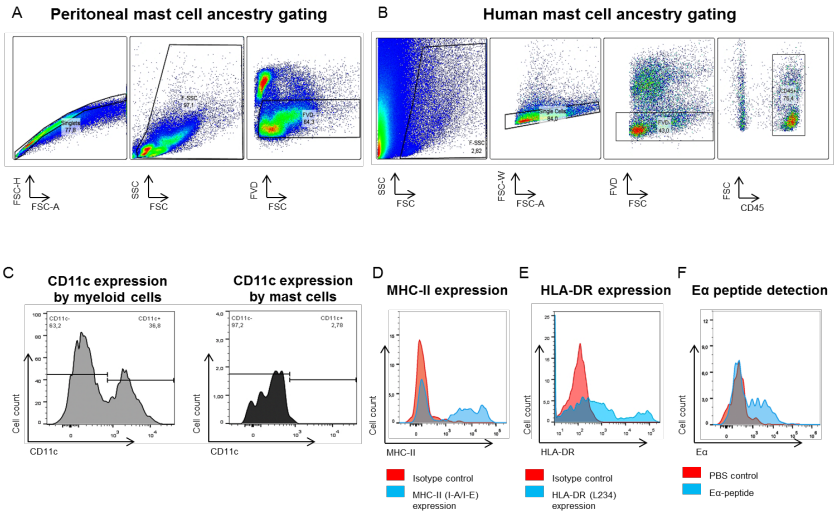
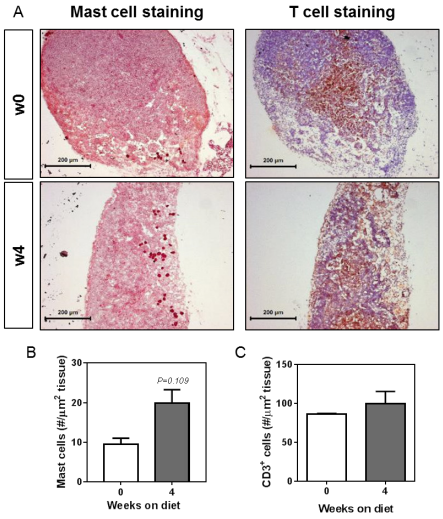
Supplementary Information



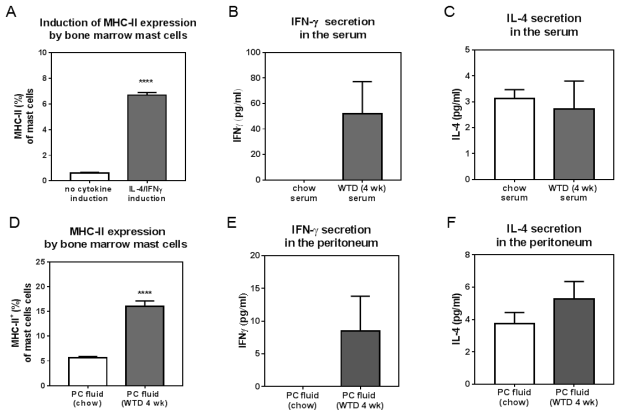
Supplemental Figure 1: **Experimental set-up for the *in vivo* experiments.** Time-course experiment using LDLr$^{-/-}$ mice fed 4 to 8 weeks Western-type diet or chow diet fed mice to compare their peritoneal mast cell populations (**A**). Eα-peptide presentation experiment performed on LDLr$^{-/-}$ mice fed a WTD for 4 weeks. Eα-peptide or sterile PBS was injected 24 hours prior sacrifice, in order to analyze the presentation capacity of peritoneal mast cells (**B**). Analysis of the aortic CD4^{+} T cell content in mast cell deficient apoE$^{-/-}$ mice, as compared to their wild-type counterparts, upon WTD feeding (**C**).



Supplemental Figure 2: **Gating strategies & controls of flow cytometry experiments.** Representative ancestry gating of mouse peritoneal mast cells and human intraplaque mast cells discriminated for their singularity based on cell height (FSC-H) or width (FSC-W) and area (FSC-A) as well as size (FSC) and granularity (SSC). The cells were further separated from the dead population which stained positive for a viability marker (FVD) (**A**) and human cells were further stained for the pan-leukocyte marker (CD45) (**B**). Peritoneal mast cell population did not show any CD11c expression in comparison to peritoneal myeloid cells (**C**). Isotype control for MHC-II antibody clone (I-A/I-E) and HLA-DR antibody clone used in flow cytometry experiments (**D** and **E**). Eα-peptide expression on Eα-injected mice as compared to their PBS counterparts (**F**).



Supplemental Figure 3: **Mast cells increase in numbers inside the para-aortic draining lymph nodes of LDLr$^{-/-}$ upon 4 weeks of Western-type diet.** Representative pictures of para-aortic lymph nodes of LDLr$^{-/-}$ mice stained with Naphthol-chloroacetate esterase for mast cells (red) and with αCD3^{+} for T cells (brown) (**A**). The mast cell numbers were found increased in the para-aortic lymph nodes of LDLr$^{-/-}$ mice upon 4 weeks of WTD (**B**). The T cell numbers within the para-aortic lymph nodes of LDLr$^{-/-}$ were not altered upon 4 weeks of WTD as compared to control chow diet (**C**). All values (n=3/grp) are depicted as mean±SEM.



Supplemental Figure 4: Cytokine mediated induction of MHC-II on the mast cell surface: Bone marrow mast cells express MHC-II only upon induction by cytokines IL-4 (10ng/ml) and IFNγ (20ng/ml) (**A**). WTD serum isolated by LDLr$^{-/-}$ mice after being fed a WTD for 4 weeks contains a combination of both IFNγ and IL-4 cytokines as compared to chow serum (**B** and **C**). Bone marrow derived mast cells repeatedly incubated with fluid isolated from the PC-cavity of mice under 4 weeks of WTD increase their MHC-II expression as compared to chow PC-fluid (**D**). IFNγ and IL-4 cytokines are present in the peritoneal cell fluid of LDLr$^{-/-}$ mice fed 4 weeks of WTD (**E** and **F**). All values are shown as mean±SEM; ****p<0.001.*