The Journal of Immunology

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J Immunol published online 25 January 2019
http://www.jimmunol.org/content/early/2019/01/24/jimmunol.1800648

Supplementary Material http://www.jimmunol.org/content/suppl/2019/01/24/jimmunol.1800648.DCSupplemental

The Journal of Immunology is published twice each month by The American Association of Immunologists, Inc., 1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Published January 25, 2019, doi:10.4049/jimmunol.1800648

Hypercholesterolemia Induces a Mast Cell–CD4$^+$ T Cell Interaction in Atherosclerosis

Eva Kritikou,* Thomas van der Heijden,* Maarten Swart,* Janine van Duijn,* Bram Slütter,* Anouk Wezel,† Harm J. Smeets,† Pasquale Maffia,‡,§,¶ Johan Kuiper,* and Ilze Bot*

Mast cells (MCs) are potent innate immune cells that aggravate atherosclerosis through the release of proinflammatory 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 APCs. Recently it has been proposed that these two cell types can interact in a direct manner. However, no indication of such an interaction has been investigated in the context of atherosclerosis. In our study, we aimed to examine whether MCs can act as APCs in atherosclerosis, thereby modulating CD4$^+$ T cell responses. We observed that MCs increased their MHC class II expression under hyperlipidemic conditions both in vivo and in vitro. Furthermore, we showed that MCs can present Ags in vivo via MHC class II molecules. Serum from high-fat diet–fed mice also enhanced the expression of the costimulatory molecule CD86 on cultured MCs, whereas OVA peptide–loaded MCs increased OT-II CD4$^+$ T cell proliferation in vitro. The aortic CD4$^+$ and T_{H1} cell content of atherosclerotic mice that lack MCs was reduced as compared with their wild-type counterparts. Importantly, we identified MCs that express HLA-DR in advanced human atheromata, indicating that these cells are capable of Ag presentation within human atherosclerotic plaques. Therefore, in this artice, we show that MCs may directly modulate adaptive immunity by acting as APCs in atherosclerosis. ***The Journal of Immunology*****, 2019, 202: 000–000.**

Atherosclerosis is a chronic, lipid-mediated, autoimmune disease of the medium and large-sized arteries. The disease is established through circulation of low-density lipoproteins (LDL) at excessive levels, a process termed hypercholesterolemia (1). LDL molecules can damage and penetrate the arterial wall and upon oxidation give rise to lipid-rich, highly inflamed atherosclerotic plaques (2). Atherosclerosis development involves both innate and adaptive immune responses (3). Communication between the innate and adaptive immune system is achieved through professional APCs, such as dendritic cells, that can specifically activate T lymphocytes with Ags taken up from the atherosclerotic plaque (4). It has been established that during progression of atherosclerosis, Ags from oxidized LDL (oxLDL) can elicit immune responses by activating an array of T cell subsets (5, 6). This occurs within secondary lymphoid tissues, such as the spleen and local draining lymph nodes where Ags are presented through MHC class I or MHC class II (MHC-II) (7). Subsequently, activated T cells translocate to the atherosclerotic plaque and influence the disease outcome locally (8). The effect evoked by different T cell subsets, such as T_{H1} or T_{H2} cells, is mainly attributed to their distinct cytokine profile. For example, T_{H1} cells, the most abundant Th cell type in progressing atherosclerotic plaques (9), release large amounts of IFN-γ, whereas T_{H2} cells are found at lower numbers and secrete mainly IL-4, IL-5, and IL-13 (10). CD4$^+$ T_{H1} cells and IFN-γ have been proven to be proatherogenic (11, 12), whereas T_{H2} cell function is more difficult to define (13, 14). IL-5 shows an atheroprotective action (15), whereas IL-4 appears to induce atherosclerosis (16). However, these cytokines cannot be strictly appointed to T_{H2} cells alone. Interestingly, blockade of costimulatory molecule OX40L, which specifically lowered T_{H2} responses, reduced atherosclerosis levels (17). Thus, the exact role of T_{H2} cells in atherosclerosis is still under debate. It is clear, however, that the diversity of T cell subsets, in terms of both quality and quantity, can exert inverse effects on the progression of atherosclerosis. Although effector T cells have in principle a pre-established fate upon entering the vessel wall, local costimulation in the plaques (18, 19) may alter their behavior.

Mast cells (MCs) are tissue-resident innate immune cells that have long been established to contribute to atherosclerosis progression (20). Their accumulation within the atherosclerotic tissue upon plaque development, and their subsequent classical mode of activation, results in the release of proinflammatory mediators, such as proteases and cytokines (21, 22). Yet increasing evidence

*Division of BioTherapeutics, Leiden Academic Centre for Drug Research, Leiden University, 2300 RA Leiden, the Netherlands; †Department of Surgery, Haaglanden Medical Center Westeinde, 2501 CK The Hague, the Netherlands; ‡Centre for Immunobiology, Institute of Infection, Immunity and Inflammation, College of Medical, Veterinary and Life Sciences, University of Glasgow, Glasgow G12 8TA, United Kingdom; §Institute of Cardiovascular and Medical Sciences, College of Medical, Veterinary and Life Sciences, University of Glasgow, Glasgow G12 8TA, United Kingdom; and ¶Department of Pharmacy, University of Naples Federico II, 80138 Naples, Italy

ORCIDs: 0000-0002-0331-8757 (E.K.); 0000-0002-5097-0713 (J.v.D.); 0000-0003-3996-0503 (B.S.); 0000-0002-4370-1611 (H.J.S.); 0000-0003-3926-4225 (P.M.); 0000-0002-1242-1959 (I.B.).

Received for publication May 4, 2018. Accepted for publication December 20, 2018.

This work was supported by a Dr. Dekker Senior Postdoc grant from the Netherlands Heart Foundation (2012T083 to E.K. and I.B.). This work was also supported by the Netherlands CardioVascular Research Initiative: The Dutch Heart Foundation, the Dutch Federation of University Medical Centres, the Netherlands Organisation for Health Research and Development, and the Royal Netherlands Academy of Sciences, for the GENIUS project "Generating the Best Evidence-Based Pharmaceutical Targets for Atherosclerosis" (CVON2011-19). P.M. is supported by British Heart Foundation Grants PG/12/81/29897 and RE/13/5/30177.

Address correspondence and reprint requests to Dr. Eva Kritikou at the current address: Department of Pathology, Brigham and Women's Hospital–Harvard Medical School, 77 Avenue Louis Pasteur, 02115 Boston, MA. E-mail address: ekritikou@bwh.harvard.edu

The online version of this article contains supplemental material.

Abbreviations used in this article: LDL, low-density lipoprotein; LDLr, LDL receptor; MC, mast cell; MHC-II, MHC class II; oxLDL, oxidized LDL; WTD, Western-type diet.

suggests that MCs can also adopt an Ag-presenting phenotype that may influence the immune response inside tissues (23). MCs are not classical APCs like dendritic cells or macrophages. However, peritoneal MCs have been found to upregulate MHC-II in the presence of cytokines IL-4 and IFN-γ (24), as well as inside the lymph nodes of mice injected with LPS (25). Importantly, mouse bone marrow–derived MCs have been reported to present peptides of the model Ag OVA to CD4$^+$ T cells (26). Likewise, human cultured MCs can directly present Ags to CD4$^+$ T cells through the human MHC-II cell surface receptor, HLA-DR (27, 28). However, to date, it is still unknown whether MCs can directly influence the adaptive immune response during atherosclerotic plaque progression. Therefore, we aimed to study the Ag presentation capacity of MCs to CD4$^+$ T cells in atherosclerosis. We found that MCs are capable of presenting Ags through their MHC-II machinery and modify the CD4$^+$ T effector cell response during hyperlipidemia and atherosclerosis development.

Materials and Methods

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 being provided with food and water ad libitum. At experimental end point, mice were s.c. anesthetized with an injection mix of ketamine (100 mg/ml), Sedazine (25 mg/ml), and atropine (0.5 mg/ml), and their vascular system was perfused with PBS upon heart puncture in the left ventricle.

Western-type diet time-course experiment

To examine the effect of Western-type diet (WTD) on the Ag presentation capacity of MCs, we performed a time-course experiment (Supplemental Fig. 1A). Male LDL receptor (LDLr)$^{-/-}$ mice (n = 10 per group) with an average age of 8–10 wk were placed on a WTD (0.25% cholesterol, 15% cocoa butter; Special Diet Services, Essex, U.K.) for either 4 or 8 wk or kept on a chow diet until the experimental end point. Mouse peritoneal cells were collected by peritoneal lavage, using 10 ml PBS, to study the MC peritoneal population according to their characteristic expression of markers FcεRIα and CD117. Notably, the peritoneal MC population was also examined for the expression of the myeloid cell marker CD11c, to ensure that the observed population were not MHC-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 (29, 30). The para-aortic lymph nodes of these mice were isolated at the experimental end point and fixed in formalin for 24 h. Subsequently, all lymph nodes were embedded in OCT medium (Sakura), and 6-μm cryosections were collected. MC detection was performed using a Naphthol AS-D Chloroacetate Esterase kit (Sigma-Aldrich). The T cell population in the para-aortic lymph nodes was detected through Ab staining against CD3 at a 1:150 concentration (clone SP7; Thermo Fisher Scientific). Determination of all MC and CD3$^+$ T cell numbers was performed by blinded and independent manual quantification of two collected sections per mouse. Representative pictures were obtained using a Leica DMRE microscope (Leica Systems).

Eα presentation experiment

To study the ability of MCs to present Ags through their MHC-II molecule, we made use of the Eα-GFP/Y-A*e* 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 Abs against Eα fragments (31). We performed an Eα-GFP complex presentation experiment (32, 33), as described in Supplemental Fig. 1B, in which male LDLr$^{-/-}$ mice (n = 14 per group, average age 13 wk) were placed on a WTD for 4 wk. Twenty-four hours prior to the end of the experiment, one group was injected i.p. with Eα-GFP (100 μg/mouse), whereas 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 (200 μg/ml) for either 3 or 24 h and compared with peritoneal cells isolated from the PBS-injected mice. The expression levels of an Y-A*e*–streptavidin Ab against Eα were determined by flow cytometry in comparison with the control-PBS expression.

Bone marrow–derived MC stimulation

To investigate the Ag presentation function of MCs in vitro, bone marrow–derived MCs were cultured from isolated bone marrow cells in RPMI 1640 medium containing 25 mM HEPES (Lonza) supplemented with 10% FCS, (1×) minimal essential medium nonessential amino acids (Life Technologies), 60 μM 2-ME (Sigma-Aldrich), 1% mix of penicillin/streptomycin (Lonza), 1% sodium pyruvate (Sigma-Aldrich), and 2% L-glutamine (Lonza) for 4 wk with cytokine IL-3 (5 ng/ml; ImmunoTools). MC purity was assessed by the expression of FcεRIα and CD117 and routinely found to be above 98%. Mature MCs kept in culture for weeks 4–6 were used for all experiments. MHC-II–induced MCs were obtained by a mix of cytokines IL-4 (20 ng/ml) and IFN-γ (50 ng/ml). Subsequently, to study the effect of hyperlipidemic serum, MCs were stimulated for 24 h with 10% serum isolated from either chow-fed or 4 wk WTD–fed LDLr$^{-/-}$ mice and all conditions were renewed for an additional period of 24 h. MCs were additionally stimulated with 40% peritoneal fluid, which was obtained by peritoneal lavage with 10 ml PBS and after centrifugation at 1.600 RPM for 5 min. After a period of 24 h, all conditions were renewed accordingly. Cytokine IFN-γ and IL-4 secretion was measured by ELISA (BD Biosciences) according to the manufacturer's protocol.

MC–OT-II CD4$^+$ T cell coculture

To study the direct presentation of Ags by MCs to CD4$^+$ T cells in vitro we made use of the OVA presentation system. In a coculture experiment, MCs were repeatedly treated with 10% chow/WTD serum, as described above, in the presence of a chicken OVA 323–339 peptide (10 μg/ml) that can directly bind the MHC-II (I-A^b) surface molecules and is directly presented to the TCR without requiring internalization. CD4$^+$ T cells were isolated from genetically modified OT-II mice (34) using a magnetic bead isolation kit (Miltenyi Biotec) and activated with anti-CD3 (1 μg/ml) and anti-CD28 (0.5 μg/ml) for 24 h. Subsequently, after washing off the stimulants, MCs and T cells were placed together in a coculture system. Cells were seeded in quintuplicate per condition at a 1:1 ratio for 72 h. At the end of the experiment, all samples were prepared for flow cytometry. For the proliferation assay, radioactive [^{3}H]thymidine (0.5 μCi/well; PerkinElmer) was added to the samples after 48 h and the incorporation rate was measured 15 h later using a liquid scintillation analyzer (Tri-Carb 2900R).

MC deficiency experiment

To investigate the effect of MCs on the T cell population in atherosclerosis, male MC-deficient apoE$^{-/-}$/Kit$^{W\text{-}sh/W\text{-}sh}$ mice as well as control apoE$^{-/-}$ mice (Supplemental Fig. 1C) (n = 16 group, average age 16 wk), were fed a WTD for 6 wk. The intraplaque cell content was obtained through enzymatic digestion of the aortic tissue with a mix of collagenase I (450 U/ml; Sigma-Aldrich), XI (250 U/ml Sigma-Aldrich), DNase (120 U/ml; Sigma-Aldrich), and hyaluronidase (120 U/ml; Sigma-Aldrich), and samples were further processed using flow cytometry.

Human specimens

To explore the ability of human intraplaque MCs to present Ags, 20 anonymous atherosclerotic plaques were collected postoperatively from carotid or femoral artery endarterectomy surgeries performed between July 2016 and December 2016 at the Haaglanden Medical Center, 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 (35). In short, cell suspensions from human atherosclerotic plaques were obtained upon digestion with collagenase IV (Life Technologies) and DNase (Sigma-Aldrich) for 2 h at 37°C prior to single-cell separation through a 70-μm cell strainer. All human WBC populations were characterized by flow cytometry, based on the expression of the panleukocyte marker CD45. The MCs were identified by the FcεRIα and CD117 Abs.

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 Fig. 2. Cells were stained with Abs against extracellular proteins or fixated and permeabilized using a transcription factor kit (eBioscience) for intracellular stainings (Table I). Flow cytometry measurements were performed on a FACS Canto II and data were analyzed using FlowJo software.

Statistics

All data are presented as mean ± SEM. Values within groups were tested for normal distribution and corrected with a Bonferroni posttest for

multiple comparisons. In the event of one-variable analysis between two groups, a two-tailed Student *t* test was used, whereas 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 correlation was used to estimate the association between two variables in human MCs. The p (α) value for all tests was set to 0.05, with differences lower than this considered significant ($p < 0.05$).

Results

WTD increases the MHC-II expression on peritoneal MCs

To determine the Ag presentation properties of MCs in the course of high lipid diet, we fed LDLr$^{-/-}$ mice a WTD for a period of 4 or 8 wk, during which time these mice developed high cholesterol levels in their blood because of ineffective LDL clearance (36), and compared the peritoneal MC population with LDLr$^{-/-}$ mice fed a chow diet. Exposure to WTD for a prolonged time significantly increased the proportion of MCs in the peritoneum (Fig. 1A; chow: 0.39 ± 0.1% versus WTD 4 wk: 1.97 ± 0.4%, $p = 0.014$; chow versus WTD 8 wk: 2.02 ± 0.3%, $p = 0.011$). Interestingly, we observed that during 4 wk of WTD, the peritoneal MC population showed enhanced expression of the Ag-presenting protein MHC-II (Fig. 1B; chow MCs: 30.38 ± 1.7% versus WTD 4 wk MCs: 52.48 ± 5.9%, $p = 0.014$). Furthermore, the para-aortic lymph nodes of these mice showed a mild increase in the number of MCs after 4 wk of WTD, as compared with the chow-fed mice (Supplemental Fig. 3A, 3B). However, no difference was observed between the groups in the number of T cells in the lymph nodes (Supplemental Fig. 3C).

MHC-II molecules on peritoneal MCs can present Ags in vivo

We next sought to examine whether the MHC-II molecules expressed by peritoneal MCs are functional and capable of presenting Ags in vivo. To that end, we used the Eα-GFP/Y-A*e* presentation system, which is designed to detect MHC-II–restricted Ag presentation (37). Specifically, the Eα-GFP/Y-A*e* system can identify Ag presentation in vivo through the ability of Y-A*e* Ab to recognize the Eα peptide in the context of MHC-II binding (I-A^b) (31, 32). We therefore injected the Eα peptide or PBS control in the peritoneum of WTD-fed LDLr$^{-/-}$ mice. After 24 h we observed that the MHC-II expression was higher in the Eα-injected MCs as compared with control (Fig. 2A; PBS: 10.99 ± 2.13% Eα: 21.17 ± 3.8% $p = 0.037$). Importantly, Eα peptide fragments were detected on the MC surface of the Eα-injected group, using Y-A*e* (Fig. 2B; PBS: 2.30 ± 0.2% versus Eα: 7.35 ± 1.7%, $p = 0.0160$). We further examined the capacity of peritoneal MCs to present the Eα peptide ex vivo. Three hours after peptide addition, the Eα-GFP$^+$ signal was detected in peritoneal MCs, indicating that the peptide is internalized but not yet processed in the lysosomal compartment (Fig. 2C; PBS: 0 versus Eα [3 h]: 7.25 ± 1.6%, $p = 0.0003$; versus Eα [24 h]: 5.25 ± 1.8% $p = 0.004$). After 24 h, Eα peptide fragments were detected on the peritoneal MC surface, indicating that during this period the Eα peptide was fully processed by the lysosomes and its fragments were presented on the MC surface through the MHC-II molecule (Fig. 2D; PBS: 2.52 ± 0.9% versus Eα [24 h]: 26.02 ± 9.1%, $p = 0.0033$). As a control reference for the Eα-GFP/Y-A*e* presentation system, we examined the Eα peptide presentation efficiency in dendritic cells from the peritoneal cavity treated with Eα-GFP ex vivo (Supplemental Fig. 4A, 4B).

WTD serum enhances the presentation capacity of bone marrow–derived MCs in vitro

Because WTD-induced hypercholesterolemia seemed to enhance the presentation machinery of MCs, we aimed to further investigate the direct effect of hyperlipidemic serum on the MHC-II expression

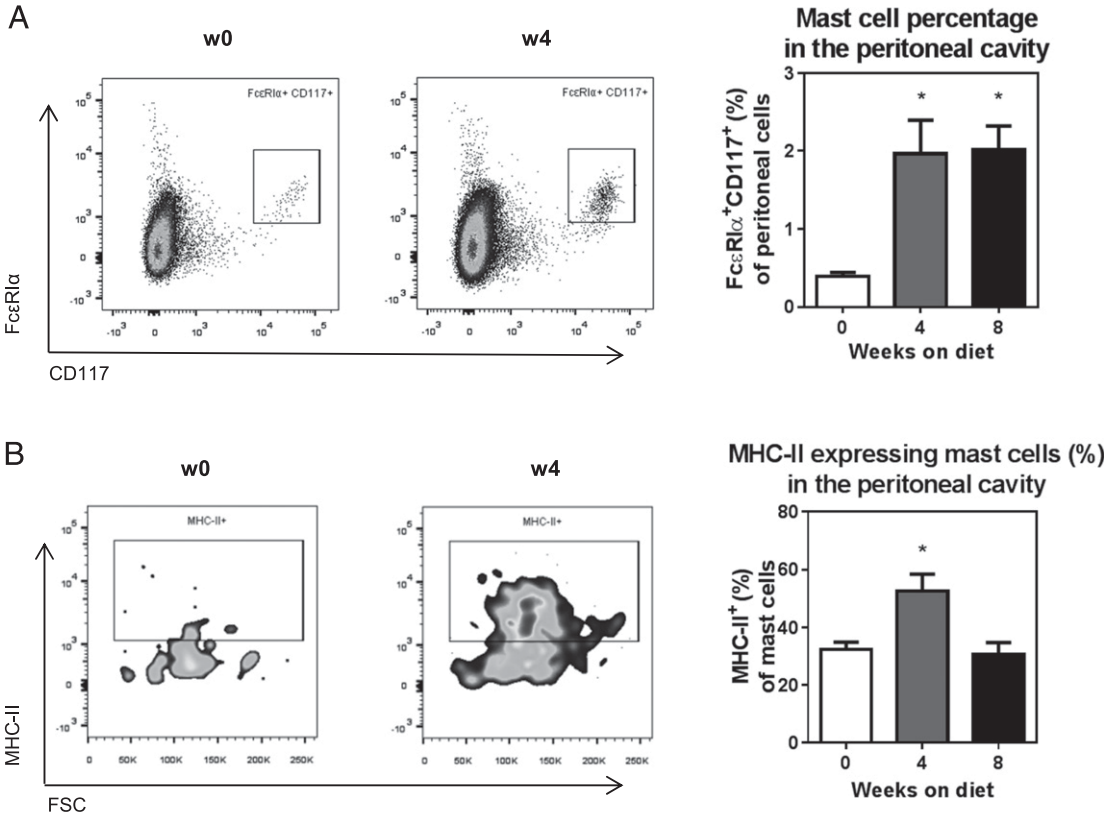

FIGURE 1. Peritoneal MCs of LDLr$^{-/-}$ mice increase in amount and MHC-II expression after 4 wk of WTD. Representative flow cytometry plots are shown, with the MC population defined by the expression of receptors FcεRIα and CD117. The MC percentage in the peritoneal cavity of LDLr$^{-/-}$ mice increased after 4 and 8 wk of WTD (**A**). Characteristic dot plots of the MHC-II expression on the MC population show the peritoneal MCs that express protein MHC-II (**B**). All values ($n = 5$ per group) are depicted as mean ± SEM. *$p < 0.05$.

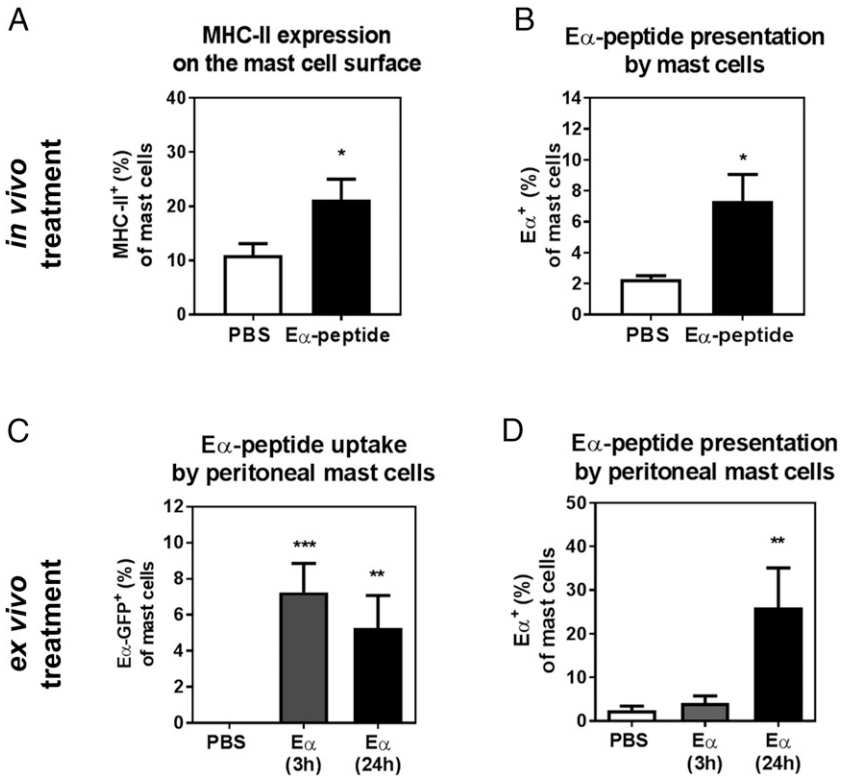

FIGURE 2. The MHC-II protein on the peritoneal MC surface is able to present epitopes of the Eα peptide upon WTD. LDLr$^{-/-}$ mice, fed a WTD for 4 wk, increased their MHC-II expression levels 24 h after i.p. injection of Eα-GFP, as compared with PBS-injected mice (**A**). The Eα peptide fragments were detected in vivo on the surface of peritoneal MCs, 24 h after Eα administration (**B**). Peritoneal cells isolated from the PBS and Eα-administered mice were further treated ex vivo with Eα-GFP$^+$ protein. Eα-GFP$^+$ uptake by peritoneal MCs was detected at 3 and 24 h after addition (**C**). E peptide fragments (Y-Ae^+) were observed 24 h later, on the peritoneal MC surface (**D**). All values (n = 10 per group) are depicted as mean ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

of MCs. We therefore used bone marrow–derived MCs to examine the effects of atherogenic serum from LDLr$^{-/-}$ mice fed a WTD for 4 wk compared with serum from chow-fed LDLr$^{-/-}$ mice. Unlike peritoneal MCs (38), bone marrow–derived MCs are not able to constitutively express MHC-II (39) unless induced with specific stimulation factors (24, 25). For that reason, we induced MHC-II expression on the MCs using IL-4 and IFN-γ (Supplemental Fig. 4C). This is not necessary in vivo, potentially because the inflammatory status of hyperlipidemic serum already contains these cytokines (40). Indeed, we observed that upon repeated treatment of MCs with WTD (4 wk) serum for 48 h, MCs increased their MHC-II expression levels by 2-fold as compared with chow serum (Fig. 3A; chow MC: 3.12 ± 0.3% versus WTD MC: 10.25 ± 0.1%; $p < 0.0001$). Furthermore, the costimulatory protein CD86, which is required for efficient T cell activation (41), was also increased on WTD MCs, as compared with chow MCs (Fig. 3B; chow MC: 1.73 ± 0.1% versus WTD MC: 2.53 ± 0.1%, $p = 0.0012$). Interestingly, when MCs were treated with fluid collected from the peritoneal cavity of LDLr$^{-/-}$ mice fed either chow or WTD (4 wk), they showed a marked increase in their MHC-II expression (Supplemental Fig. 4D). We measured the IL-4 and IFN-γ levels in the peritoneal cell fluid and observed that both cytokines are present (Supplemental Fig. 4E, 4F), indicating that the peritoneal cavity locally possesses the necessary signals for MHC-II induction on the MCs in vivo.

OVA-specific CD4$^+$ T cell proliferation is enhanced upon coculture with WTD-treated MCs

Next, we aimed to detect the direct effect of MC-mediated Ag presentation onto CD4$^+$ T cells. We isolated CD4$^+$ T cells from genetically modified OT-II mice that specifically recognize chicken OVA peptide in the context of MHC-II (34), and after 24 h of preactivation with anti-CD3/CD28 we placed them in a coculture with MCs preloaded with an OVA peptide in the temporary presence of 10% chow or WTD diet serum. OT-II CD4$^+$ T cells cocultured with both chow and WTD MCs increased their proliferation rate upon OVA peptide presentation, based on the incorporation rate of [^{3}H]thymidine. However, this increase was enhanced by 2-fold when OT-II CD4$^+$ T cells were cocultured with OVA peptide–loaded WTD MCs, as compared with coculture with OVA peptide–loaded chow MCs (Fig. 4A; chow MCs: 9033 ± 2558 dpm versus WTD MCs: 25331 ± 2889 dpm, $p < 0.0001$). The WTD effect of OVA peptide–loaded MCs on the proliferation rate of CD4$^+$ T cells was further confirmed by flow cytometry upon detection of the intracellular proliferation marker Ki-67 on the CD4$^+$ T cell population (Fig. 4B; chow MCs: 17.76 ± 0.09% versus WTD MCs: 25.56 ± 1.7%, $p = 0.004$).

Aortic CD4$^+$ T cells show reduced proliferation in MC-deficient mice

To verify whether MCs affect the CD4$^+$ T cell population in the atherosclerotic plaque, we placed MC-deficient apoE$^{-/-}$/Kit$^{W\text{-}sh/W\text{-}sh}$

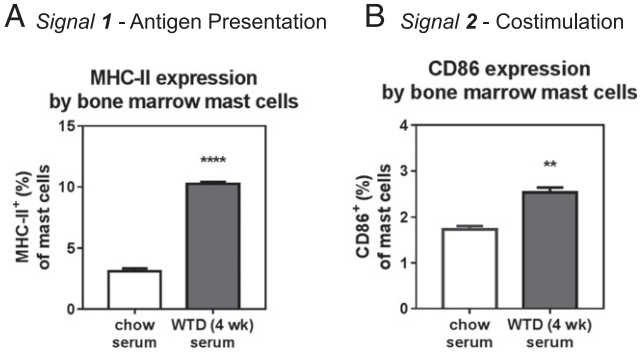

FIGURE 3. Inducible bone marrow–derived MCs repeatedly treated with WTD serum possess the signals required for Ag presentation. The expression of MHC-II on the surface of bone marrow MCs increased upon repeated (48 h) in vitro treatment with 10% serum isolated from LDLr$^{-/-}$ mice that were fed a WTD for 4 wk, as compared with chow serum (**A**). Costimulatory molecule CD86 was enhanced on the surface of MCs after treatment with 10% WTD (4 wk) serum from LDLr$^{-/-}$ mice in comparison with chow serum (**B**). All values are shown as mean ± SEM. **$p < 0.01$, ****$p < 0.0001$.

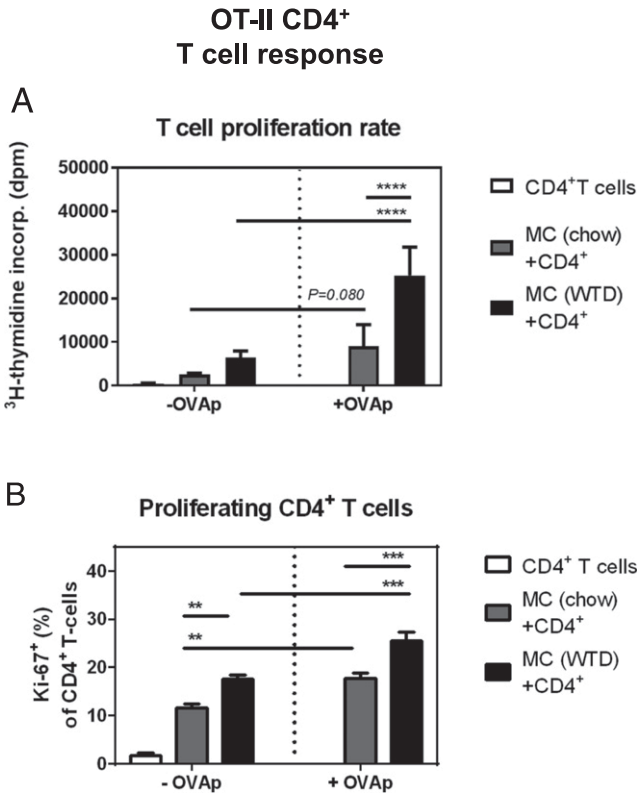

FIGURE 4. Bone marrow MCs, treated with WTD serum and loaded with OVA peptide, enhance the proliferation rate of preactivated OT-II $CD4^+$ T cells. OT-II $CD4^+$ T cells increased their proliferation upon presentation of OVA peptide (OVAp) by MCs treated with WTD, as compared with chow-treated MCs, based on radioactive thymidine incorporation and expression of proliferation marker Ki-67^+ (**A** and **B**). All values ($n = 5$ per group) are shown as mean ± SEM. $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

mice, which show significantly elevated (V)LDL cholesterol levels in their blood (36), on a WTD and compared the $CD4^+$ T cell content in the aortic arch with that from WTD-fed control $apoE^{-/-}$ mice. Using flow cytometry, we observed a reduction in the percentage of $CD4^+$ T cells in the aorta of atherosclerotic MC-deficient mice, as compared with control (Fig. 5A; $apoE^{-/-}$: 4.72 ± 0.8% versus $apoE^{-/-}/Kit^{W\text{-}sh/W\text{-}sh}$: 2.37 ± 0.7, $p = 0.028$). Moreover, the proportion of proliferating aortic $CD4^+$ T cells, as defined by the expression of Ki-67, was significantly reduced in atherosclerotic-deficient mice as opposed to control (Fig. 5B; $apoE^{-/-}$: 30.91 ± 5.2% versus $apoE^{-/-}/Kit^{W\text{-}sh/W\text{-}sh}$: 13.07 ± 1.2, $p = 0.013$). A similar trend was observed when examining the absolute number of proliferating $CD4^+$ T cells (Fig. 5C; $apoE^{-/-}$: 1725 ± 567 cells versus $apoE^{-/-}/Kit^{W\text{-}sh/W\text{-}sh}$: 389 ± 59 cells, $p = 0.056$). Interestingly, a significant decline in the number of T_{H1} cells was also detected in the aortas of MC-deficient $apoE^{-/-}$ mice as compared with control $apoE^{-/-}$ mice (Fig. 5D; $apoE^{-/-}$: 148 ± 25 cells versus $apoE^{-/-}/Kit^{W\text{-}sh/W\text{-}sh}$: 45 ± 16 cells, $p = 0.0042$).

Human intraplaque MCs express HLA-DR

Finally, to establish whether our murine data are relevant to the human disease setting, we analyzed the MC content of 20 end-arterectomy plaques collected from carotid or femoral arteries. Of note, we used flow cytometry for the detection of human intraplaque MCs. Specifically, in femoral as well as carotid arteries, MCs were detected by the expression of receptors FcεRIα and CD117 (Fig. 6A). Focusing on the Ag presentation capacity of MCs within atherosclerotic plaques, we stained the MCs for the expression of HLA-DR. We observed that MCs inside human atherosclerotic plaques express HLA-DR in both femoral and carotid arteries (Fig. 6B). Interestingly, the HLA-DR–expressing cells showed a negative association with the activated MC population, as detected by marker CD63 (42), which comprises the majority of MCs within the atherosclerotic plaque (Fig. 6C, Table I).

Discussion

In this project, we examined whether MCs are able to contribute to MHC-II–restricted activation of $CD4^+$ T effector cells in atherosclerosis. We observed that hyperlipidemic inflammatory conditions induced the expression of MHC-II by peritoneal MCs and, furthermore, that MCs were capable of presenting Ags in vivo and in vitro. Within the atherosclerotic plaques, MCs appear to augment the local T_{H1} response because their absence resulted in reduced $CD4^+$ T cell proliferation and a lower T_{H1} aortic cell content. Importantly, we showed in this study that MCs express HLA-DR in human atherosclerotic plaques and may therefore be capable of Ag presentation in human subjects.

The capacity of MCs to modulate effector T cell responses when influenced by inflammatory signals in their microenvironment is a process that has recently gained attention in the field (43, 44). Here we observed that mouse MCs increase their MHC-II levels, both in vivo and in vitro, in a WTD-specific manner. In accordance, we detected high levels of IFN-γ in the peritoneal fluid of hyperlipidemic mice. Furthermore, the absence of MCs significantly reduced the number of T_{H1} cells, the main producers of IFN-γ inside the atherosclerotic plaques (45). In the past, a study reported that rat MCs incubated with IFN-γ show increased MHC-II expression, arguing that aside from their classical role as degranulating effector cells, MCs may also be capable of presenting Ags and thus affecting T cell function (46). Additionally, peritoneal MCs in the presence of IFN-γ and IL-4 (24) have been shown to increase their MHC-II expression levels. The regulation of MHC-II by IFN-γ has previously been established in macrophage cell lines (47) but also in human MCs (48). Interestingly, human plaque tissue contains high amounts of IFN-γ (49), and patients suffering from coronary heart disease show increased levels of IFN-γ in their serum (50). This may explain why MCs increase their MHC-II expression specifically within an atherosclerotic environment.

In addition, we noticed that MCs can also express costimulatory signals, in the form of CD86, which are indispensable for Ag presentation. Specifically, WTD conditions increased CD86 expression. However, other costimulatory molecules could also participate in the described interaction with $CD4^+$ T cells. The presence of multiple costimulatory molecules on the MC surface, such as OX40L and CD80, was suggested to mediate direct communication with $CD4^+$ T cells (25, 51).

Importantly, in this study we establish that the MHC-II protein on the surface of MCs is a fully functional molecule that can bind and present Ags 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. Nonetheless, MCs are poor activators of naive T cells (25). In atherosclerosis, however, the majority of T cells infiltrating the plaque or the peritoneal cavity are previously activated and thus can be directly influenced by MCs. In this rationale, we aimed to study the direct interaction of MCs with preactivated $CD4^+$ T cells. Additionally, although the Eα and OVA peptides are model Ags that serve in exploring the presentation capacity of a cell, the WTD-specific induction of MHC-II raises the possibility of lipid-specific Ag presentation by MCs. It

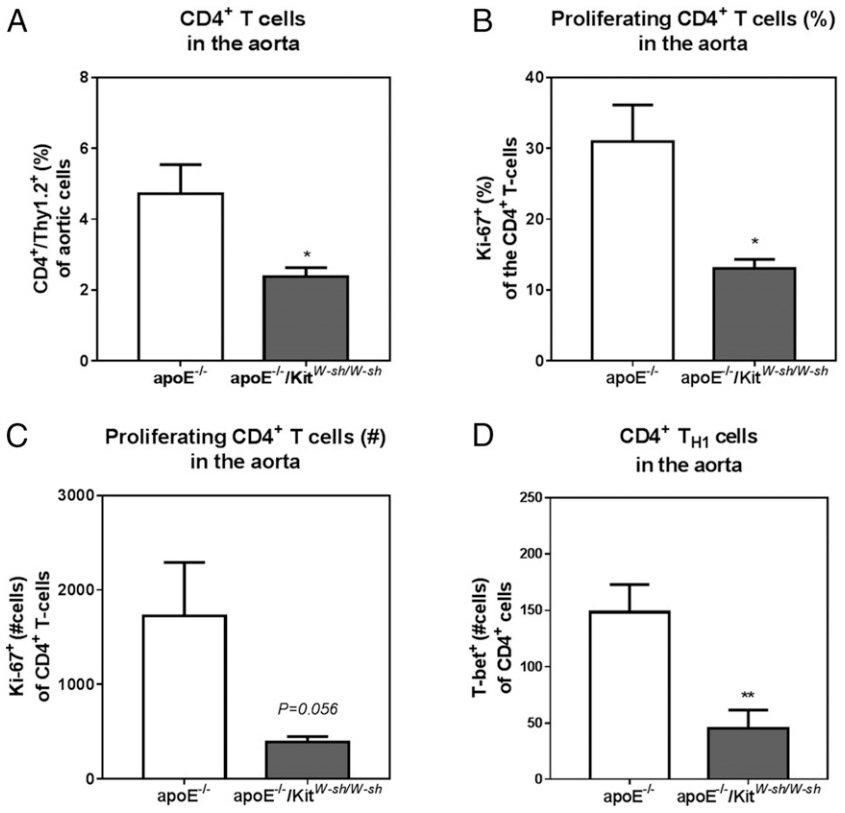

FIGURE 5. MC-deficient apoE$^{-/-}$/Kit$^{W\text{-}sh/W\text{-}sh}$ mice show a reduction in the proliferation of CD4$^+$ T_{H1} cells in the aortic arch. The CD4$^+$ T cell content was significantly decreased in the aortas of WTD-fed apoE$^{-/-}$ mice upon the absence of MCs (**A**). The percentage 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 wk as compared with apoE$^{-/-}$ mice (**B** and **C**). The number of T_{H1} cells in the aorta of MC-deficient apoE$^{-/-}$ mice was decreased, as measured by the expression of transcription factor T-bet on the aortic CD4$^+$ T cell population (**D**). All values (n = 8 per group) are depicted as mean ± SEM. *$p < 0.05$, **$p < 0.01$.

is worth mentioning that oxLDL is known to activate both macrophages and MCs (52). Also, oxLDL immune complexes can induce proatherogenic cytokine secretion by the MCs (53). In fact, the way by which MCs are activated within the atherosclerotic tissue has not yet been fully deciphered. Although the classical FcεR pathway is a plausible explanation, oxLDL can activate

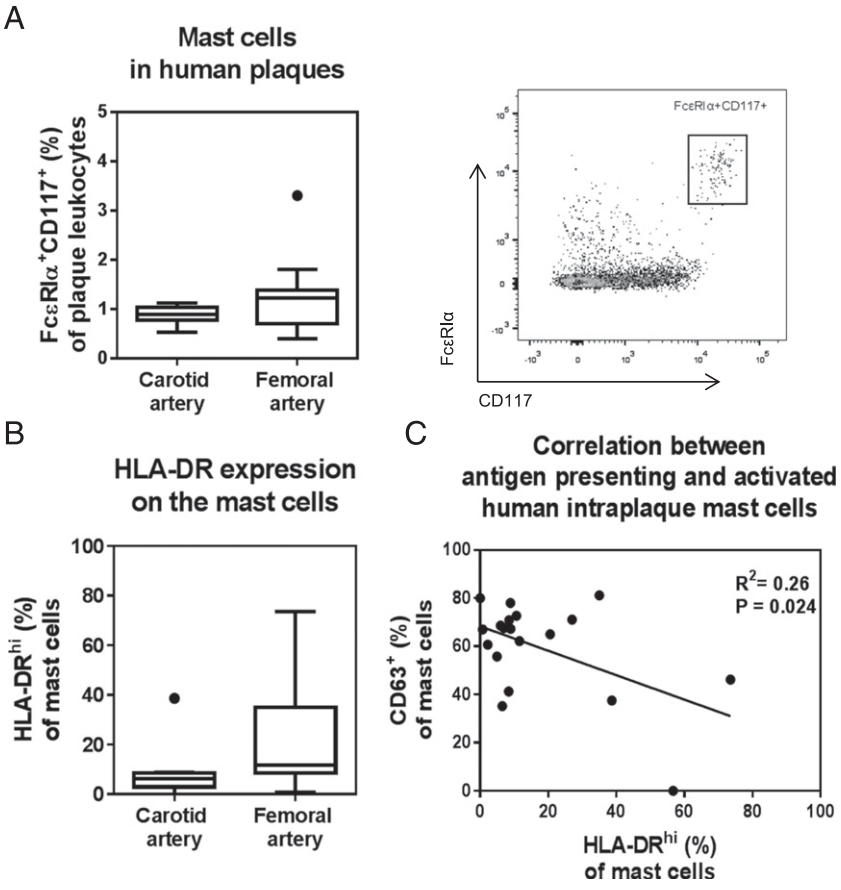

FIGURE 6. Human MCs from carotid and femoral atherosclerotic plaques express HLA-DR. The MC population in the plaques of human subjects, obtained from endarterectomy surgery, is detected based on the high expression of FcεRIα and CD117. Representative flow cytometry plots of human MCs are shown, gated according to the expression of receptors FcεRIα and CD117 (**A**). Human intraplaque MCs expressed HLA-DR in the femoral artery and carotid artery samples (**B**). The HLA-DR^{hi} MCs showed a negative correlation with the activated CD63$^+$ MC population in the atherosclerotic arteries (**C**).

Table I. List of extracellular and intracellular Abs used in all flow cytometry experiments

Targeted Species	Ab	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	Allophycocyanin/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/allophycocyanin	GL1	0.1 μg/sample	eBioscience
Mouse	Ea52-68	Biotin	EBioY-Ae	0.5 μg/sample	eBioscience
Mouse	Streptavidin	Allophycocyanin		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 600	eBio4B10	0.3 μg/sample	eBioscience
Mouse	*Ki-67*	FITC	SoIA15	0.3 μg/sample	eBioscience
Human	CD45	PE Cy7	2D1	0.3 μg/sample	eBioscience
Human	FcεRIα	Allophycocyanin	AER-37	0.1 μg/sample	eBioscience
Human	CD117	PercP Cy5.5	104D2	0.1 μg/sample	BD Biosciences
Human	HLA-DR	PE	L243	0.3 μg/sample	eBioscience
Human	CD63	PE	H5C6	0.1 μg/sample	eBioscience

Italics indicate intracellular Abs.

human MCs in a TLR4-dependent mode (54). This is important, considering that TLR4 signaling on APCs enhances Ag presentation (55). Interestingly, MCs have been previously reported to influence both the innate (56) and the adaptive immune response through TLR signaling (57). It would therefore be intriguing to see if an oxLDL/TLR-mediated pathway is shaping the Ag specificity of presenting MCs. Overall, it is interesting to investigate the means through which MCs may take up Ags from their surrounding atherosclerotic environment as, to our knowledge, there are no reports of MCs advancing into a foam cell state. Nonetheless, MCs have been reported in the past to affect intraplaque macrophages (58), whereas they were found to also interact with dendritic cells and actively exchange intracellular material and Ags (59) or even MHC-II molecules (60). In such a way, MCs can shape T cell responses in as much a direct as an indirect manner.

To gain a translational impression on the MC Ag presentation capacity, we applied flow cytometry to study human MCs within atherosclerotic plaques. It is known that human and mouse MCs share a multitude of characteristics, and although human MCs show a more complex secretome (61), their origin and tissue maturation processes are similar (62, 63). Furthermore, atherosclerosis studies on both murine and human MCs (64) highlight akin functions that may permit translation from rodents to humans. One example is the association of MCs with intraplaque hemorrhage reported in both mice (65) and humans (66). In our study, we observed that intraplaque MCs show high levels of CD63, an activation marker linked to the FcεR-mediated degranulation (42). However, their CD63 expression was negatively correlated to the HLA-DR MC-expressing population. Although this does not indicate a causal relationship, it suggests that MCs engaging in the presentation of Ags may not be activated at the same time. In the future, it would be of interest to explore this relationship between MC presentation and degranulation and to further address the differences in MC functionality between normal chow diet and high-fat diet conditions. Furthermore, HLA-DR expression by human MCs has also been previously reported in human tonsils and the authors suggest that it mediates Ag presentation by MCs to $CD4^+$ T cells (27). Importantly, a very recent study investigating the presentation capacity of human skin MCs provides additional evidence that these cells can also express HLA-DM in an IFN-γ–specific manner and directly present Ags to $CD4^+$ T cells, further shaping thus the T_{H1} response (67).

Finally, it is worth mentioning that examination of the Ag presentation capacity of MCs shows slight discrepancies in different studies with regard to their costimulatory molecule expression and influence on $CD4^+$ T cell responses (43). This diversity may originate from the differential in vitro generation of MCs from bone marrow progenitors but also from the different MC-deficient animal models. It is important to remember that MCs are found only within tissues, whereupon they undergo the last step of differentiation, influenced by local stimuli.

In conclusion, this study shows that MCs are capable of presenting Ags in a hypercholesterolemic environment and this pathway can potently modulate $CD4^+$ T effector cells toward a proatherogenic T_{H1} phenotype within atherosclerotic plaques. The Ag-presentation capacity of MCs in both mice and humans indicates that these cells can directly shape the adaptive immune responses, apart from exacerbating the innate inflammatory pathways. Further research on how to modulate this interaction may lead to novel ways to limit atherosclerosis development.

Disclosures

The authors have no financial conflicts of interest.

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