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Hyaluronan synthase 3 promotes plaque inflammation and atheroprogression

Susanne Homann[a,†], **Maria Grandoch**[a,†], **Lena S. Kiene**[a], **Yanina Podsvyadek**[a], **Kathrin Feldmann**[a], **Berit Rabausch**[a], **Nadine Nagy**[a,b], **Stefan Lehr**[c], **Inga Kretschmer**[a], **Alexander Oberhuber**[d], **Paul Bollyky**[b], and **Jens W. Fischer**[a,e]

[a]Institut für Pharmakologie und Klinische Pharmakologie, University Hospital, Heinrich-Heine-University Düsseldorf, Düsseldorf, Germany

[b]Division of Infectious Diseases and Geographic Medicine, Department of Medicine, Stanford Immunology, Stanford, USA

[c]Institute of Clinical Biochemistry and Pathobiochemistry, German Diabetes Center at the Heinrich-Heine-University Duesseldorf, Leibniz Center for Diabetes Research, Düsseldorf, Germany

[d]Department of Vascular and Endovascular Surgery, University Hospital Düsseldorf, Düsseldorf, Germany

[e]CARID, Cardiovascular Research Institute Düsseldorf, University Hospital, Heinrich-Heine-University Düsseldorf, Düsseldorf, Germany

Abstract

Objective—Hyaluronan (HA) is a prominent component of the provisional extracellular matrix (ECM) present in the neointima of atherosclerotic plaques. Here the role of HA synthase 3 (HAS3) in atheroprogression was studied.

Approach and results—It is demonstrated here that HAS isoenzymes 1, -2 and -3 are expressed in human atherosclerotic plaques of the carotid artery. In *Apolipoprotein E* (*Apoe*)-deficient mice *Has3* expression is increased early during lesion formation when macrophages enter atherosclerotic plaques. Importantly, HAS3 expression in vascular smooth muscle cells (VSMC) was found to be regulated by interleukin 1 β (IL-1β) in an NF*k*B dependent manner and blocking antibodies to IL-1β abrogate *Has3* expression in VSMC by activated macrophages. *Has3/Apoe* double deficient mice developed less atherosclerosis characterized by decreased Th1-cell responses, decreased IL-12 release, and decreased macrophage-driven inflammation.

Conclusions—Inhibition of HAS3-dependent synthesis of HA dampens systemic Th1 cell polarization and reduces plaque inflammation. These data suggest that HAS3 might be a promising therapeutic target in atherosclerosis. Moreover, because HAS3 is regulated by IL-1β, our results suggest that therapeutic anti-IL-1β antibodies, recently tested in human clinical trials

Correspondence to Jens W. Fischer: Institut für Pharmakologie und Klinische Pharmakologie, Universitätsklinikum der Heinrich-Heine-Universität Düsseldorf, Moorenstr. 5, 40225 Düsseldorf, Germany. jens.fischer@uni-duesseldorf.de.

[†]Equal contribution.

(CANTOS), may exert their beneficial effects on inflammation in post-myocardial infarction patients in part via effects on HAS3.

Keywords

Inflammation; Macrophage; T-cells; Atherosclerosis; Hyaluronan; Interleukin 1 beta

Subject codes

Vascular Biology

Introduction

Atherosclerosis is a chronic inflammatory disease initiated by endothelial dysfunction and LDL-triggered inflammation of the artery wall [1]. Inflammation in atherosclerosis is known to reflect interactions between cells, cytokines and chemokines, and the extracellular matrix. Monocytes/macrophages are the effector cells of the plaque associated chronic inflammation and a primary target in attempts to develop anti-inflammatory treatment options [2]. In the recent years disease modifying roles for neutrophils [3], T cells [4], and B cells [5] in plaque inflammation have been identified as well. Important factors that initiate, drive, and orchestrate the chronic inflammation are chemokines and cytokines. Among those interleukin (IL-)1β is now being targeted in clinical trials in an attempt to treat the “residual inflammatory risk” of patients that already receive the standard therapy according to international guidelines for patients at high risk for cardiovascular events [6].

The extracellular matrix (ECM) of atherosclerotic plaques has been well studied with respect to collagen and its contribution to the stability of the fibrous cap. However, important roles of non-collagenous matrix components such as sulfated glycosaminoglycans, proteoglycans, and hyalur-onan (HA) are emerging. HA is a matrix component that received so far less attention with respect to plaque inflammation but that could indeed be importantly involved in various aspects of the development of complex atherosclerotic lesions. So far it is known that accumulation of HA occurs in all clinically relevant manifestations of atherosclerosis such as primary symptomatic atherosclerosis, restenotic lesions, and eroded lesions [7–10].

HA is a unique glucosaminoglycan because it is not attached to a core protein as compared to e.g. proteoglycans and because it is synthesized at the plasma membrane – not in the Golgi apparatus and thereby rapidly creates an HA rich pericellular matrix. The HA synthesis is mediated by transmembrane HA synthase (HAS) isoenzymes 1–3. Work in smooth muscle cells (SMC) has shown in vitro that an HA rich microenvironment supports the migration of SMCs and their proliferation. Therefore, mainly these two functions have been attributed to HA during neointimal hyperplasia and atherosclerosis: proliferation and migration of SMC [11]. Evidence from animal studies support this role by showing proliferating SMC in HA-rich areas of the neointima [7] and decreased neointimal hyperplasia in *Has3*-deficient mice [12]. Therefore, it is thought that HA creates a permissive matrix for SMC-mediated neointimal hyperplasia.

The role of different HAS isoenzymes in models of atherosclerosis is only beginning to be understood. So far HAS2 overexpression in differentiated SMC has been shown to increase atherosclerosis and restenosis by affecting medial SMC which is in line with pro-proliferative and pro-migratory effects of HA as mentioned above [13,14]. On the other hand, pharmacologic inhibition of all isoforms of HAS, by use of 4-methylumbelliferone, caused damage of the endothelial glycocalyx and subsequently endothelial dysfunction and enhanced plaque inflammation leading to more atherosclerosis [15].

Importantly, in atherosclerosis also inflammation might be affected by the interstitial HA matrix. This concept has however not been explored so far and is the aim of the current study. The evidence that HA might be an important mediator of inflammation in atherosclerosis originates from investigations in other diseases, primarily lung injury and inflammatory bowel disease [16–18].

Therefore, we attempted in this study to identify HAS isoenzymes that convey important functions in the interstitial plaque matrix and may be involved in the modulation of plaque inflammation. This study shows that HAS3 is induced by IL-1β in vascular SMC and that HAS3 is critically involved in early macrophage driven inflammation. As proof of concept atherosclerosis is strongly decreased in *Has3*$^{-/-}$/*Apoe*$^{-/-}$ mice.

Results

HAS3 is stimulated by macrophages and IL-1β during atherosclerosis

Expression of all HAS isoenzymes was detected in human carotid atherectomy specimens of the carotid artery by immunohistochemistry and qPCR (Fig. 1A). These data suggest that HA is produced by multiple HAS isoforms in human atherosclerosis.

To better investigate the role of HA and HA synthases in the progression of atherosclerosis we next examined murine atherosclerotic lesions of *Apoe*-deficient mice. The time course of HAS isoenzyme expression, HA accumulation, and macrophage accumulation in these animals was monitored from 6 weeks until 44 weeks of age (Fig. 1). Interestingly, during early atherosclerosis primarily *Has3* is induced (Fig. 1B). *Has1* and *Has2* mRNA are increased much later during the time course of atherosclerosis (around 20 weeks) when *Has3* mRNA already steeply declined (Fig. 1B). This analysis also revealed that HA builds up within the lesions reaching a plateau at around 20 weeks and continues to be present at high levels (Fig. 1C). Of note the appearance of Mac2 positive macrophages precedes the strong induction of HAS3 at 10 weeks of age (Fig. 1D). Furthermore, IL-1β caused a rapid and dose dependent increase of especially HAS3 in human vascular SMC (Fig. 2A). The response to 10 ng/ml IL-1β in different isolates of human coronary SMC was in the range between 30 and 70 fold increase in comparison to unstimulated controls 3 h after stimulation. Other cytokines had much smaller effects on HAS3 mRNA expression. The second strongest effect was found for TNFα whereas IL-6 and IL-10 (Supplemental Fig. I) did not affect *HAS3* mRNA expression to a considerable degree. Accordingly, a blocking IL-1β antibody abrogated HAS3 induction by the supernatant of activated U937 cells in co-culture with human vascular SMC (Fig. 2B). HAS3 induction by IL-1β was mediated through NFκB as shown by the inhibitory effect of the inhibitor of IkBa phosphorylation,

Bay 11-7082, (Fig. 2B,C). The results led to our hypothesis that HAS3 is induced in atherosclerotic lesions by macrophages releasing cytokines such as IL-1β. Considering the proposed importance of HA during progression of atherosclerosis, HAS3 might be a novel and important target gene of therapeutic antibodies against IL-1β currently being tested in patients at high cardiovascular risk.

Has3$^{-/-}$/*Apoe*$^{-/-}$ mice develop less atherosclerosis

To follow up on the proposed importance of HAS3 during atherosclerosis, mice double deficient in *Has3* and *Apoe* (*Has3*$^{-/-}$/*Apoe*$^{-/-}$) were bred and compared to *Apoe* deficient (*Apoe*$^{-/-}$) littermates.

These mice showed no alterations in circulating LDL/VLDL and even decreased HDL concentrations (Supplemental Fig. 2). Importantly, *Has3/Apoe* double deficient mice developed considerably less atherosclerosis as evidenced by reduced atherosclerotic plaque score in the aorta and reduced lesion size in the brachiocephalic artery (Fig. 3A, B, C). To address possible mechanisms leading to reduced lesion size in the absence of HAS3 first the morphology of plaques at the aortic root was characterized at the end of the study in 23 week-old mice. The staining of the macrophage marker Mac2 was not affected in these mature plaques and the amount, distribution, and quantity of alphaSMA positive cells was also unaffected (Supplemental Fig. 3). This was confirmed by flow cytometric analysis of the aorta in 23 week old *Has3/Apoe* double deficient mice revealing no differences in the major leukocyte populations (Supplemental Fig. 4). A representative gating scheme of aortic immune cells is shown in Supplemental Fig. 5. Total collagen content (Supplemental Fig. 3), necrotic core size, and cell density (data not shown) were unchanged as well.

Reduced macrophage infiltration during early atherosclerosis

The results above showed that *Has3* induction occurs early in association with the macrophage appearance in murine atherosclerotic aortas and that - despite reduced plaque score - the plaque composition was unaffected in mature lesions of *Has3/Apoe* double deficient mice. Therefore, it was considered next that during early stages of atherosclerosis macrophage-mediated inflammation was affected by *Has3* deletion which might then be responsible for reduced atheroprogression seen in 23 week-old mice. For this purpose a detailed analysis of aortic immune cell subsets was performed after feeding a Western type diet for 4 weeks.

Indeed, FACS analysis of aortic immune cells after 4 weeks of feeding Western Diet (12 weeks of age) revealed a strong anti-inflammatory effect of the *Has3* deletion (Fig. 4). In particular, less aortic monocytes/macrophages and less neutrophils were detected in *Has3/Apoe* double-deficient mice (Fig. 4). This reduction in aortic macrophages occurred without changes in macrophage polarization as determined by FACS analysis of CD86 versus CD206 (Fig. 4H). In contrast to the aorta, circulating numbers of Ly6C^{low}, Ly6C^{intermediate} and Ly6C^{high} monocytes were unaffected (Fig. 5A). In order to test whether reduced monocyte/macrophage driven inflammation is specific for atherosclerosis in *Has3/Apoe* deficient mice, monocyte recruitment into the peritoneal fluid in response to thioglycollate

was investigated. Monocyte recruitment was indeed also reduced in this acute model of inflammation, as depicted in Supplemental Fig. 6.

The Th1-cell response is alleviated in *Has3*$^{-/-}$/*Apoe*$^{-/-}$ mice

Given that the impact of HAS3 absence on leukocyte recruitment was observed within multiple immune compartments, we sought to better define the impact of this deletion on systemic immunity. In the blood, monocytes as well as general B cell and T cell populations were not affected (Fig. 5A,B). However specifically circulating Th1 cells were strongly diminished in *Has3/Apoe* double-deficient mice (Fig. 5C) suggesting an effect on T cell polarization. In line with this effect on Th1 cells the concentrations of circulating IL-12 were reduced in plasma 15 week old *Has3/Apoe* double-deficient mice (Fig. 5E). In the past, the Th1 cell response has been shown to drive macrophage-mediated inflammation in atherosclerosis [19] and could therefore represent an underlying mechanism how deficiency of HAS3 decreases inflammation during atherosclerosis. To better characterize the decrease in Th1 cells specifically in the aorta of *Has3/Apoe* double-deficient mice, mRNA expression of key genes typical for the Th1 cell response were analyzed in the aorta of 12 weeks old *Has3/Apoe*-mice. We observed reduced expression of *Cd3e* and trends towards reduced *Tbx21* and *Il12b* were detected (Fig. 6) providing first evidence for reduced aortic Th1 cell responses. Furthermore, *Ccl5* and *Csf1r* mRNA were reduced confirming reduced monocyte recruitment.

Given the potential importance of T cells in the present study, HAS isoenzyme expression was determined in $CD4^+$ T cells and specifically in Th1 cells (Fig. 7A,B). We found *Has3* was expressed more strongly than *Has1* and *Has2* and *Has3* was more abundant in Th1- compared to Th2 cells (Fig. 7B). Whether IL-1β is a regulator of HAS3 expression in T cells was tested in isolated murine T cells from the spleen, which react to IL-1β with a rapid and robust upregulation of *Has3* (Fig. 7C). To test whether these findings could be explained by reduced activation, splenic T cells were isolated from WT- and *Has3/Apoe*-deficient mice and activated by anti-CD3/-CD28 beads (Fig. 8). No differences were observed in $CD4^+$ and $CD8^+$ T cells with respect to the activation markers CD25, CD44 or CD62L (Fig. 8) indicating that the phenotype of isolated T cells ex vivo is normal especially with respect to the response to these stimuli. Further, these results suggest that HAS3-mediated HA synthesis may regulate T cell function either in cooperation with non T cells such as dendritic cells [20] or that cross talk with other cells is required.

Discussion

Inflammation is a key process during progression of atherosclerosis from intimal thickening into complicated, symptomatic plaques. Among non-immune cells vascular SMC are essential because they interact with immune cells, transdifferentiate into macrophages [21], switch their phenotype towards a synthetic phenotype, and contribute to intimal hyperplasia by migration and proliferation. Therapeutically, the pharmacologic lowering of LDL cholesterol has been a breakthrough since the 4S-trial [22] and is complemented by the consequent treatment and/or avoidance of additional risk factors such as hypertension, obesity, diabetes mellitus type 1 and -type 2 as well as cigarette smoking. However, many

patients treated with statins still face the so called “residual cholesterol and inflammatory risk” [23].

The former calls for more aggressive LDL lowering and complementary treatment regimens optimizing lipid profiles whereas the latter led to the design of clinical trials addressing the effectiveness of primarily anti-inflammatory agents. As such methotrexate (Cardiovascular Inflammation reduction Trial, CIRT) and the use of the blocking IL-1β antibody Canakinumab (Anti-Inflammatory Thrombosis Outcomes Study, CANTOS) are currently being tested to reduce the event rate in patients at high risk of cardiovascular events. However, in addition to the general anti-inflammatory treatment options the exploration of novel targets within the ECM appears promising, because ECM modulates inflammation and in addition other crucial aspects of the pathophysiology of atherosclerosis such as plaque stability and lipid retention. ECM shapes the plaque microenvironment and thereby modulates the activity of inflammatory cells and determines the SMC phenotype. Furthermore, the stability and thrombogenicity of the plaque is critically dependent on the composition of the plaque ECM such as the collagen content of the fibrous cap. Up to now less is known about the composition and function of ECM in eroded plaques. However, interesting in the context of the findings in this manuscript is the fact that the luminal part of eroded plaques is devoid of collagen but rich in HA [9].

Here we show that HAS3 is an IL-1β target and that deletion of HAS3 strongly inhibits macrophage mediated plaque inflammation and atherosclerosis. Mechanistically, in *Apoe*$^{-/-}$-mice macrophages, HA accumulation, and HAS3 expression occur at the same time, suggesting that either macrophages stimulate HAS3 expression by SMC and/or that HAS3 further sustains macrophage-driven inflammation. On the one hand, the current data are in line with a local effect of macrophage-released IL-1β on HAS3 expression in vascular SMC. In the light of the previous results that HAS3 promotes neointimal hyperplasia in vivo and migration and proliferation of SMC in vitro [12] it is likely that IL-1β thereby also promotes SMC dependent aspects of atheroprogression because HAS3 will create an HA- rich microenvironment.

On the other hand, direct pro-inflammatory effects of HAS3 might be even more important for the progression of atherosclerosis in the current model. Our data suggest that these pro-inflammatory effects likely occur both locally in the plaque microenvironment and systemically. First, HA might serve as a TLR ligand within the plaque matrix and might directly stimulate the inflammatory response through toll like receptors (TLR) 2 and -4. The pro-inflammatory action of the HA/TLR2 axis [16] has been established in lung injury and the pro-atherosclerotic function of TLR2 and -4 has already been shown in atherosclerosis by use of TLR2 and TLR4 knock outs [24,25]. Furthermore, HA matrix in inflamed tissue might itself be adhesive and pro-inflammatory for macrophages due to the formation of supramolecular structures containing HA, versican, and hyaladherins. These cable-like HA structures that macrophages adhere to have been first described in intestinal SMC in response to viral mimetics and in inflammatory bowel disease [17] and have later also be discovered in vascular SMC [26,27]. Also T cells adhere to HA rich matrices provided by fibroblasts [28].

In addition to local pro-inflammatory effects the current data clearly indicate that HAS3 deletion in *Apoe*-deficient mice causes systemic anti-inflammatory effects by affecting T-cell polarization. Specifically, reduced circulating Th1 cells, reduced circulating IL-12, and reduced aortic expression of T cell and Th1 cell related genes were detected. In the past the Th1 cell response has been shown to drive macrophage mediated inflammation in atherosclerosis [19] and could therefore represent an underlying mechanism how deficiency of HAS3 decreases inflammation during atherosclerosis. Reduced Th1 responses could therefore be part of the underlying mechanisms for decreased macrophage recruitment to the lesions of *Has3/Apoe*-mice [19,29,30]. In addition, we show here that Th1 cells mainly express HAS3. The role of HAS3 in T cell mediated immunity is supported by the involvement of HA in the immune synapse and the binding of T-cells to antigen presenting cells [20]. Furthermore, in line with the present results it was shown that activation of autoreactive T cells and subsequent Th1 polarization is dependent on HA [31] in autoimmunity. Therefore, HAS3 could be required to provide the HA-matrix that is co-stimulatory for Th1 cell polarization in atherosclerosis. Interestingly, we show here that IL-1β also induces HAS3 in T cells which suggests that IL-1β induces a proinflammatory HA matrix response also in T cells themselves. This might be considered as a pro-inflammatory feed forward mechanism and might thereby represent an additional mechanism how IL-1β promotes inflammation in atherosclerosis.

In conclusion, HAS3 is crucial for atherogenesis in mice as shown in *Has3/Apoe* double-deficient mice. Mechanistically, the present data suggest that (i) HAS3 promotes macrophage-driven plaque inflammation likely by enhancing the Th1 cell response and (ii) HAS3 expression in the plaque is stimulated by IL-1β which might additionally drive inflammation and SMC-mediated plaque. This pro-inflammatory effect of the IL-1β/HA axis might be perpetuated by adherence of T cells and macrophages to the HA-rich plaque matrix and by the stimulation of HAS3 expression by IL-1β in SMC and T cells. All considered, HAS3 might be an important enzyme that shapes a pro-inflammatory and a pro-hyperplastic matrix microenvironment in atherosclerotic plaques and in lymphoid organs. Of translational importance appears the fact that HAS3 is an IL-1β target that could contribute to effects of therapeutic inhibition of IL-1β and should therefore be considered when interpreting the results of the current clinical trials on IL-1β inhibition.

Experimental procedures

Mice

In this study, male mice double-deficient for *apolipoprotein E* and *hyaluronan synthase 3* (*Has3*$^{-/-}$/*Apoe*$^{-/-}$) and respective control mice (*Apoe*$^{-/-}$) were used. *Has3*-deficient mice were generated by genOway (Lyon Cedex, France) as described by Kiene et al. [12] and then crossbred with *Apoe*-deficient mice (Taconic; Hudson, NY, USA).

All animals had open access to food and pathogen-free drinking water and were kept at a regular 12-hour day/night rhythm. The authorizations for conducting animal experiments were granted by the "*Landesamt für Natur, Umwelt und Verbraucherschutz* (*LANUV*) *Nordrhein-Westfalen, Bezirksregierung Düsseldorf*" according to § 8 of the animal welfare act as amended on May 18th 2006 (*Aktenzeichen* 84-02.04.2013.A434).

Male *Apoe*-deficient mice were kept on normal chow for up to 44 weeks of age. Mice were sacrificed at 6, 8, 10, 14, 19, 22, 25, 30, and 44 weeks of age and the aorta was excised from the aortic arch to the renal arteries for RNA isolation and qPCR analysis.

Starting at 8 weeks of age, male $Apoe^{-/-}/Has3^{-/-}$ mice and respective littermate $Apoe^{-/-}$ control mice were fed a western-type diet (WD) containing 21% fat and 0.15% cholesterol (S8200-E010; Ssniff Spezialdiäten GmbH, Soest, Germany). After feeding the mice with WD for 4, 7, and 15 weeks, respectively, mice were sacrificed and organs were harvested.

Thioglycollate injection

After 4 weeks of WD feeding, male $Has3^{-/-}/Apoe^{-/-}$ mice and respective littermate $Apoe^{-/-}$ control mice were injected with 3% sterile thioglycollate intraperitoneally (i.p.) to induce an acute inflammatory response. After 5 days, invaded cells were isolated from the peritoneal cavity by injecting 3 ml PBS i.p. followed by aspirating the fluids. Isolated cells were analyzed by flow cytometric measurements using CD45, CD11b, and Ly6C as monocyte markers.

Cell culture

For T cell isolation, axillary lymph nodes and the spleen of $Apoe^{-/-}$ and $Has3^{-/-}/Apoe^{-/-}$ mice were harvested and directly processed. To gain a single cell suspension, lymph nodes were incubated for 15 min at 37 °C under rotation in an enzymatic digestion mix and in parallel the spleen was dissociated in PEB buffer using the gentleMACS™ Dissociator (Miltenyi Biotec, Bergisch Gladbach, Germany). Afterwards, tissue suspensions were combined, filtered through 40 μm nylon filters (Greiner Bio-One, Frankfurt, Germany), and centrifuged at 500 ×*g* for 10 min at 4 °C. The cells were resuspended in PEB buffer and the cell number was determined using a Neubauer chamber. All following steps for T cell isolation were performed according to manufacturer instructions described for the Pan T cell isolation Kit II for murine cells (Miltenyi Biotec, Bergisch Gladbach, Germany).

Directly after T cell isolation, cells were cultured at a final density of 1×10^6 cells per ml and stimulated using the T Cell Activation/Expansion Kit for mouse T cells (Miltenyi Biotec, Bergisch Gladbach, Germany). The bead preparation and T cell activation was performed as described in the manufacturer's protocol. These particles are able to mimic T cell activation by antigen-presenting cells. The beads were applied in a bead-to-cell ratio of 1:1 so that 1×10^6 beads were added to 1×10^6 T cells. As a negative control, no beads were added to the T cells. Both stimulated and unstimulated T cells were cultivated for 24 h and 48 h and then harvested for flow cytometric measurements.

Isolation of human T cells from peripheral blood mononuclear cells, generation of T_H1 and T_H2 clones and quantitative qPCR were performed as described before [20].

Human coronary artery vascular smooth muscle cells

Human coronary artery smooth muscle cells (vascular smooth muscle cells, VSMCs) were obtained from PromoCell (Heidelberg, Germany) and were used between passages 3 to 9.

VSMCs were grown in Smooth Muscle Cell Growth Medium-2 (PromoCell) containing 10% FCS according to the instructions of the manufacturer.

VSMCs were stimulated with recombinant human interleukin-1β (Calbiochem, San Diego, CA, USA), tumor necrosis factor alpha (Biomol, Hamburg, Germany), interleukin 10 (Biomol), or interleukin 6 (Biomol), respectively. For co-culture experiments, differentiated U937 cells (PMA 100 nmol/l) were seeded in permeable transwell inserts (0.4 μm pore size) and cultured with VSMCs seeded in the bottom layer. U937 cells were stimulated with LPS (10 μg/ml) alone or in combination with control IgG, anti-IL-1β (10 μg/ml; R&D Systems, Minneapolis, MN, USA), BAY11-7082 (10 μm) or the combination of IL-1β and BAY-11-7082 followed by mRNA analysis in VSMCs. Primary murine T cells were stimulated with recombinant murine IL-1β from Peprotech (Rocky Hill, NJ, USA).

Flow cytometry

For flow cytometric analyses of immune cells, a broad spectrum of lymphoid, non-lymphoid tissues, and isolated cells were harvested and directly processed to get a single cell suspension. The preparation of a single cell suspension of the aorta was performed according to the protocol of Butcher et al. [32] with minor changes. To disrupt tissue structures and receive conditions for optimal epitope staining different buffers were used. To identify immune cells within the single cell suspension, antibodies were used in different combinations. A list of antibodies, clone, and manufacturer is depicted in Supplemental Table 1.

Circulating cytokines

EDTA-anti-coagulated (final concentration: 10 mM) blood was centrifuged for 15 min at 800 ×*g* at 4 °C. The supernatant was carefully removed and centrifuged for another 5 min at 15,700 ×*g* at 4 °C. Finally, the plasma was collected and stored at −80 °C for later analysis. Circulating cytokines and chemokines were analyzed in murine plasma using multiplex analyses to examine a broad variety of different cytokines within one sample. The Bio-Plex Pro™ Mouse Cytokine 23-plex Assay (BioRad, Hercules, CA, USA) was used according to the provided instructions with plasma samples diluted 1:4 to analyze cytokines concentrations. The measurement was performed with a Bio-Plex® 200 System (BioRad, Hercules, CA, USA) and the calculation of the concentrations were done with the Bio-Plex Manager Software™ (BioRad, Hercules, CA, USA).

Plasma lipids

The analyses of LDL/VLDL cholesterol, and HDL cholesterol were performed as specified by the manufacturer using the HDL and LDL/VLDL Qualification Colorimetric/ Fluorometric Kit (BioVision Inc., Milpitas, CA, USA).

Histology and immunohistochemistry

The heart and the brachiocephalic artery (BCA) were harvested and fixed for 24 h at 4 °C in Roti®-Histofix 4% (Carl Roth GmbH & Co KG, Karlsruhe, Germany). Subsequently, they were dehydrated and embedded in paraffin. Embedded hearts were sliced into tissue sections of a thickness of 5 μm and BCAs into 10 μm sections.

The picrosirius red staining, previously described by Puchtler et al. [33], was used to depict collagen within atherosclerotic plaques [15].

The primary and secondary antibodies for mac2 and α-smooth muscle actin stainings are listed in Supplemental Table 2.

Oil Red O (ORO) staining of whole aortas

After fixation in Roti®-Histofix 4%, aortas were cleaned from surrounding adipose tissue and stained with Oil-Red-O solution (Sigma-Aldrich, St. Louis, MO, USA). For analyzing lipid deposition, aortas were opened longitudinally and red staining was quantified using the ImageJ 1.45s Software (National Institutes of health, USA).

Gene expression

For gene expression analysis in murine cells, total RNA was isolated and qPCR was performed in a 96-well-plate using the Platinum® SYBR® Green qPCR SuperMix-UDG (Applied Biosystems, Foster City, CA, USA) containing a polymerase, SYBR® Green, dNTPs, and buffer. Primer pairs are given in Supplemental Table 3.

Total RNA was harvested from T-cells and DC using the RNeasy mini kit from Qiagen (Valencia, CA). cDNA was prepared from 350 ng total RNA reverse transcribed in a 40 μl reaction mix with random primers using the High-Capacity cDNA Archive Kit according to manufacturer's instructions. Relative quantification of TGFbeta-1 gene expression was performed using Taqman Gene Expression Assay Mm03024053_m1 and eukaryotic 18S rRNA Endogenous Control part no.4333760. Briefly, 1.2 μl cDNA was amplified in 1XTaqman Fast Universal PCR Mix with 250 nM Taqman probe in a 20 μl reaction using the Fast program for 50 cycles on an ABI7900HT thermocycler. All qPCR reagents were from Applied Biosystems, Foster City, CA. All samples were done in duplicate and data were analyzed using the Comparative Ct Method with software from Applied Biosystems. Estimated copy numbers were generated from a standard curve created by using a selected reference cDNA template and Taqman probe [29].

Human atherectomy specimens

Human atherectomy specimens were derived from patients undergoing an elective carotid endarterectomy after informed consent (Ethics approval number: 3944). Harvested tissues were directly transferred into liquid nitrogen for isolation of total RNA or formalin-fixed (4% paraformaldehyde) and embedded into paraffin. Paraffin embedded sections of atherectomy specimens were stained for the HAS isoenzymes HAS1, HAS2, and HAS3 in consecutive sections using HAS1 (G-17, sc-34021), HAS2 (S-15, sc-34067), HAS3 (E-15, sc-34204) primary antibodies (Santa Cruz, Dallas) and secondary anti-goat antibody (Vector). 3,3′-diaminobenzidine (DAB; Zytomed, Berlin, Germany) was used for the detection.

Statistical analysis

Statistical evaluation was performed using the GraphPad Prism Software Version 7.0 (GraphPad Software, La Jolla, CA, USA). All data are presented as mean ± standard error

mean (SEM) calculated from n independently performed experiments. Statistical outliers that were computed by means of the Grubb's test ($\alpha = 0.05$) were excluded from the analysis. For all data sets a Gaussian distribution was assumed. When comparing two experimental setups, data sets were analyzed using the unpaired, two-tailed student's *t*-test. If the f-test, which compares variances between two groups, was significant, the parametric, unpaired, two-tailed Welch's correction test was performed. To analyze two or more independent data sets over time, e.g. immune cell changes during 15 weeks of WD feeding, the two-way ANOVA was taken for statistical calculations. $p < 0.05$ was considered statistically significant. If not stated otherwise n represents independent in vitro experiments or individual animals.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.matbio.2017.09.005.

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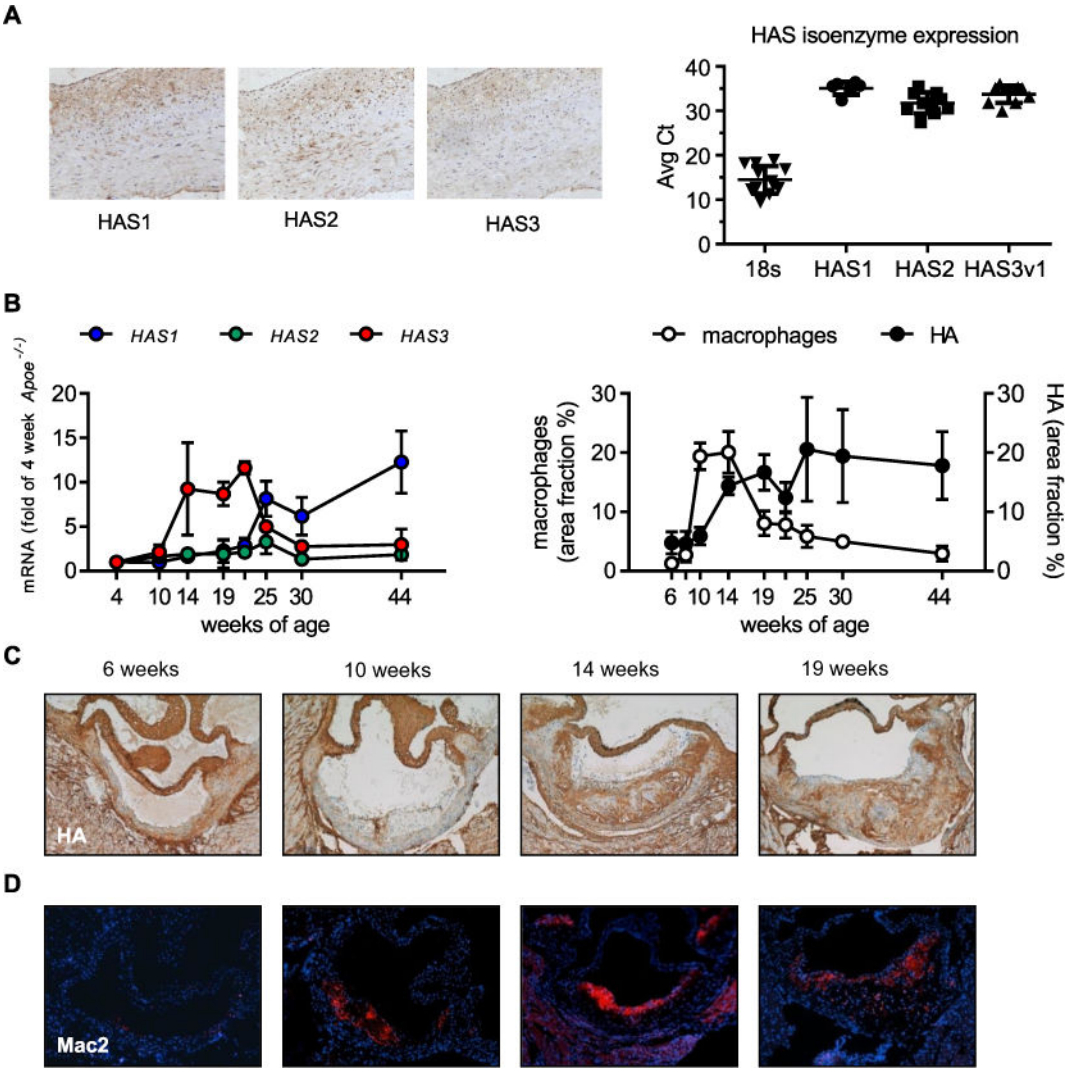

Fig. 1.

Detection of HAS isoenzymes in human atherosclerotic lesions and association of *Has3* expression with early macrophage accumulation in murine lesions. A Representative images of HAS1,-2 and -3 detection via immunohistochemical stainings and Ct values of *18S*, *HAS1*, *HAS2*, and *HAS3v1* in human atherectomy specimen as determined by qPCR; mean ± SEM; $n = 6–12$. B Left, mRNA expression of *Has1, Has2, and Has3* in aortas of $Apoe^{-/-}$ mice at different ages; $n = 2–3$; means ± SEM. Right, quantification of the area fraction of HA staining and Mac2 in aortic root sections at different ages of $Apoe^{-/-}$ mice; mean ± SEM; $n = 3–7$. C,D Depiction of HA (C) and Mac2 (D) stainings of aortic roots of 6-, 10-, 14-, and 19-week-old $Apoe^{-/-}$ mice.

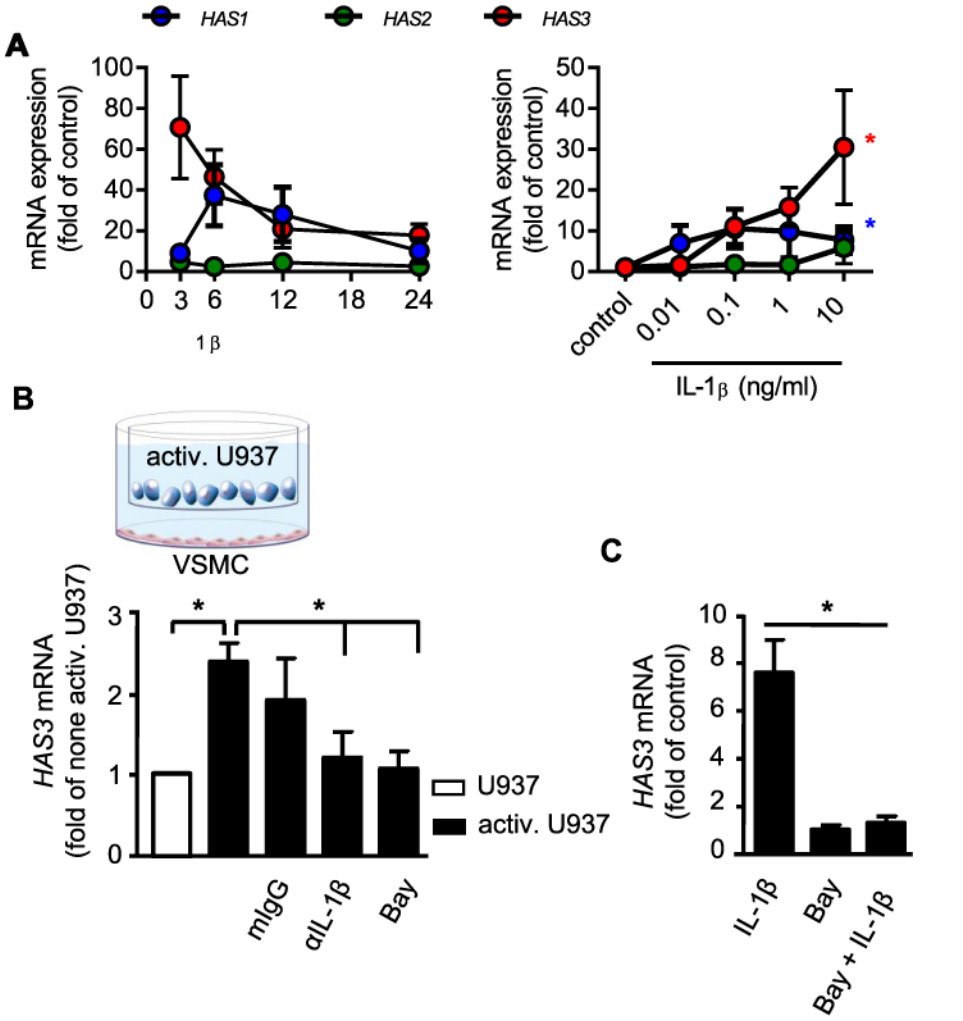

Fig. 2.

Activated macrophages induce *HAS3* expression in human vascular SMCs via IL-1β and NF-κB signaling. A Human vascular SMCs (VSMC) were stimulated with IL-1β and subsequently RNA was extracted and analyzed via qPCR. *Has* isoenzyme mRNA expression is expressed as fold of unstimulated controls. Left, *Has* mRNA expression after 3, 6, 12, and 24 h of stimulation with IL-1β (10 ng/ml). Right, IL-1β dose-dependent *Has* isoenzyme expression. RNA was isolated after 3 h of IL-1β stimulation; means ± SEM, n = 3–10; mRNA expression is expressed as fold of unstimulated controls; $*p < 0.05$ vs. control. B In a transwell insert LPS-activated U937 macrophages were co-cultured with VSMCs in the presence and absence of a control mIgG (10 μg/ml), a neutralizing IL-1β antibody (10 μg/ml), and the NF-κB inhibitor BAY11-7082 (10 μM), respectively. After 24 h of co-culture, *Has3* mRNA expression was analyzed in VSMCs; means ± SEM; $n = 3$; $*p < 0.05$. C VSMCs were incubated for 3 h with IL-1β, Bay11-7082 (Bay), or Bay11-7082 and IL-1β. Thereupon, *Has3* expression was assessed using qPCR; n = 3; $*p < 0.05$. Data are shown as mean ± SEM.

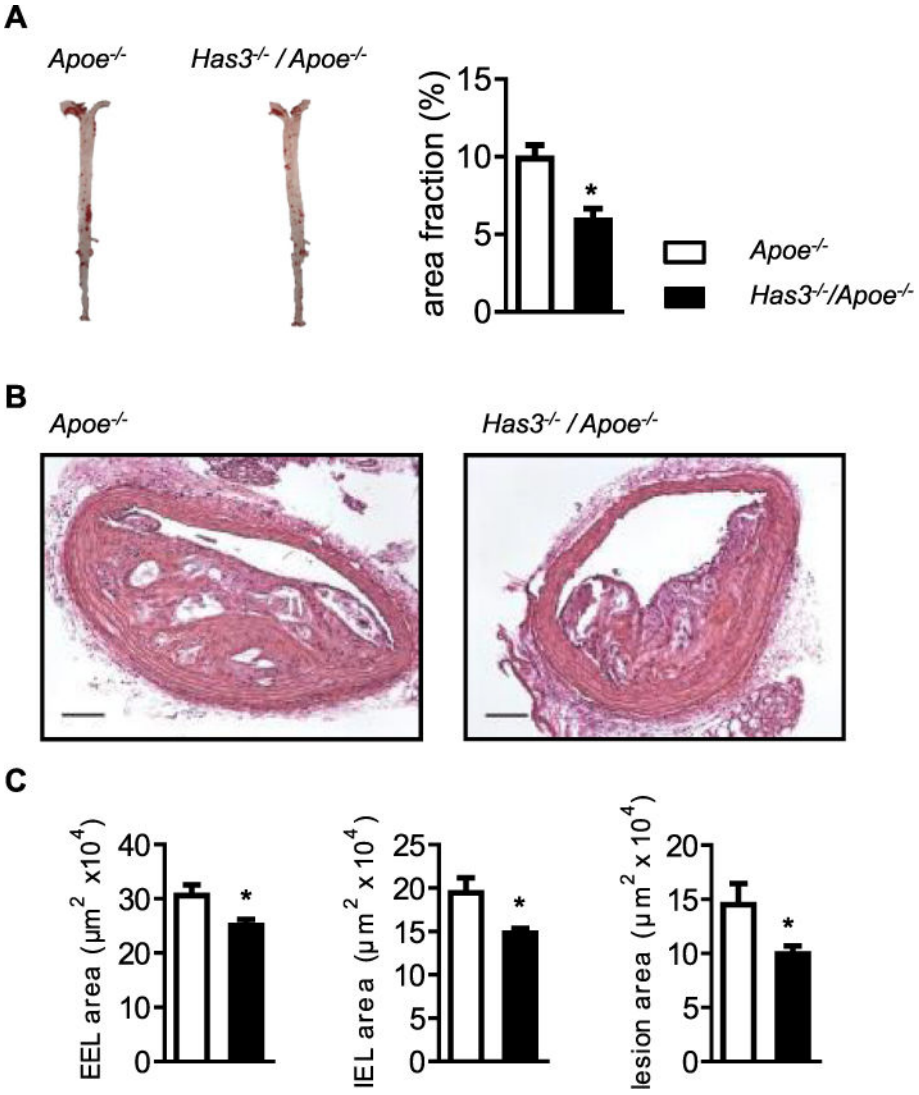

Fig. 3.

Has3$^{-/-}$/*Apoe*$^{-/-}$ mice have reduced aortic plaque burden and reduced lesion size A Aortic plaque score was determined by Oil-Red-O staining of aortas from *Apoe*$^{-/-}$ and *Has3*$^{-/-}$/*Apoe*$^{-/-}$ mice after 15 weeks of feeding WD (23 weeks of age). Representative images and quantification of *en face* and Oil-Red-O stained aortas are depicted; $n = 7$; mean ± SEM; $*p < 0.05$. B Representative images of an H&E stained brachiocephalic artery section from *Apoe*$^{-/-}$ and *Has3*$^{-/-}$/*Apoe*$^{-/-}$ mice, respectively. Scale bar: 100 µm. C Quantification of the area within the external elastic lamina (EEL), the internal elastic lamina (IEL), and the lesion area. Data are shown as mean ± SEM; $n = 6/5$; $*p < 0.05$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

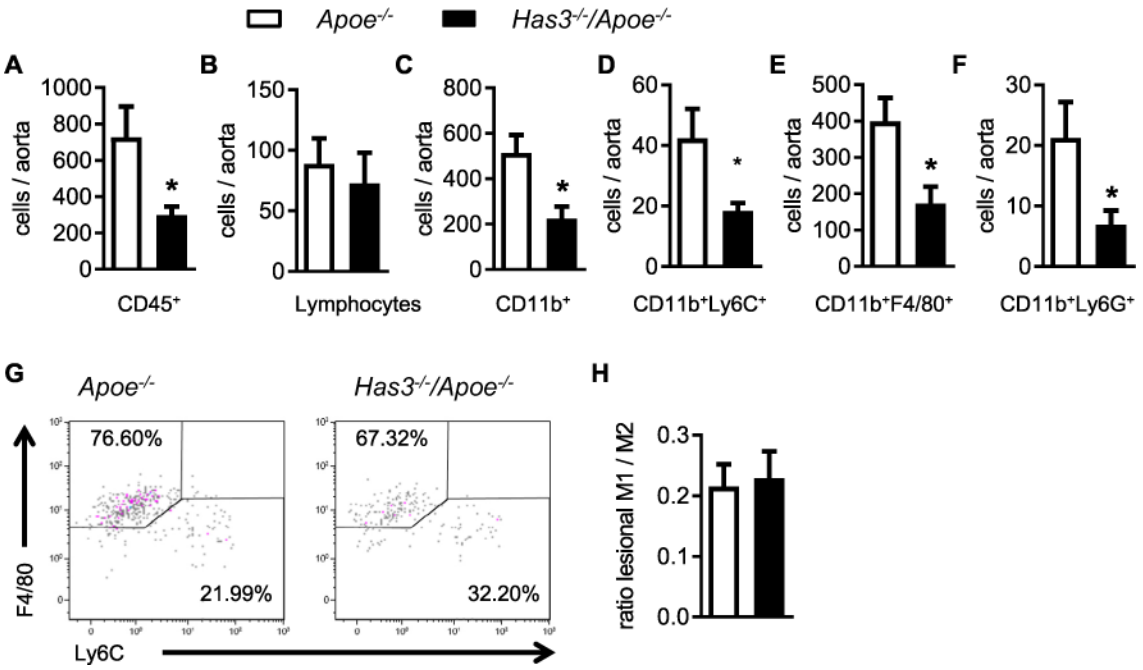

Fig. 4.

In early atherosclerotic lesions of *Has3/Apoe*-double deficient mice monocyte/macrophage accumulation is reduced After 4 weeks of WD feeding (12 weeks of age), aortic immune cell accumulation was analyzed in $Apoe^{-/-}$ and $Has3^{-/-}/Apoe^{-/-}$ mice by flow cytometry. Immune cells were detected by surface CD45 expression and then further discriminated into (A) $CD45^{+}SSC^{low}$ lymphocytes (B) and $CD11b^{+}$ myeloid cells. $CD11b^{+}$ cells were subdivided into $CD11b^{+}Ly6C^{+}$ monocytes (D), $CD11b^{+}F4/80^{+}$ macrophages (E), and $CD11b^{+}Ly6G^{+}$ neutrophils (F). (G) Representative plots of the flow cytometric analyses are depicted. (H) Ratio of $F4/80^{+}CD86^{+}$ M1 macrophages and $F4/80^{+}CD206^{+}$ M2 macrophages in atherosclerotic lesions was calculated. Data are represented as mean ± SEM; n = 3–7; $*p < 0.05$.

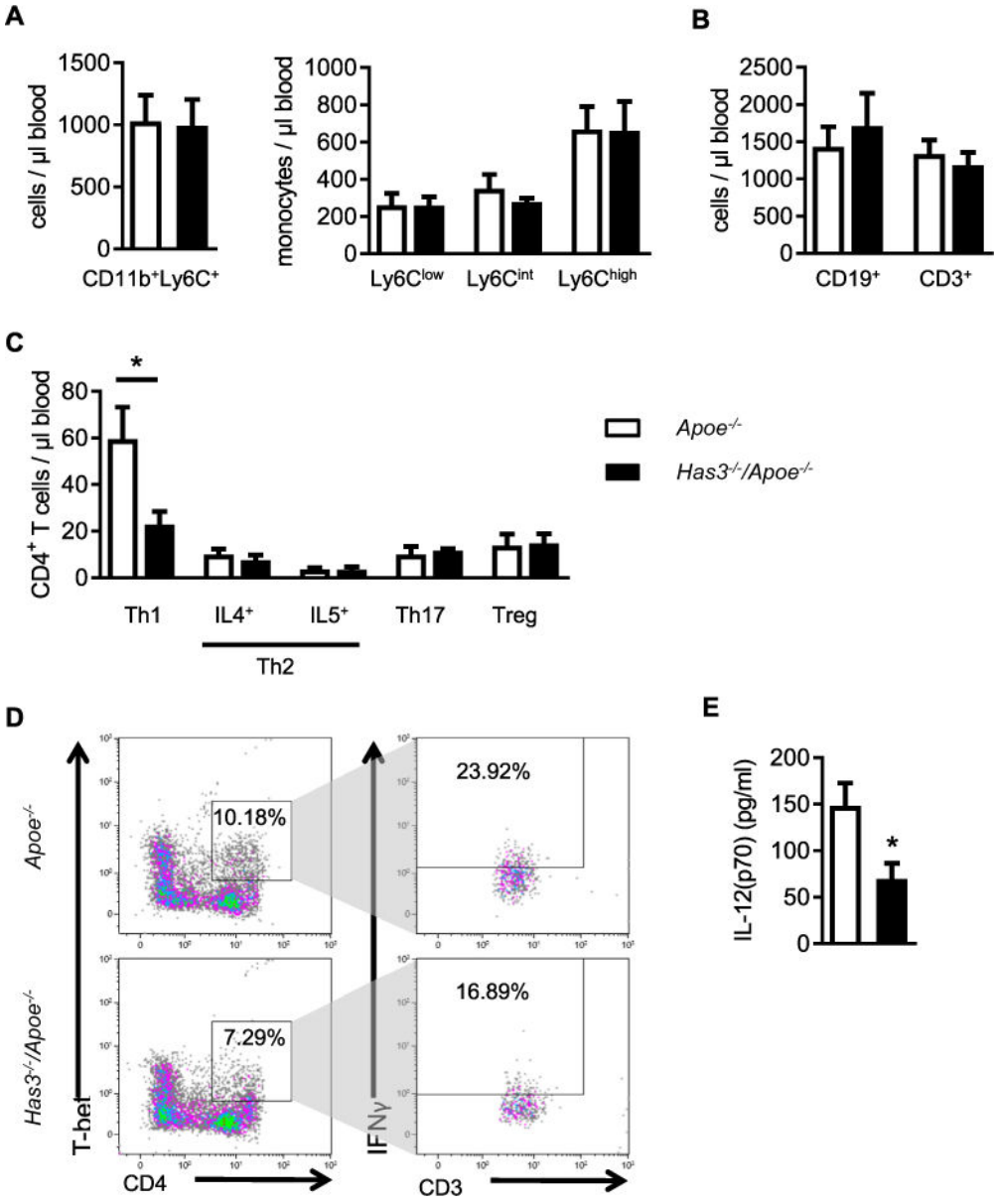

Fig. 5.

Reduced circulating Th1 cells in *Has3*$^{-/-}$/*Apoe*$^{-/-}$ mice Immune cells in the blood were investigated after 4 weeks of WD by flow cytometry. (A) The number of circulating CD11b^{+}CD115^{+} monocytes and the three monocyte subsets that differentially express Ly6C are depicted. (B) CD19^{+} B cells and CD3^{+} T cells; (C) different CD4^{+} T cell subsets, such as T-bet^{+}IFNγ^{+} Th1 cells, Gata3^{+}IL4^{+} and Gata3^{+}IL5^{+} Th2 cells, RORγT^{+}IL17A^{+} Th17 cells, and FoxP3^{+} T_{reg} cells. Data are shown as mean ± SEM; $n = 5–7$; *$p < 0.05$. (D) Representative plots for the determination of Th1 cells by flow cytometric analysis. (E) Plasma concentration of IL-12(p70) was analyzed after 7 weeks of WD feeding in *Has3*$^{-/-}$/*Apoe*$^{-/-}$ and control mice. Data are shown as mean ± SEM; n = 7,9; *$p < 0.05$.

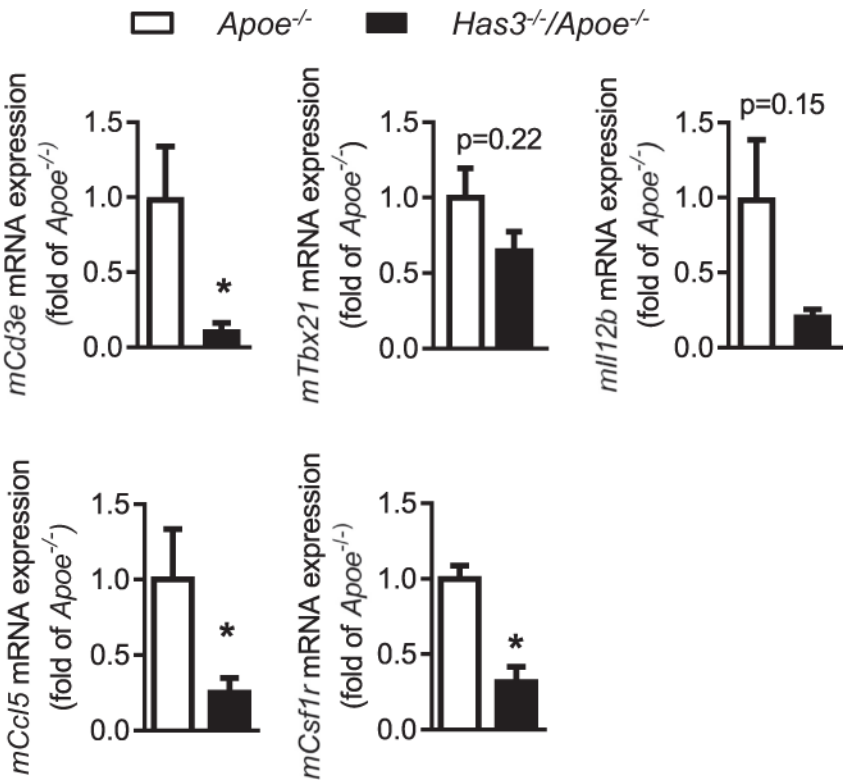

Fig. 6.

Has3-deficiency affects the cytokine expression in aortas. After 4 weeks of WD feeding, RNA was extracted from aortas of *Has3*$^{-/-}$/*Apoe*$^{-/-}$and *Apoe*$^{-/-}$ mice and analyzed using qPCR. (A) *Cd3e*, (B) *Tbx21*, (C) *Il12b*, (D) *Ccl5*, (E) *Csfr1*; n = 3–5; data are shown as mean ± SEM; *$p < 0.05$.

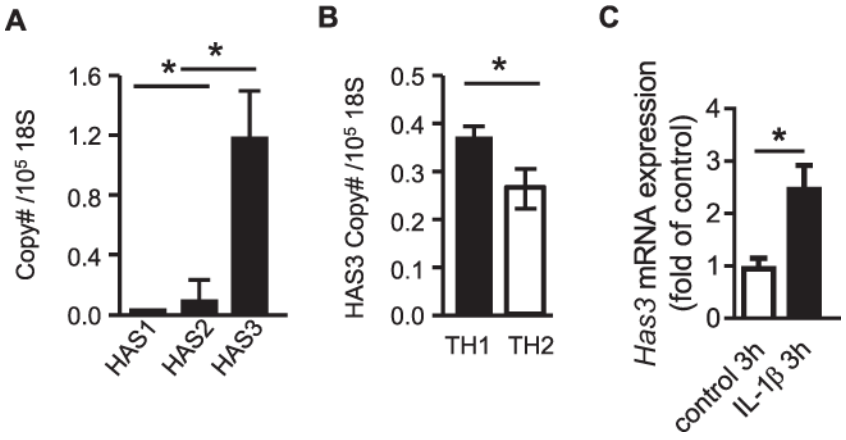

Fig. 7.

HAS3 in human and murine T cells. A, mRNA expression of the three hyaluronan synthases, *Has1*, *Has2*, and *Has3* by activated T cells normalized to 18S expression levels. Data are for pooled $CD4^+$ T cells isolated from 4 mice, activated using anti-CD3/28 beads, and measured in triplicate. B, *HAS3* mRNA expression by activated, well characterized T_H1 and T_H2 clones. The clones were previously described in Bollyky et al., Cellular and Molecular Immunology, 2010. Panel A includes 12 measurements for each gene; 3 mRNA assessments from each of 4 animals. The error bars shown represent the standard error of the mean. Panel B includes a total of 12 measurements; 3 mRNA assessments from a total of 4 human T-cell clones; 2 clones for each condition. The error bars shown represent standard deviation; $*p < 0.05$. C, $CD3^+$ T-cells were isolated from spleens of $Has3^{-/-}/Apoe^{-/-}$ deficient mice and $Apoe^{-/-}$ mice (8–12 weeks old) and stimulated with IL-1β (10 ng/ml) for 3 h. Subsequently *Has3* mRNA was analyzed by qPCR; means ± SEM; n = 6; $*p < 0.05$.

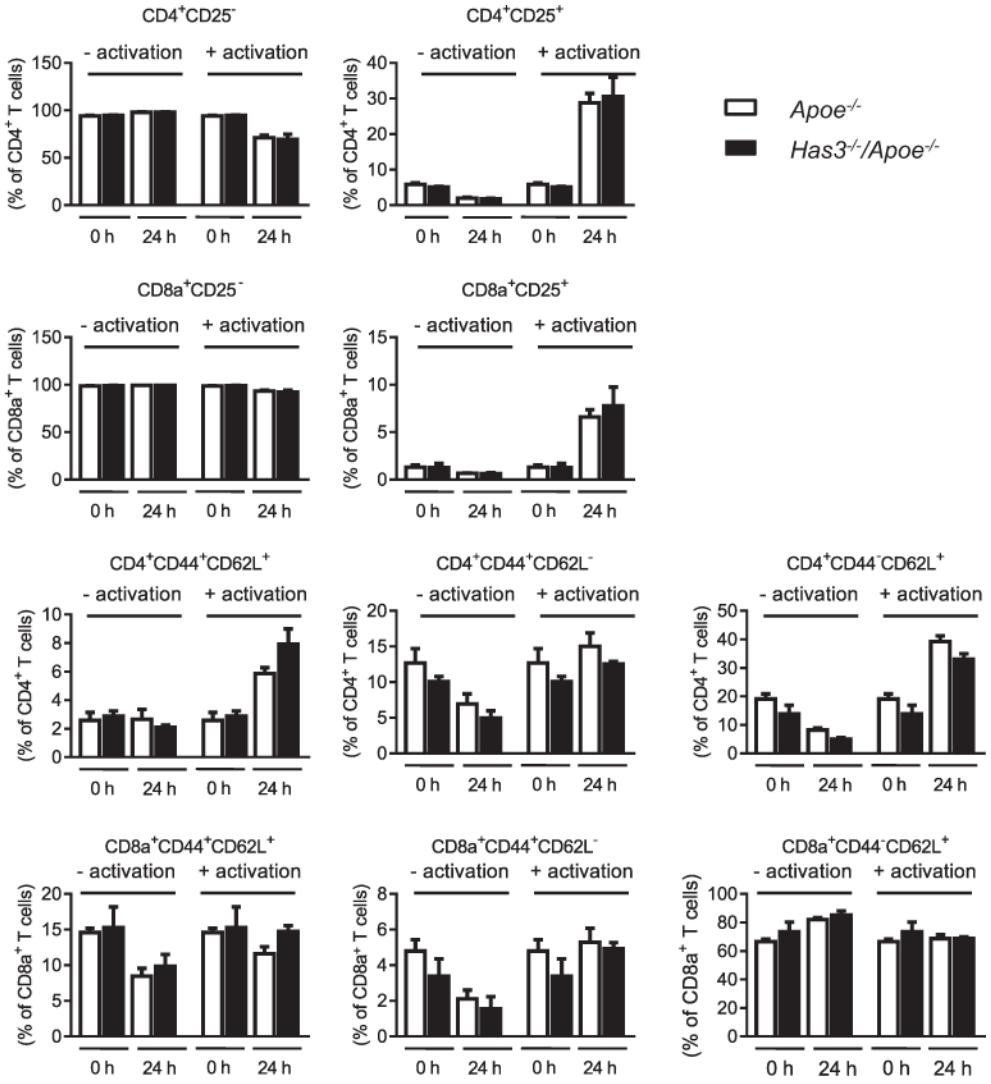

Fig. 8.

Has3-deficiency does not affect CD3-/CD28-induced stimulation of isolated T cells. In 12 week old mice (4 weeks on WD), T lymphocytes were isolated from the spleen and axillary lymph nodes of $Apoe^{-/-}$ and $Has3^{-/-}/Apoe^{-/-}$ mice and stimulated for 24 h with anti-CD3/CD28 beads. Activation of $CD4^+$ and $CD8a^+$ T cells was assessed by flow cytometric measurements of the surface antigens. As controls T cells were cultured without anti-CD3/CD28 beads for 24 h. Data are shown as mean ± SEM; $n = 4–3$.