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Resolving Lipid Mediators Maresin 1 and Resolvin D2 Prevent Atheroprogression in Mice

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ABSTRACT

_Rationale__:_ Atheroprogression is a consequence of non-resolved inflammation and currently a comprehensive overview of the mechanisms preventing resolution is missing. However, in acute inflammation, resolution is known to be orchestrated by a switch from inflammatory to resolving lipid mediators. Therefore we hypothesized that lesional lipid mediator imbalance favors atheroprogression.

_Objective__:_ To understand the lipid mediator balance during atheroprogression and to establish an interventional strategy based on delivery of resolving lipid mediators.

_Methods and Results__:_ Aortic lipid mediator profiling of aortas from *Apoe*$^{-/-}$ mice fed a high fat diet for four weeks, eight weeks, or four months revealed an expansion of inflammatory lipid mediators, Leukotriene B4 (LTB4) and Prostaglandin E2 (PGE2), and a concomitant decrease of resolving lipid mediators, Resolvin D2 (RvD2) and Maresin 1 (MaR1), during advanced atherosclerosis. Functionally, aortic LTB4 and PGE2 levels correlated with traits of plaque instability while RvD2 and MaR1 levels correlated with signs of plaque stability. In a therapeutic context, repetitive RvD2 and MaR1 delivery prevented atheroprogression as characterized by halted expansion of the necrotic core and accumulation of macrophages along with increased fibrous cap thickness and smooth muscle cell numbers. Mechanistically, RvD2 and MaR1 induced a shift in macrophage profile towards a reparative phenotype which secondarily stimulated collagen synthesis in smooth muscle cells.

Conclusions: We present evidence for the imbalance between inflammatory and resolving lipid mediators during atheroprogression. Delivery of RvD2 and MaR1 successfully prevented atheroprogression suggesting that resolving lipid mediators potentially represent an innovative strategy to resolve arterial inflammation.

Nonstandard Abbreviations and Acronyms:

DHA	docosahexaenoic acid
EPA	eicosapentaenoic acid
HFD	high fat diet
LTB4	Leukotriene B4
MaR1	Maresin 1
PG	Prostaglandin
RvD2	Resolvin D2
VPI	vulnerability plaque index

INTRODUCTION

Atherosclerosis is a chronic inflammation of the arterial vessel wall that can lead to severe clinical events. In advanced atherosclerosis a series of mechanisms involved in inflammation resolution fail: (1) the recruitment of inflammatory leukocytes is continuous and (2) accompanied by macrophage proliferation, whose phenotype exhibits pro-inflammatory traits and (3) hampered efferocytic ability. The concomitant increase in accumulation of apoptotic cells builds up a necrotic core which is followed by fibrous cap thinning, further adding to plaque vulnerability and eventually leading to rupture[1-4]. Resolution of inflammation not only involves limiting the cellular traffic, but also removal of apoptotic cells (by efferocytosis), cell phenotype alterations, tissue healing and return to homeostasis. In accordance, several resolving lipid mediators, in particular Resolvin D2 (RvD2) and Maresin 1 (MaR1), have been reported to have an effect on macrophage phenotype, favoring M2 markers[5, 6] and increasing efferocytic[6, 7] and phagocytic capacity[8], as well as increasing tissue regeneration[7]. Indeed with the identification of resolving lipid mediators the resolution process is no longer regarded as passive but rather dynamic[9], opening a new window for therapeutic intervention. In this context, the strong potential of pro-resolution strategies in atherosclerosis was recently shown by administration of the Annexin A1-derived Ac2-26 fragment[10, 11], a peptide that shares the FPR2 receptor with Resolvin D1[12]. However, regarding resolving lipid mediators, their possible mode of action and a general comprehensive view in atherosclerosis are lacking, in particular for Resolvins of D-series and Maresins, which are recent members of the lipid mediator family. Overall clinical trials and animal studies report on atheroprotective traits of Omega-3 fatty acids, particularly of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), the precursors of most resolving lipid mediators, namely lipid-lowering effects, and reduced lesional accumulation of macrophages and foam cell numbers[13-15]. Indeed DHA, the precursor for Resolvins D1 and D2 (RvD2)[16, 17] as well as Maresin 1 (MaR1)[8], reduces atherosclerosis in mice[18] and plasma DHA levels associate with reduced atheroprogression in coronary arteries in humans[19]. Similarly, 12/15 lipoxygenase, the enzyme responsible for the synthesis of Lipoxin A4 (LXA₄), RvD1 and MaR1 was also shown to be protective towards atherosclerosis[20], setting the stage for the indication that progression of atherosclerosis may be a consequence of failed resolution[21]. Given the associative evidence for atheroprotective effects of the precursors of resolving lipid mediators, and more recently of Resolvin E1 in ApoE*3Leiden mice [22], we here hypothesized that atheroprogression may partly be explained by an imbalance of the arterial lipid mediator profile. Therefore we set to investigate how the balance of these mediators changes during the course of atherosclerosis, aiming at identifying relevant resolution-inducing candidates. Our data indicate that arterial levels of MaR1 and RvD2 decrease during atheroprogression and that delivery of these two lipid mediators promotes plaque stability.

METHODS

Mice.

Apoe^{-/-} mice on C57Bl/6 background were fed a high-fat diet (HFD) containing 21% fat (ssniff) for 4 or 8 weeks, 3 months, or 4 months as indicated in the respective experimental description. For treatment studies, after 3 months of HFD a combination of MaR1 and RvD2 (each 100 ng, i.p., every second day) or vehicle was administered during additional 4 weeks of HFD feeding. The doses for MaR1 and RvD2 were chosen based on previously published studies.[23] All animal experiments were approved by the local ethical committee.

Liquid chromatography/tandem mass spectrometry (LC-MS/MS).

Aortic lipid mediators were measured by LC-MS/MS as previously described[24].

Statistics.

All data are expressed as mean±SEM. Statistical calculations were performed using GraphPad Prism 5 (GraphPad Software Inc.). After calculating for normality by D`Agostino Pearson omnibus test, unpaired Student's t-test, one-way ANOVA with Tukey' multiple comparison test, Mann Whitney test, Wilcoxon Signed-rank test, and Pearsons correlations were used as specified in each figure legend.

For detailed Materials and Methods, please consult the supplemental materials.

RESULTS

Atheroprogression is characterized by imbalance of inflammatory and resolving lipid mediators.

Apoe^{-/-} mice were fed a high fat diet (HFD) for four weeks, eight weeks, or four months, thus allowing for development of early[25] and advanced stages of atherosclerosis in mice[26]. To determine lipid mediator levels in atherosclerotic arteries at different stages of atherosclerosis, we subjected snap-frozen aortas from the *Apoe*^{-/-} mice fed a HFD to LC-MS/MS analysis (Figure 1). At all three time points we detected inflammatory lipid mediators including Prostaglandins (PGE2, PGD2, PGA1, 15d-PGJ2, and 8iso-PGA2), and Leukotriene B4 (LTB4). In addition, we could identify the resolution-inducing lipid mediators Resolvin D2 (RvD2) and Maresin 1 (MaR1), while Resolvin D1, Lipoxin A4, or Protectin Dx were below detection limit. Comparison of the measured lipid mediators during the course of atherosclerosis development showed a significant increase for most of the identified inflammatory lipid mediators with a 4.5-fold increase of LTB4 and a 3-fold increase of PGE2 between the 4 weeks and the 4 months high fat diet (Figure 1A). In contrast, resolving lipid mediators significantly decreased with prolonged high fat diet feeding with a 5-fold decrease of RvD2 and a 2.5-fold decrease of MaR1 between the earlier and the later stage of atherosclerosis (Figure 1B/C). Together these data indicate that the progression of atherosclerosis in hypercholesterolemic mice is associated with an imbalance between inflammatory and resolving lipid mediators.

Imbalance of aortic lipid mediators correlates with signs of plaque stability.

To relate aortic lipid mediator signatures to characteristics of atherosclerotic lesions, we assessed signs of lesion stability in aortic root sections in *Apoe*^{-/-} mice fed a high fat diet for four months and quantified lipid mediators in the aortas of the same mice. Herein, we measured lesion and necrotic core sizes, macrophages and smooth muscle cells, lesional collagen, and fibrous cap thickness. When integrating these parameters into a vulnerability plaque index (VPI), essentially a quotient of destabilizing (macrophages, necrotic core size) and stabilizing (smooth muscle cells, collagen) parameters, an integral

impression of plaque stability can be derived[27]. In addition, endothelial activation was assessed by measurement of ICAM1- and VCAM1-positive endothelial lining.

When correlating lipid mediator levels with the VPI, we found a significant positive correlation for PGE2 and LTB4, whereas the correlation between VPI and RvD2 as well as MaR1 was found to be negative (Online Table I). No correlation was found for VPI and PGD2, PGA1, 15d-PGJ2, or 8iso-PGA2. When assessing individual plaque characteristics we observed that PGE2 and LTB4 primarily correlated with lesion size and macrophage accumulation (Figure 2A/B & Online Table I). In stark contrast, RvD2 and MaR1 correlated positively with the number of lesional smooth muscle cells, collagen deposition, and fibrous cap thickness (Figure 2C/D & Online Table I). In addition, RvD2 and MaR1 correlated negatively with necrotic core sizes. Thus, these data suggest that aortic levels of LTB4 and PGE2 associate with signs of plaque instability, while aortic RvD2 and MaR1 levels associate with signs of plaque stability.

Administration of Resolvin D2 and Maresin 1 prevents atheroprogression.

The decline of both RvD2 and MaR1 during advanced stages of atherosclerosis as well as the correlation of RvD2 and MaR1 with traits of plaque stability led us to question whether a combined therapeutic delivery of RvD2 and MaR1 would allow arresting plaque progression. Therefore, we subjected hypercholesterolemic *Apoe*^{-/-} mice to an atheroprogression protocol (Figure 3A). Herein the baseline group was euthanized after three months of high fat diet feeding, time after which treatment was administered to the other two groups: a second group that received RvD2 and MaR1 (100 ng each, i.p., every second day) during a fourth month of high fat diet feeding, and a third group, treated with vehicle control instead. In mice receiving vehicle control, lesions increased in size and the VPI became progressively worse, a circumstance attributable to an expansion of the necrotic core and the accumulation of macrophages (Figure 3B-I). In contrast, treatment with RvD2 and MaR1 halted atheroprogression as assessed by VPI. Immunohistomorphologically, treatment with resolving lipid mediators reduced the formation of the necrotic core, decreased plaque macrophage content and increased the numbers of smooth muscle cells (Figure 3B-I). In line with the latter are the significantly higher levels of total collagen in the group receiving lipid mediators (Figure 3D), possibly contributing to a thicker fibrous cap (Figure 3E). No difference was observed in endothelial VCAM1 or ICAM1 expression (Online Figure I). Treatment with RvD2 and MaR1 did also not affect body weight, plasma lipid levels, nor blood cell counts as well as differential white cell counts in spleen, bone marrow, and lymph nodes (Online Figures II-V). Taken together, these data indicate that treatment with RvD2 and MaR1 suppresses atherosclerotic plaque progression.

Resolvin D2 and Maresin 1 dampen macrophage-instructed inflammation.

To address the mechanism of halted atheroprogression following MaR1/RvD2 treatment, we first assessed if the effect was systemic or local. Plasma levels of TNF, IL6, and IL13 were not significantly different between the two groups, whereas TGFβ was enhanced in the MaR1/RvD2-treated group (Online Figure VI). The latter finding may be supportive of pronounced lesional collagen deposition in these mice. In agreement with non-altered levels of inflammatory cytokines, the concentration of serum amyloid A, an acute phase protein, was not affected by MaR1/RvD2 administration (Online Figure VI). In addition, no changes in phenotypic markers on leukocyte subpopulations in the spleen, the bone marrow, and the blood were found (Online Table II). Together with unaltered leukocyte counts in blood, bone marrow, and spleen, these data suggest that the observed lesion phenotype does not primarily rely on a systemic but rather a local, lesion-inherent effect.

Therefore, and to better understand the lesional microenvironment, we conducted a whole transcriptome analysis of aortic RNA extracts. Following bioinformatics analysis, we identified changes indicative of a macrophage M2 polarization in MaR1/RvD2-treated mice (Online Figure VII). To assess macrophage phenotypes in atherosclerotic lesions in more detail, we stained aortic root sections with

antibodies to inducible NO synthase (iNOS) and CD206, indicators of an inflammatory M1 or a reparative M2 polarization, respectively. When comparing aortic root section from mice receiving a HFD for 4 weeks with those of 4 months we evidence a clear decrease in the fraction of M2-polarized macrophages with a concomitant increase in M1 macrophage numbers (Online Figure VIII), thus shifting the balance between these polarization patterns. Comparison of lesions of mice receiving resolving lipid mediators with lesions from vehicle-treated mice then revealed a clear increase of the M2 macrophage fraction upon MaR1/RvD2 treatment (Figure 4A). This phenotypic switch was further confirmed by qPCR of aortic RNA when quantifying *Retnla* (also known as *Fizz1*) and *Nos2* (Figure 4B). The action of MaR1 and RvD2 on macrophage phenotype was additionally studied in vitro. Herein, macrophages were treated with TNF and IFNγ to generate a lesion-mimicking macrophage profile. Multiplex cytokine analysis showed that a combination of MaR1 and RvD2 decreased the secretion of TNF and IL6 by macrophages, while increasing that of TGFβ (Figure 4C). Gene expression studies confirmed the pro-resolving effect of these lipid mediators, revealing a decreased expression of *Nos2* accompanied by enhanced expression of *Arg1* and *Tgfb1* (Figure 4D) in MaR1/RvD2-treated macrophages.

As treatment of atherosclerotic mice with MaR1/RvD2 affected not only lesional macrophages but also smooth muscle cells and collagen deposition (Figure 3), we questioned whether the resolving lipid mediators act directly on smooth muscle cells or if the effect is rather mediated through macrophages. In vitro studies in aortic smooth muscle cells showed that the mRNA expression of genes related to collagen expression and maturation (*Col1a1, Col3a1, P4ha1*), as well as inflammation (*Vcam1, Tgfb1*) was not directly affected by MaR1/RvD2 treatment (Figure 4E). However, when aortic smooth muscle cells were treated with the supernatant of TNF-activated macrophages or of macrophages activated with TNF in presence of MaR1/RvD2, we observed an increase in mRNA levels of genes involved in collagen production (*Col1a1, Col3a1*) in smooth muscle cells receiving the supernatant harvested from reparative macrophages (Figure 4F). In line herewith, collagen production was found to be increased in aortic smooth muscle cells after treatment with the supernatant of reparative macrophages when compared to the treatment with inflammatory macrophages (Figure 4G). Interestingly, when blocking TGFβ receptor on smooth muscle cells much of the effect of the supernatant derived from reparative macrophages was lost indicative of the importance of macrophage-borne TGFβ during collagen production (Figure 4G). Altogether, these data suggest that in the present disease model, MaR1 and RvD2 locally act on macrophages, dampening the local inflammatory phenotype and favoring a pro-resolving milieu. This in turn acts on the surrounding smooth muscle cells and induces the production of collagen that stabilizes the atherosclerotic plaque.

DISCUSSION

Atheroprogression has long been regarded as a chronic inflammation however recent concepts also view atherosclerosis as a consequence of failed resolution[3, 28]. Resolution is crucially orchestrated by a switch from inflammatory to resolving lipid mediators in the affected tissue[29], an event that is possibly impaired during atheroprogression.[21] Here we show that as atherosclerosis progresses inflammatory lipid mediators overwhelm their resolving counterparts. In the current disease model, increased levels of LTB4 and PGE2 directly correlate with plaque vulnerability whereas RvD2 and MaR1 are associated with plaque stability. With the decline of RvD2 and MaR1 during atheroprogression we chose to repetitively administer these lipid mediators as experimental therapeutic strategy. This setup allowed to halt atheroprogression as observed by reduced necrotic core sizes, thicker fibrous caps, and expansion of smooth muscle cells accompanied by reduction of macrophage accumulation. Central to this were changes in the macrophage profile from an inflammatory towards a reparative phenotype secondarily stimulating atheroprotective effects in smooth muscle cells (Online Figure IX).

In contrast to acute inflammatory situations, atheroprogression is characterized by the persistence of the initiating stimulus, including hypertension, hyperlipidemia, and oxidative stress[3, 28]. Interestingly, the synthesis of LTB4, driven by 5-lipoxygenase, depends on reactive oxygen species and is inhibited by nitric oxide[30], a milieu typically prevailing in progressing atherosclerotic lesions. In addition, the local cytokine environment favors the accumulation of inflammatory over reparative macrophages[31]. LTB4 and PGE2 are produced in abundance at sites of inflammation[32-34], suggesting their production by inflammatory macrophages. In accordance, metabololipidomic studies on human monocyte-derived macrophages show that M1 macrophages contain higher levels of LTB4 and PGE2, and, importantly, favor the synthesis of inflammatory lipid mediators, in comparison to their M2 counterpart.[35] Thus, and albeit pro-resolving lipid mediators are being temporarily synthesized throughout the course of chronic inflammation, the arterial inflammatory milieu during atheroprogression crucially shapes the imbalance of arterial lipid mediators. Such an observation is also herein grounded by the prevailing M1 macrophage phenotype found in the atherosclerotic plaque of vehicle-treated mice, in comparison to the M2 phenotype found in MaR1/RvD2-treated group as well as the content of reparative macrophages found within the lesion at early stages of the disease (mice subjected to HFD feeding for four weeks). Mechanistically, LTB4 potentiates atherosclerosis by increasing the expression of fatty acid translocase/CD36 and of CCL2 hereby inducing a positive feed-back loop to recruit leukocytes[36, 37]. PGE2, as well as PGD2, on the other hand have been reported to have a dual, and paradoxical, role, functioning as pro-inflammatory and pro-resolving mediator, depending on its location.[38] In the context of atherosclerosis, PGE2 is associated with disease progression, mediated by activation of platelets[33] and consequently monocyte recruitment [39]. In general lipid mediators are mostly produced by myeloid cells, where often the same cell can be source of both classes of lipid mediators. Under inflammatory conditions neutrophils[40, 41] and macrophages[35, 42] preferentially synthesize LTB4 and PGE2 over MaR1 and RvD2. Also endothelial[43] and epithelial cells[44] add to the pool of LTB4 and PGE2 production. Upon a switch towards resolution, macrophages (reparative) as well as neutrophils (possibly second wave neutrophils) also synthesize Maresin-1[8] and Resolvin D2[35, 45]. However, during the course of a chronic inflammation, such as atherosclerosis, it is likely that overall the inflammatory environment prevails, favoring the synthesis of inflammatory lipid mediators.

The observed imbalance of arterial lipid mediators raised the question whether the administration of the decreased resolving mediators (MaR1 and RvD2) prompts inhibition of atheroprogression, thus possibly disclosing a novel therapeutic concept in the context of atherosclerosis. Although MaR1 and RvD2 share leukocyte-related functions few observations point out in the direction of distinct roles for these pro-resolving lipid mediators[9]. Hence, we have administered a combination of MaR1 and RvD2 as a therapeutic approach in our disease model. Indeed mice receiving resolving lipid mediators exhibited lesions with stability traits comparable to the baseline group, with additional improved smooth muscle cells numbers, collagen content, fibrous cap thickness and increased numbers of reparative macrophages. In line with previous reports [5, 6, 8], our in vitro studies confirmed that RvD2 and MaR1 have the ability to skew macrophage phenotype towards resolution-like, with increased TGFβ production and secretion. In accordance, and much like the lesion phenotype observed in the present study, macrophage-borne TGFβ gives rise to lesions characterized by smooth muscle cell accumulation, collagen deposition, and lower macrophage numbers[46]. In vitro, MaR1 and RvD2 reduced the secretion of pro-inflammatory cytokines by macrophages, an effect that was earlier reported for Resolvin D2[17]. Hence, overall the actions of Maresin-1 and Resolvin D2 on macrophages fully support a dampening of the inflammatory milieu within the lesion. Resolving lipid mediators are mostly found in self-resolving exudates, suggesting their synthesis by M2 macrophages.[5, 16] In agreement, M2 macrophages produce more pro-resolving lipid mediators than M1.[35] These observations then suggest a positive feedback loop, by the administration of MaR1 and RvD2, that further feeds resolution of inflammation and halts atheroprogression. Indeed, positive loops of resolution have been previously reported[47], such as the stimulation of synthesis of Lipoxin A4 by Resolvin E1[48]. MaR1 and RvD2-treated mice exhibited lesions with increased levels of collagen, as compared to vehicle-treated group. Collagen within the atherosclerotic plaque mainly originates from smooth muscle cells. Despite MaR1 and RvD2 being able to directly exert effects on smooth muscle cells[23], in the current study, the

increased collagen levels observed within the lipid mediator treated-group were explained by direct actions of MaR1 and RvD2 on the macrophages, which in turn, by changing the surrounding milieu, stimulated collagen production by smooth muscle cells.

Despite the success of lipid-lowering statins, anti-platelet or anti-hypertensive compounds, mortality from cardiovascular disease remains high and development of specific plaque stabilization therapies is a major challenge in preventive cardiology. The current inflammation focused understanding of atherosclerotic plaque destabilization has led to the emergence of numerous pre-clinical developments. However, recent examples of obstacles in the clinical phase epitomize the pitfalls inherent to cardiovascular drug development. *E.g.* neutralization of TNF, a signature cytokine released from activated macrophages, does not reduce the risk for acute coronary syndrome. There are many such examples available[4], showing that recent strategies targeting inflammation in the context of plaque destabilization are full of intrinsic obstacles and alternative approaches are required. In addition anti-inflammatory therapies mostly antagonize specific pathways engaged upon inflammation, and in spite several ongoing clinical trials and their beneficial effects at early stages of inflammation, anti-inflammatory approaches can be immunosuppressive and limit repair.[29, 49, 50] The transfer of concepts of resolution from bench to bedside requires a shift from inhibitory therapy to replacement therapy, *i.e.* from antagonism to agonism. While anti-inflammatory actions are limited to an inhibitory response, pro-resolution involves stimulation and initiation of processes such as apoptosis, efferocytosis, changes in macrophage phenotype and repair. Moreover, pro-resolution mediators have been associated with positive feedback loops, where one pro-resolving mediator induces another.[47] On the contrary, anti-inflammatory drugs, such as those inhibiting COX-2, have been reported to delay resolution of inflammation, by disrupting the production of anti-inflammatory prostaglandins and lipoxins [51-54]. Thus, our study is in support of the potential translational relevance of resolution-inducing agonists in cardiovascular disease progression.

In summary, delivery of resolving lipid mediators halts inflammation possibly by instructing a positive feed-back loop centered on the induction of reparative macrophages giving rise to a homeostasis-restoring environment promoting plaque stability.

DISCLOSURES
None.

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FIGURE LEGENDS

Figure 1: Imbalance of aortic lipid mediators fosters atheroprogression. *Apoe*^{-/-} mice were fed a high fat diet for 4 weeks, 8 weeks or 4 months and inflammatory (A) or resolving (B) lipid mediators were quantified in aortic lipid extracts using liquid chromatography and tandem mass spectrometry (LC-MS/MS). Representative chromatographs for Resolvin D2 and Maresin 1 at 4 weeks and 4 months are displayed (C). Data are expressed as mean±SEM. One way ANOVA with Tukey' multiple comparison test. * p<0.05, ** p<0.01, *** p<0.001 compared to 4 weeks high fat diet. n=24 aortas for 4 weeks high fat diet, 16 for 8 weeks and 19 for 4 months high fat diet. LTB4, Leukotriene B4; MaR1, Maresin 1; PGA1, Prostaglandin A1; 8iso-PGA2, 8iso-Prostaglandin A2; PGD2, Prostaglandin D2; PGE2, Prostaglandin E2; 15d-PGJ2, 15-deoxy-$\Delta^{12,14}$-Prostaglandin J2; RvD2, Resolvin D2.

Figure 2: Aortic lipid mediators correlate with signs of plaque stability. *Apoe*^{-/-} mice were fed a high fat diet for 4 months and lipid mediators were quantified in aortic lipid extracts using liquid chromatography and tandem mass spectrometry. Lipid mediator levels were correlated with atherosclerotic lesion characteristics assessed in aortic root sections of the same mice: lesion size (HE staining), macrophage number (Mac2 staining), endothelial activation (endothelial ICAM1 and VCAM1 staining), necrotic core size (HE staining), smooth muscle cell numbers (αSMA staining), collagen amount (Sirius red staining), and fibrous cap thickness (Sirius red staining). Displayed are radar charts where the axis represents the Pearson correlation coefficient r (-1 indicates maximal negative correlation, 0 indicates no correlation, +1 maximal positive correlation) for each lesion characteristic. Axes are only labelled when a significant correlation (p≤0.05) was found. Shown are radar charts for Prostaglandin E2 (PGE2, A), Leukotriene B4, (LTB4, B), Maresin 1 (MaR1, C), and Resolvin D2 (RvD2, D). Detailed explanation of figure outline is displayed in (E). Representative images for HE staining, Sirius red staining, Mac2 staining, and αSMA staining of plaques obtained from mice with high amount of inflammatory lipid mediators (top) or with high amount of resolving lipid mediators (bottom) are shown in (F). Note that different intensities in HE and Sirius red staining do not impact on quantification, as area and not intensity is measured. Black scale bars indicate 200 μm, white scale bars indicate 50 μm. Data are calculated as Pearson correlation from 19 mice.

Figure 3: Treatment with Resolvin D2 and Maresin 1 prevents atheroprogression. (A) Experimental outline. *Apoe*^{-/-} mice were fed a high fat diet. After three months the baseline group was harvested. The remaining two groups received high fat diet for another month. During this time one group was treated with PBS while the other group was treated with a combination of RvD2 and MaR1 (each 100 ng/mouse, i.p., every second day, last 4 weeks of high fat diet feeding) (B) Atherosclerotic lesion size. (C) Necrotic core size. (D) Lesional collagen. (E) Fibrous cap thickness. (F) Quantification of smooth muscle cells. (G) Display of lesion macrophage counts. (H) Calculation of vulnerability plaque index (VPI). (I) Representative images of HE staining, Sirius red staining, αSMA staining, and Mac2 staining are displayed. Black scale bars indicate 200 μm, white scale bars indicate 50 μm. One way ANOVA with Tukey' multiple comparison test was used to calculate statistical significance. *p<0.05, **p<0.01, ***p<0.001, n.s. not significant, HFD, high fat diet; MaR1, Maresin 1; RvD2, Resolvin D2; HE, hematoxylin and eosin stain.

Figure 4: Resolvin D2 and Maresin 1 induce a pro-resolving macrophage phenotype. (A-B) *Apoe*^{-/-} mice were fed a high fat diet for four months. During the last month mice were treated with PBS or a combination of RvD2 and MaR1 (each 100 ng/mouse, every second day, i.p.). Lesions of MaR1 and RvD2-treated mice are characterized by a pro-resolving macrophage phenotype. (A) Macrophages (CD68) in aortic root sections were costained with antibodies to iNOS (inflammatory) or CD206 (reparative). Representative images show CD68/CD206 double staining. Scale bar indicates 50μm. (B) mRNA expression of macrophage markers *Nos2* (inflammatory) and *Retnla* (reparative) in the aortas of treated mice. Data are expressed as mean±SEM. Unpaired t-test was used. n=16-17. (C-D) TNF/IFNγ-activated macrophages were treated with MaR1 and RvD2 and the cell supernatant was collected to assess cytokine

secretion (C) while the cells were harvested for mRNA expression (D). (C) Cytokine secretion of MaR1 and RvD2-treated macrophages in relative to TNF/IFNγ-activated macrophages (set to 1 for each cytokine, dashed line). Data are expressed as mean±SEM. Wilcoxon Signed-rank test. n=7. (D) Fold changes in mRNA expression of TNF/IFNγ-activated macrophages following addition of MaR1 and RvD2. Data are expressed as mean±SEM. Wilcoxon Signed-rank test. n=4-10. (E-G) MaR1 and RvD2-treated macrophages instruct smooth muscle cells to produce collagen. (E) mRNA expression in TNF-activated aortic smooth muscle cells in presence or absence of MaR1 and RvD2. Dashed line represents non-treated smooth muscle cells set to 1. (F) mRNA expression in aortic smooth muscle cells treated with the supernatant of TNF-activated macrophages (TNF_{sup}) or macrophages activated with TNF in presence of MaR1 and RvD2 ($MaR1/RvD2_{sup}$). Expression is shown as fold change in relation to treatment with the supernatant of non-activated macrophages set to 1. (G) Collagen production assessed by absorbance of Sirius red. Aortic smooth muscle cells were treated as described in (E). In some experiments SB431542 (10μM) was present to inhibit TGFβ receptor. Results are shown as fold change in relation to smooth muscle cells treated with the supernatant of untreated macrophages set to 1. Data are expressed as mean±SEM. Mann Whitney test was used to compare indicated groups. n=4-6 for each bar

Novelty and Significance

What Is Known?

- Docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) the precursors of resolving lipid mediators, have been shown to be atheroprotective.

- Atherosclerotic lesions exhibit features of failed inflammation resolution.

- Resolving lipid mediators are important regulators of resolution of inflammation.

What New Information Does This Article Contribute?

- Inflammatory lipid mediators Leukotriene B4 (LTB4) and Prostaglandin E2 (PGE2) directly correlate with traits of plaque vulnerability whereas resolving lipid mediators Resolvin D2 (RvD2), and Maresin 1 (MaR1) directly correlate with plaque stability.

- In an atheroprogression mouse model, RvD2 and MaR1 induce plaque stability by favoring a reparative macrophage phenotype accompanied by a decrease of lesional macrophage accumulation, and an increase in smooth muscle cells.

- RvD2 and MaR1 shift the profile of lesional macrophages from inflammatory to reparative, favoring a pro-resolution milieu, with increased TGFβ levels and decreased TNF and IL6.

- RvD2 and MaR1-treated macrophages instruct smooth muscle cells to produce collagen.

- The administration of RvD2 and MaR1 prevents atheroprogression suggesting these mediators as potential therapeutic candidates to avert plaque destabilization.

As a chronic inflammation, atherosclerosis also exhibits signs of failed resolution, a process which is determined by a switch in the lipid mediator class. Resolving lipid mediators, the derivatives from docosahexaenoic and eicosapentaenoic fatty acids, actively orchestrate resolution of inflammation. Clinical and animal model studies attribute protective features docosahexaenoic and eicosapentaenoic fatty acids in atheroprogression, which positively correlate with decreased inflammation and increased plaque stability. However, the actions of docosahexaenoic and eicosapentaenoic in atherosclerosis are poorly understood. The current study evidences, in a murine atheroprogression model, an imbalance between inflammatory and resolving lipid mediators that hinders resolution of inflammation. Inflammatory Leukotriene B4 and Prostaglandin E2 directly associate with increased macrophage accumulation, decreased collagen deposition and smooth muscle cell number, all traits of plaque instability. On the contrary, administration of resolving mediators Maresin 1 and Resolvin D2 in a murine model of atheroprogression results in increased lesional plaque stability. Atherosclerotic plaques of treated mice presented decreased macrophage accumulation, increased numbers of smooth muscle cells as well as collagen deposition. Moreover, in these lesions, the macrophage phenotype was primarily reparative. In vitro studies show that Maresin 1 and Resolvin D2 induce a pro-resolving phenotype in macrophages that then secrete less TNF and IL6 while increasing the TGFβ release. Interestingly, the macrophage secretome in turn acts on smooth muscle cells, stimulating collagen production. Taken together our data suggest that treatment with Maresin 1 and Resolvin D2 halts atheroprogression and promotes lesion stability.
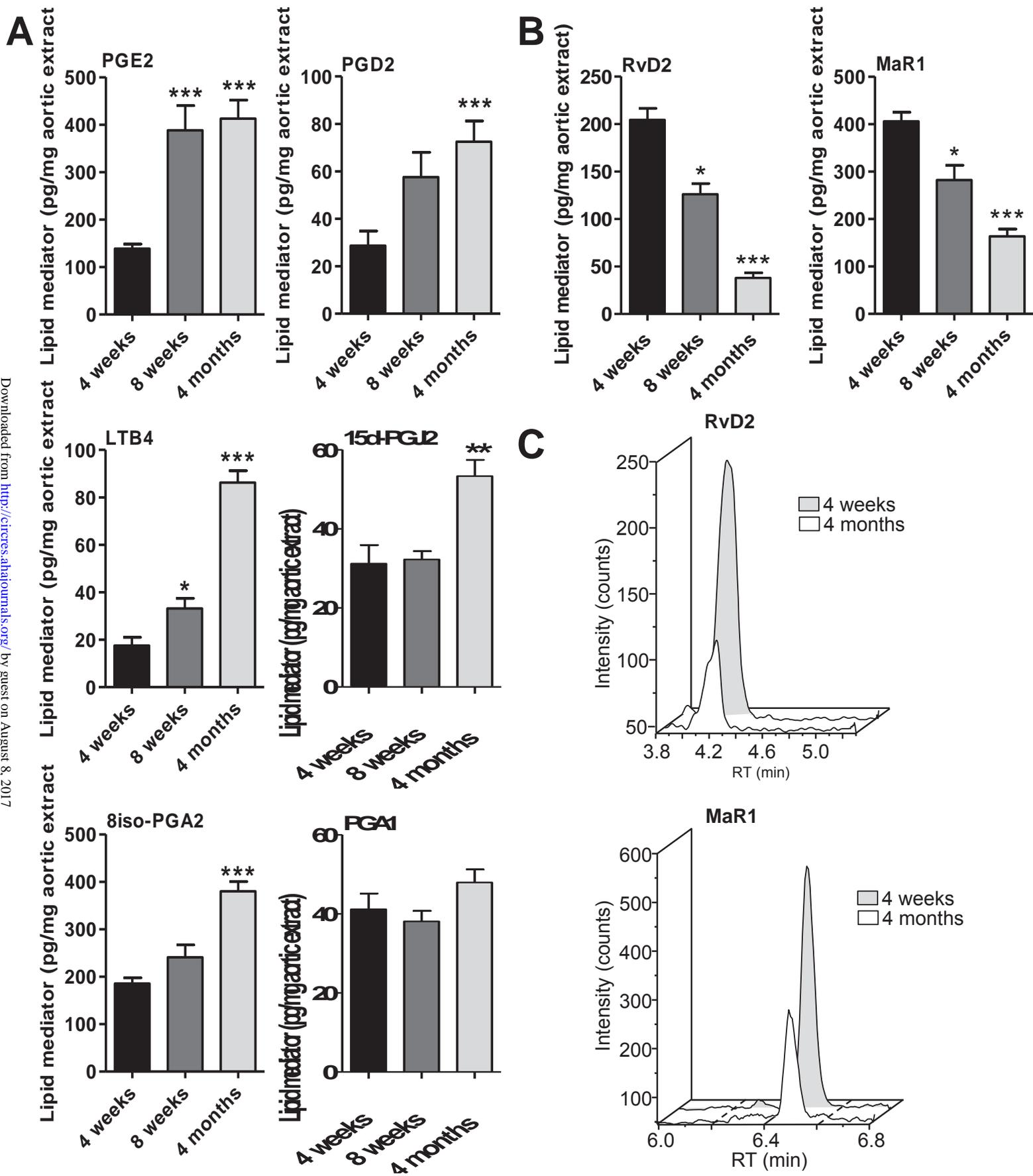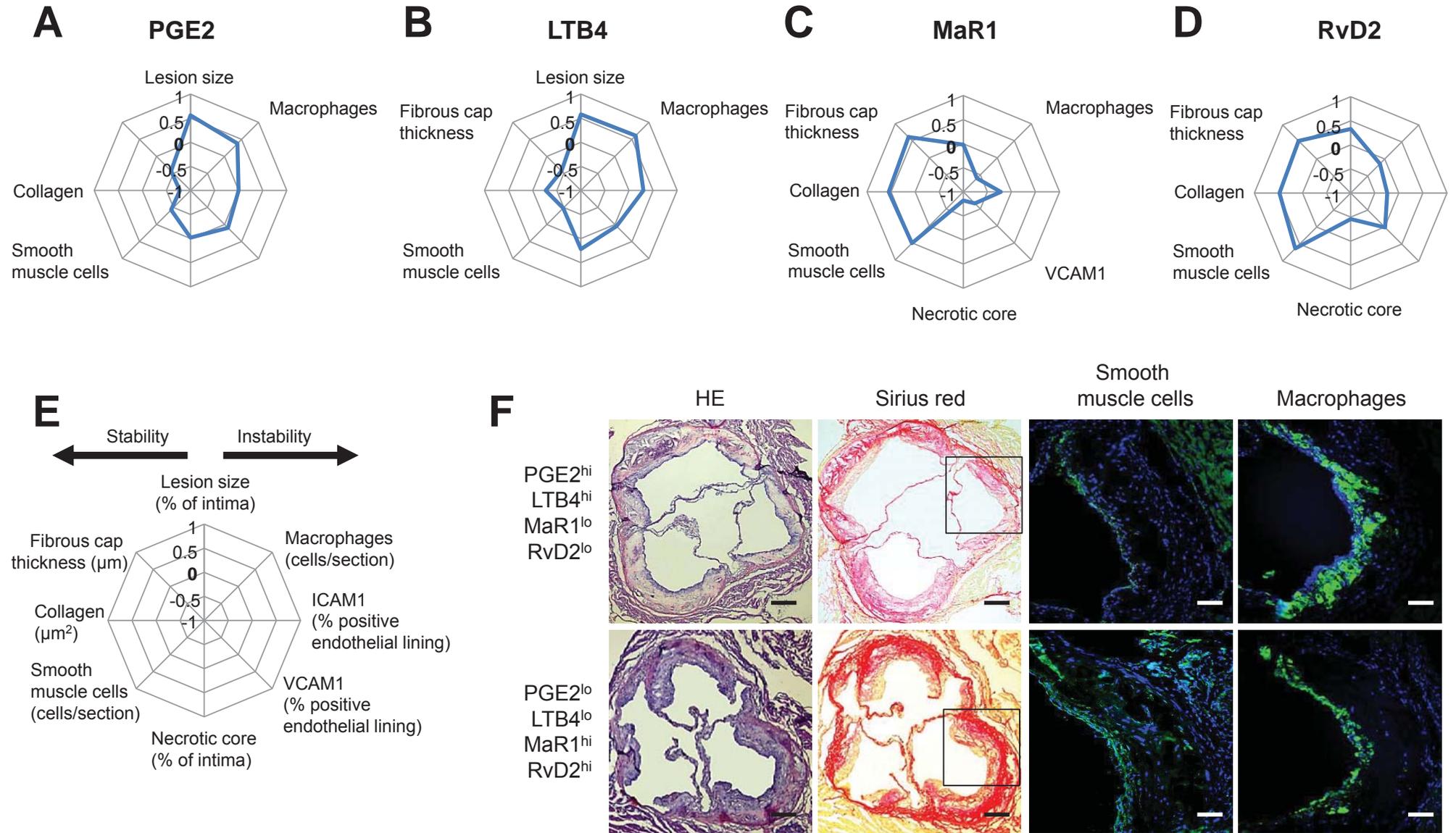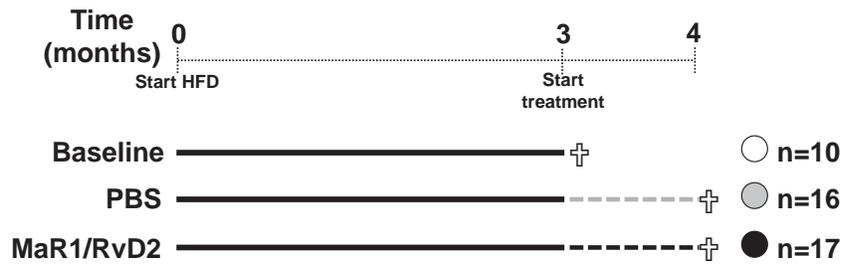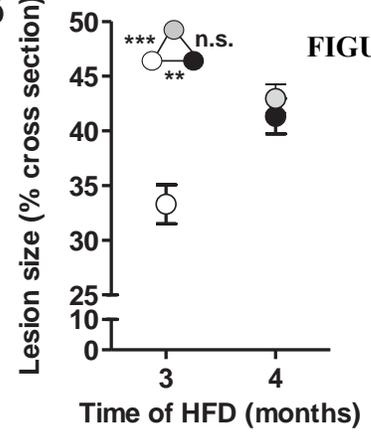
FIGURE 1
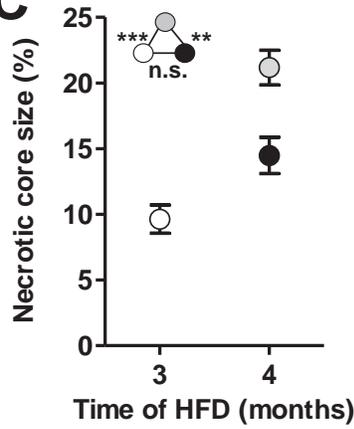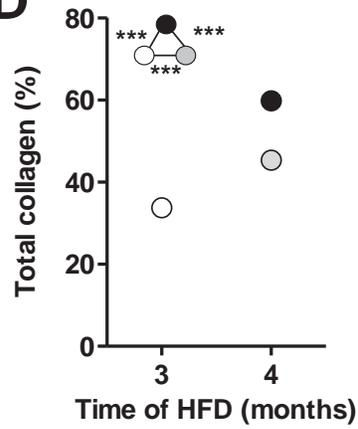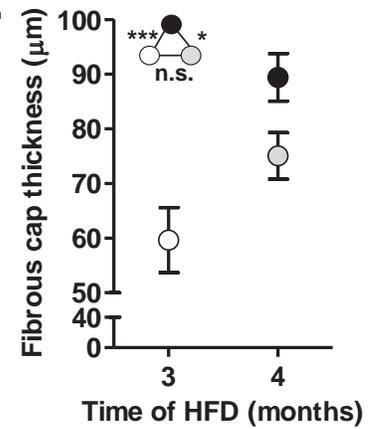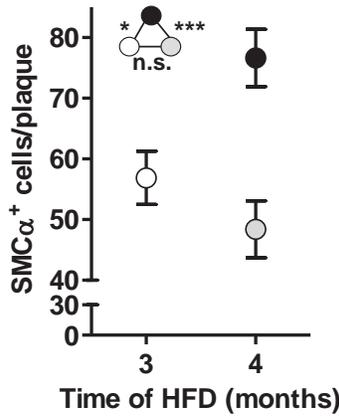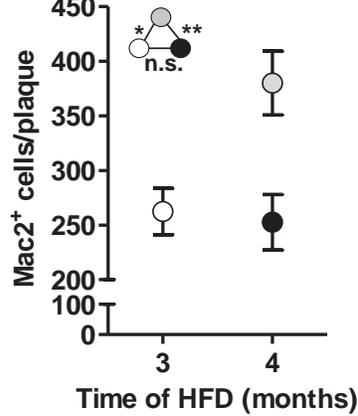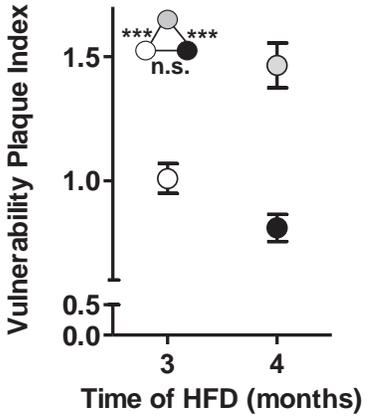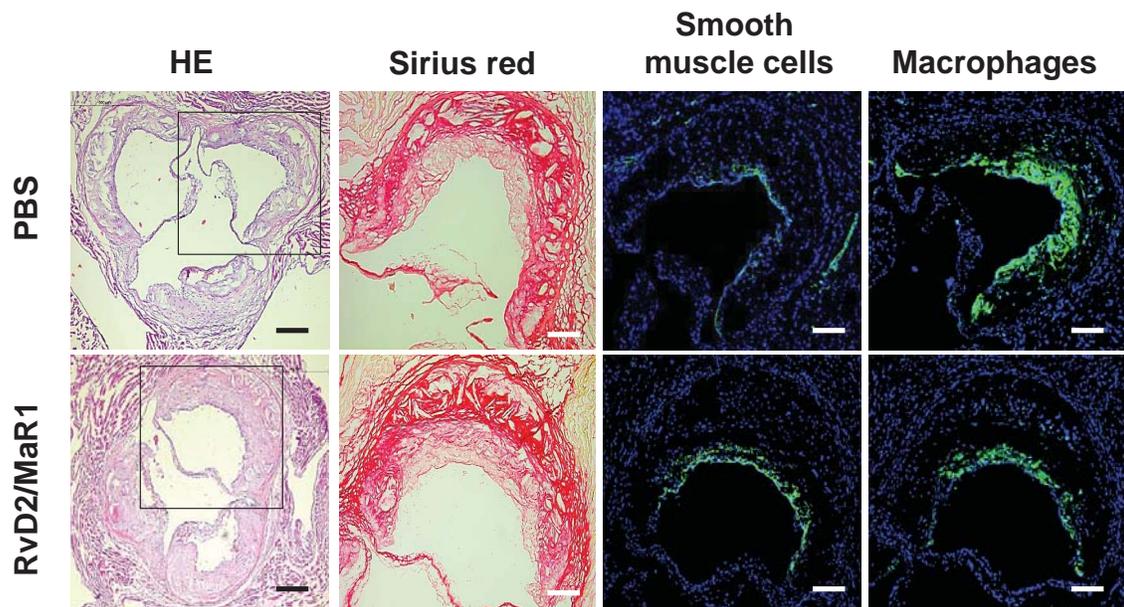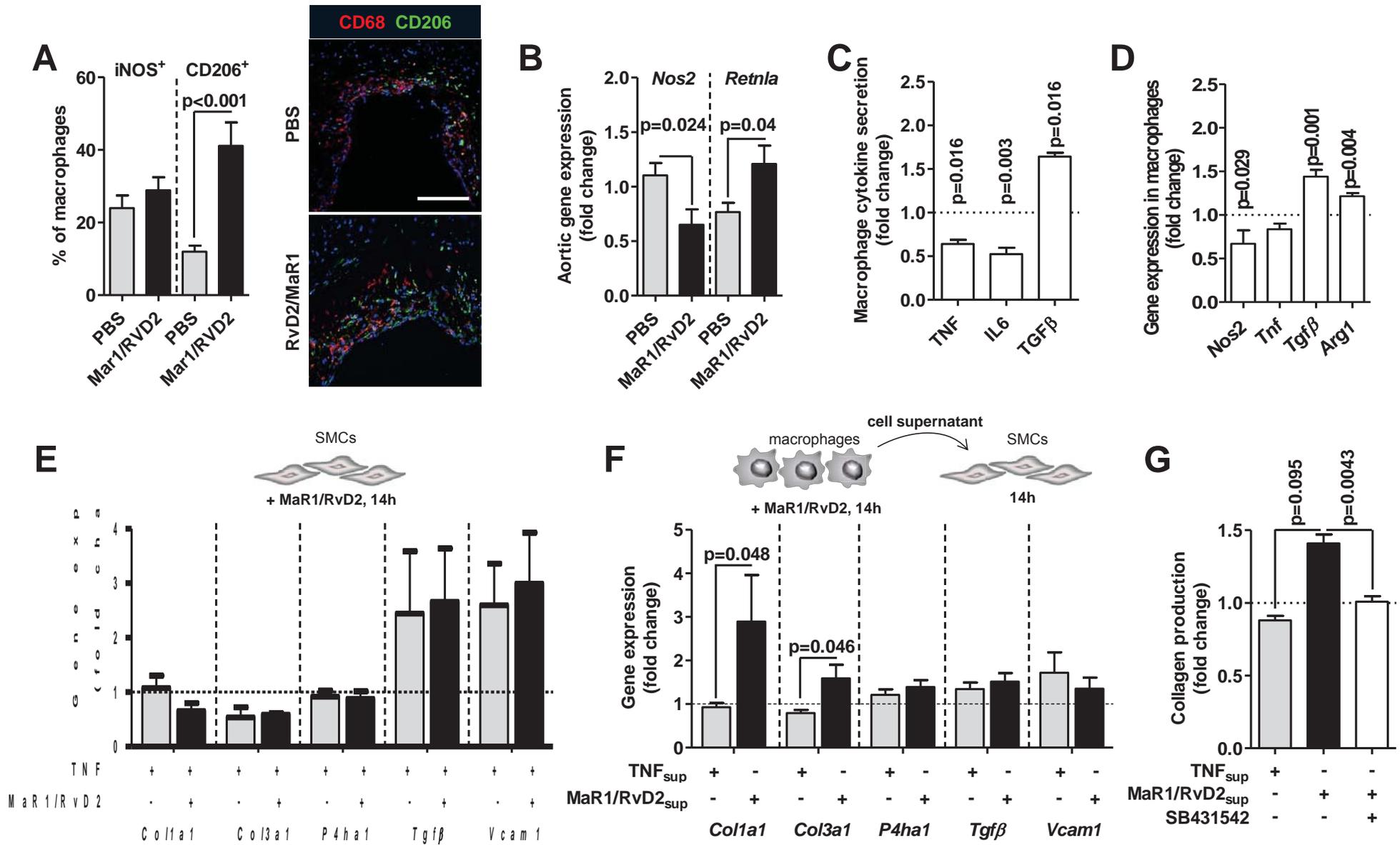
FIGURE 2

FIGURE 3

FIGURE 4

Circulation Research

Resolving Lipid Mediators Maresin 1 and Resolvin D2 Prevent Atheroprogression in Mice

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1   **Resolving lipid mediators Maresin 1 and Resolvin D2 prevent atheroprogresion in mice**
2
3   Viola: Resolving atheroprogression
4

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26
27
28   **- Supplemental Material -**
29

30 **Supplementary methods**
31
32 *Mice*
33 *Apoe*^{-/-} mice on C57Bl/6 background were fed a high-fat diet (HFD) containing 21% fat (ssniff) for 4
34 or 8 weeks, 3 months, or 4 months as indicated in the respective experimental description. For
35 treatment studies, after 3 months HFD, 100ng of MaR1 and 100ng of RvD2, or vehicle, were
36 administered, i.p, every second day, for a period of 4 weeks. Lipid mediators' dosage and treatment
37 regimen is based on earlier reports [1] and supported by comparable studies[1, 2]. At the end of the feeding
38 period, the body weight of the mice was measured and mice were euthanized by an overdose of
39 ketamine/xylazine. All animal experiments were approved by the local ethical committee.
40
41 *Plasma cholesterol and triglyceride measurements*
42 Cholesterol and triglyceride levels in mouse plasma were quantified using enzymatic assays
43 (Roche/Hitachi) according to the manufacturer's protocol.
44
45 *Assessment of blood cell counts*
46 Blood leukocyte counts were quantified by flow cytometry. Staining of single cell suspensions of
47 blood was conducted using combinations of antibodies specific for CD115-PE (eBioscience, AFS98),
48 CD11b-PerCP (BD, M1/70), CD45-APC-Cy7 (BD, 30-F11), Gr1-APC (eBioscience/BD, RB6-8C5)
49 and CD3-FITC (eBioscience, 145-2C11). Before cell staining, red blood cell lysis was performed
50 using appropriate volume of lysis buffer (150 mM NH4Cl; 10 mM KHCO3; 0.1 mM diNaEDTA, pH
51 7.4). Cells were washed with Hanks Balanced Salt Solution (HBSS) and directly analyzed by flow
52 cytometry using a FACSCantoII (BD). Absolute cell numbers were assessed by use of CountBright™
53 absolute counting beads (Invitrogen). Data were analyzed with FlowJo Software (Tree Star Inc.).
54 Erythrocytes were quantified by blood counter (ScilVet abc Plus analyser).
55
56 *Immunohistochemistry*
57 The extent of atherosclerosis was assessed in aortic roots after Hematoxylin and Eosin (HE) staining
58 followed by computerized image analysis and quantification (Leica Qwin Imaging software).
59 Minimum fibrous cap thickness (FCT) was defined as the minimum distance between the necrotic core
60 and the arterial lumen, and measured in HE stained sections. Total collagen was quantified (Fiji,
61 Image J software) after Sirius red staining, as a measure of Sirius red positive area within plaque
62 lesion. Aortic roots were stained with antibodies to Mac2 (Cedarlane), VCAM1 (429, BD
63 Biosciences), or ICAM1 (3E2, BD Biosciences), smooth muscle cell actin (1A4, Sigma-Aldrich),
64 CD68 (Abcam), iNOS (Cell signaling), or CD206 (MR5D3, BioRad). Nuclei were counter-stained by
65 4',6-Diamidino-2-phenylindol (DAPI). A Leica DM4000 microscope with a 25x objective (Leica
66 Microsystems) and a Leica DFC 365FX camera were used to capture images. Leica Qwin Imaging
67 software (Leica Ltd.) was employed for image analysis. Vulnerability plaque index (VPI) was used to
68 evaluate the overall degree of instability. The formula for VPI was expressed as $VIc(i) = (U(i)/S(i))$
69 where i represents each studied mouse. U includes the sum of necrotic core area (% of intima) and
70 Mac2^{+} area (% of intima). S consists of SMA[+] area (% of intima) and collagen[+] area (% of intima).
71
72 *Chemicals*
73 6-keto-prostaglandin F1α (6kPGF$_{1α}$), thromboxane B2 (TXB$_2$), prostaglandin E2 (PGE$_2$),
74 prostaglandin A1 (PGA$_1$), 8-iso prostaglandin A2 (8-iso PGA$_2$), prostaglandin E3 (PGE$_3$), 15-deoxy-
75 $\Delta^{12,14}$-Prostaglandin J2 (15d-PGJ$_2$), lipoxin A4 (LxA4), lipoxin B4 (LxB4), lipoxin A4 deuterated
76 (LxA4-d5), resolvin D1 (RvD1), resolvin D2 (RvD2), 7(S)-maresin (7-MaR1), leukotriene B4
77 (LTB4), leukotriene B5 (LTB5), leukotriene B4 deuterated (LTB4-d4), 10(S),17(S)-protectin (PDx),
78 18-hydroxyeicosapentaenoic acid (18-HEPE), dihydroxy-eicosatetraenoic acid (5,6-DiHETE), 15-
79 hydroxyeicosatetraenoic acid (15-HETE) and 12-HETE, 8-HETE, 5-HETE, 5-HETE-d8, 17-hydroxy-
80 docosahexaenoic acid (17-HDoHE) and 14-HDoHE, 14,15-epoxyeicosatrienoic acid (14,15-EET) and
81 11,12-EET, 8,9-EET, 5,6- EET, 5-oxoeicosatetraenoic acid (5-oxoETE) were purchased from Cayman
82 Chemicals (Ann Arbor, MI, USA).
83
84
85

86 *Aorta and plasma lipid extraction*
87 Mouse aorta were crushed with a FastPrep®-24 Instrument (MP biomedical) in 200 µL of HBSS
88 (Invitrogen) and 5 µL of internal standard mixture (LxA$_4$-d5, LTB$_4$-d4 and 5-HETE-d8 at 400 ng/mL
89 in methanol, MeOH)**.** After 2 crush cycles (6.5 m/s, 30 s), 10µL was withdrawn for protein
90 quantification and 300 µL of cold MeOH was added. Samples were centrifuged at 3000 rpm for 15
91 min at 4°C. Supernatants were collected, completed to 2 mL in H$_2$O and submitted to solid-phase
92 extraction using HRX-50 mg 96-well (Macherey Nagel). Briefly, the plate was conditioned by
93 successive washing with MeOH (2 mL) and H$_2$O/MeOH (90:10, 2 mL). The sample was loaded at
94 flow rate of 0.1 mL/min. After complete loading, the plate was washed with H$_2$O/MeOH (90:10, 2
95 mL). After drying under aspiration, lipid mediators were eluted with MeOH (2 mL). Solvent was
96 evaporated under nitrogen and samples were dissolved with MeOH and stored at -80 °C for liquid
97 chromatography/tandem mass spectrometry measurements.

99 *Liquid chromatography/tandem mass spectrometry measurements*
100 By this technique we performed the quantification of 6kPGF1α, TXB$_2$, PGE$_2$, PGA$_1$, 8-isoPGA$_2$,
101 PGE$_3$, 15d-PGJ$_2$, LxA4, LxB4, RvD1, RvD2, 7-MaR1, LTB4, LTB5, PDx, 18-HEPE, 5,6-DiHETE,
102 15-HETE, 12-HETE, 8-HETE, 5-HETE, 17-HDoHE, 14-HDoHE, 14,15-EET, 11,12-EET, 8,9-EET,
103 5,6-EET and 5-oxo-ETE in aortas[3]. To simultaneously separate lipids of interest and deuterated
104 internal standards, LC-MS/MS analysis was performed on UHPLC system (Agilent LC1290 Infinity)
105 coupled to Agilent 6460 triple quadrupole MS (Agilent Technologies) equipped with electro-spray
106 ionization operating in negative mode. Reverse-phase UHPLC was performed using ZorBAX SB-C18
107 column (2.1 mm, 50 mm, 1.8 µm) (Agilent Technologies) with a gradient elution. The mobile phases
108 consisted of water, ACN and FA (75:25:0.1;v/v/v) (A) and ACN, FA (100:0.1, v/v) (B). The linear
109 gradient was as follows: 0% B at 0 min, 85% B at 8.5 min, 100% B at 9.5 min, 100 % B at 10.5 min
110 and 0 % B at 12 min. The flow rate was 0.35 mL/min. The autosampler was set at 5°C and the
111 injection volume was 5 mL. Data were acquired in Multiple Reaction Monitoring (MRM) mode with
112 optimized conditions (ion optics and collision energy). Peak detection, integration and quantitative
113 analysis were done using Mass Hunter Quantitative analysis software (Agilent Technologies). For
114 each standard, calibration curves were built using 10 solutions at concentration ranging from 0.95
115 ng/mL to 500 ng/mL. A linear regression with a weight factor of 1/X was applied for each compound.
116 The limit of detection (LOD) and the limit of quantification (LOQ) were determined for the 27
117 compounds using signal to noise ratio (S/N). The LOD corresponded to the lowest concentration
118 leading to a signal to noise over 3 and LOQ corresponded to the lowest concentration leading to a
119 signal to noise over 5. All values under the LOQ were not considered. Importantly, blank samples
120 were evaluated, and their injection showed no interference (no peak detected), during the analysis.
121 Hierarchical clustering and heat-map were obtained with R (www.r-project.org). PUFA metabolite
122 quantities were transformed to z-scores and clustered based on 1-Pearson correlation coefficient as
123 distance and the Ward algorithm as agglomeration criterion.

125 *Microarray analysis*
126 Aortas were homogenized in 500 µL of TRIzol reagent (ambion, Life Technologies) with a T10 basic
127 Ultra Turrax disperser homogenizer (IKA). Nucleic acids were separated by addition of 100 µL
128 Chloroform (Sigma Aldrich) followed by centrifugation, and subsequently precipitated with 99%
129 ethanol and loaded into a RNA microprep purification column (Zymo Reseacrh) to proceed with
130 purification according to manufacturer's instructions. RNA quality and concentration was assessed on
131 Nanodrop 2000 (Thermo Scientific). Transcriptome studies were run in an Affymetrix GeneChip®
132 Mouse Transcriptome 1.0 Arrays used in combination with random priming and a one-color based
133 hybridization protocol. Microarray signals were detected using the Affymetrix GeneChip® 3000
134 Scanner.

136 *Bioinformatics*
137 Three replicates of PBS- and RvD2/MaR1-treated samples were analyzed using standard protocols
138 (RMA) to compute gene-wise fold changes (fc) between the conditions and the respective p-values.
139 With the limited number of macrophages in whole aortas, only small fold changes were expected for
140 even large differences in the macrophage population in the two conditions. In order to test whether our
141 data supports a shift towards M2 macrophages in the treated samples we compared the profiles with

142　data from a recent study defining gene profiles of macrophage polarization[4]. In this study (GEO data
143　set GSE69607, http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE69607), 600-700 up- and
144　down-regulated genes in M1 and about 400 each up- and down-regulated genes in M2 were identified.
145　A signature of genes was defined highly discriminating between M1 and M2 macrophages consisting
146　of 210 genes from the Jablonski data via a fold-change threshold of log2(fc) > 1.5 (about 3-fold) and a
147　p-value < 0.05. We then compare these 210 genes with our fold change data and identified subsets of
148　M1/M2 discriminating genes (signatures), which changed similarly in our data sets (**Online Figure
149　VII**). For this we computed dot product scores for genes from subsets of different sizes. The dot
150　product scores for the signature genes in our data are significantly higher as compared to their score in
151　50 similar microarray measurements randomly selected from GEO.

153　*ELISA*
154　Plasma and cell supernatant TGFβ1, TNF, IL-6 and IL-13 were assessed by a commercially available
155　Luminex assay (Affymetrix) according to the manufacturer's protocol. Plasma serum amyloid A
156　(SAA) was measured by ELISA (ICL lab).

158　**In vitro** *studies*
159　Peritoneal macrophages were plated at 80% confluence and allowed to adhere for 3h. Cells were
160　washed and treated with TNF (10 ng/ml) and IFNγ (20 ng/ml), in presence or absence of MaR1 and
161　RvD2 (10 nM) for 14h. The cell supernatant was collected and immediately frozen. Cells were further
162　washed with PBS, lysed, and treated according to manufacturer's protocol for RNA isolation (Quick-
163　RNA[TM] MicroPrep, Zymo Research). Expression of genes of interest was assessed on samples' cDNA
164　(QuantiNova Reverse Transcriptase kit, Qiagen), on an Applied Biosystems HD 7900 system, and
165　analysed by the ΔΔCT method. Primers and polymerase (QuantiNova SYBR Green PCR kit) were
166　purchased from Qiagen.
167　　　　MOVAS (murine vascular aortic smooth muscle cells) were cultured at 80% confluence and
168　serum-starved overnight. Cells were treated with TNF (10 ng/ml) for 2 hours and thereafter with
169　MaR1 and RvD2 (10nM) for 14h. Alternatively, cells were starved overnight and treated with
170　supernatant from peritoneal macrophage (untreated, TNF/IFNγ-treated or TNF/IFNγ/MaR1/RvD2) for
171　14h. Cells were harvested for RNA preparation as *supra* described. mRNA expression was analysed
172　by the ΔΔCT method. For collagen analysis in MOVAS, cells were starved overnight, and treated with
173　macrophage cell supernatant for 24 h, as *supra* described. For TGFβ receptor inhibition studies,
174　smooth muscle cells were treated with SB431542 (Tocris) (10μM) for 2h prior to treatment with
175　macrophage cell supernatant containing SB431542 (10μM). Collagen was stained as previously
176　reported.[5] Briefly, cells were washed with PBS, fixed with 4% PFA, stained with 0.1% Sirius red in
177　0.1M picric acid, and thereafter lysed with 0.01 M NaOH. Collagen was analysed by absorption
178　measured on a standard plate reader at 550nm.

179 **Supplementary references**
180
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195   *one.* 2015;10:e0145342
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198   tolerance and develop more complex atherosclerotic plaques than ldlr knockout mice.
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200 **Supplementary data**
201
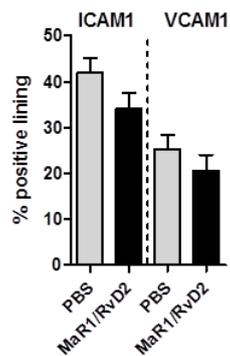
202
203 **Online Figure I: Resolvin D2 and Maresin 1 treatment does not affect endothelial ICAM1 and**
204 **VCAM1 expression.** *Apoe*$^{-/-}$ mice were fed a high fat diet for four months. During the last four weeks
205 of diet mice were injected with PBS or a combination of Maresin 1 (MaR1) and Resolvin D2 (RvD2)
206 (each 100 ng/mouse, every second day, i.p.). Aortic root sections were stained for ICAM1 or VCAM1.
207 All values are displayed as mean±SEM. Unpaired t-test. n=16 for PBS group and 17 for RvD2/MaR1
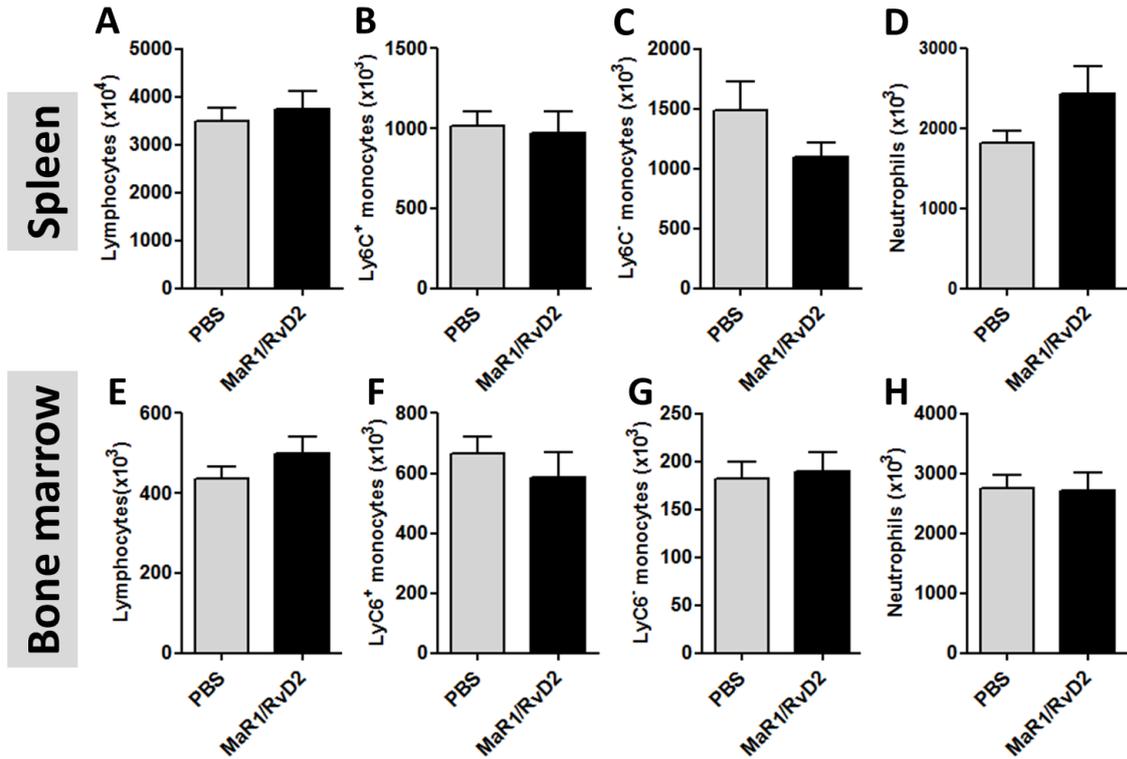208 group.

209
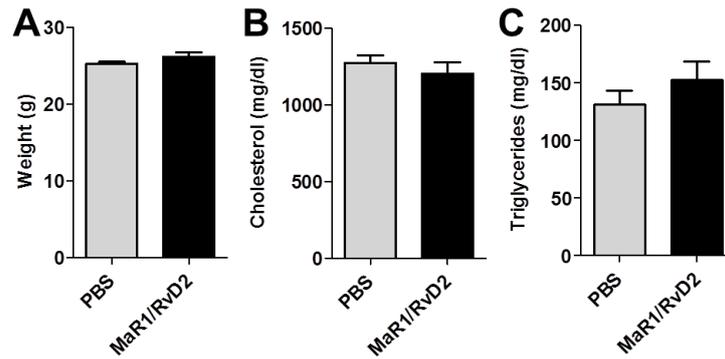
Online Figure II: Resolvin D2 and Maresin 1 treatment does not affect body weight and plasma lipid levels. *Apoe*^{-/-} mice were fed a high fat diet for four months. During the last four weeks of diet mice were injected with PBS or a combination of Maresin 1 (MaR1) and Resolvin D2 (RvD2) (each 100 ng/mouse, every second day, i.p.). At the end of the high fat diet feeding body weight was assessed (A), and cholesterol (B) and triglycerides (C) were measured in the plasma. All values are displayed as mean±SEM. Unpaired t-test. n=16 for PBS group and 17 for RvD2/MaR1 group.
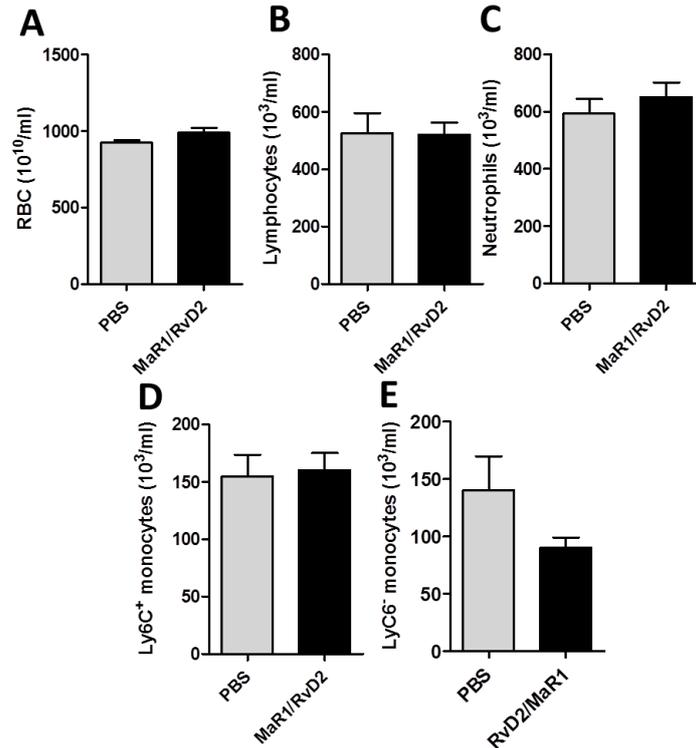
216
Online Figure III: Resolvin D2 and Maresin 1 treatment does not affect blood cell counts. *Apoe*^{-/-}
mice were fed a high fat diet for four months. During the last four weeks of diet mice were injected
with PBS or a combination of Maresin 1 (MaR1) and Resolvin D2 (RvD2). Blood was drawn at the
end of the high fat diet feeding and erythrocytes (A), lymphocytes (B), neutrophils (C), classical
monocytes (D), and non-classical monocytes (E) were quantified. All values are displayed as
mean±SEM. Unpaired t-test. n=16 for PBS group and 17 for MaR1/RvD2 group.

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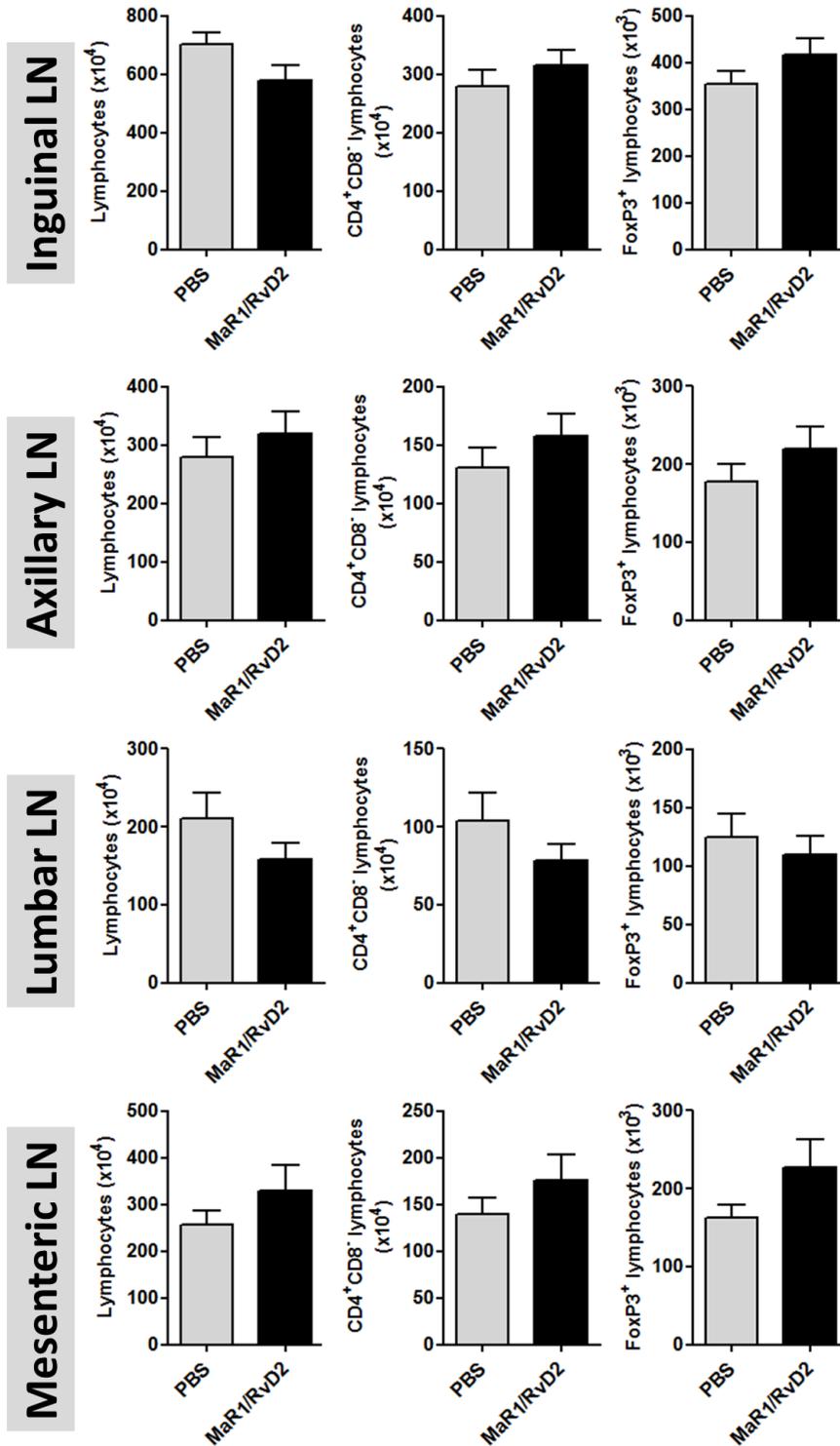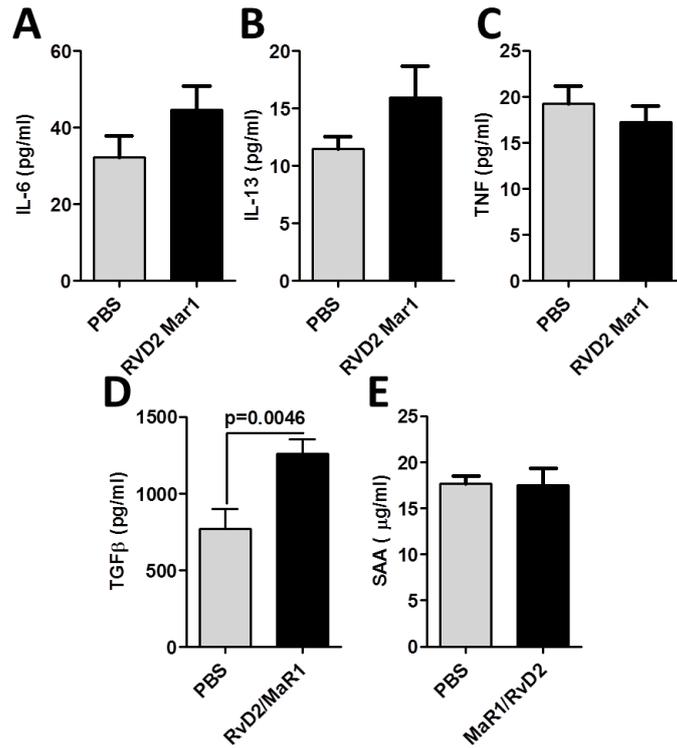225 **Online Figure IV: Resolvin D2 and Maresin 1 treatment does not affect spleen and bone marrow**
226 **(BM) cell counts.** *Apoe*^{-/-} mice were fed a high fat diet for four months. During the last four weeks of
227 diet mice were injected with PBS or a combination of Maresin 1 (MaR1) and Resolvin D2 (RvD2).
228 Spleen (A-D) and bone marrow (E-H) were collected and homogenized or flushed, respectively. Red
229 blood cells were lysed, and cells were quantified: spleen (A) and BM (E) lymphocytes, classical (B, F)
230 and non-classical (C, G) monocytes, and neutrophils (D, H). All values are displayed a mean±SEM.
231 Unpaired t-test. n=16 for PBS group and 17 for RvD2/MaR1 group.

232
Online Figure V: Resolvin D2 and Maresin 1 treatment does not affect lymph nodes counts.
Apoe^{-/-} mice were fed a high fat diet for four months. During the last four weeks of diet mice were injected with PBS or a combination of Maresin 1 (MaR1) and Resolvin D2 (RvD2). Inguinal, axillary, lumbar, and mesenteric lymph nodes (LN) were collected, homogenized and analyzed for counts of lymphocyte subpopulations. All values are displayed a mean±SEM. Unpaired t-test. n=16 for PBS group and 17 for RvD2/MaR1 group.

239
Online Figure VI: Effect of Resolvin D2 and Maresin 1 treatment on plasma cytokines and
serum amyloid A. *Apoe*^{-/-} mice were fed a high fat diet for four months. During the last four weeks of
diet mice were injected with PBS or a combination of Maresin 1 (MaR1) and Resolvin D2 (RvD2).
Plasma was collected and IL-6 (A), IL-13 (B), TNF (C), TGFβ (D), serum amyloid A (SAA) (E) were
assessed by ELISA. All values are displayed as mean±SEM. Unpaired t-test. n=16 for PBS group and
17 for RvD2/MaR1 group.
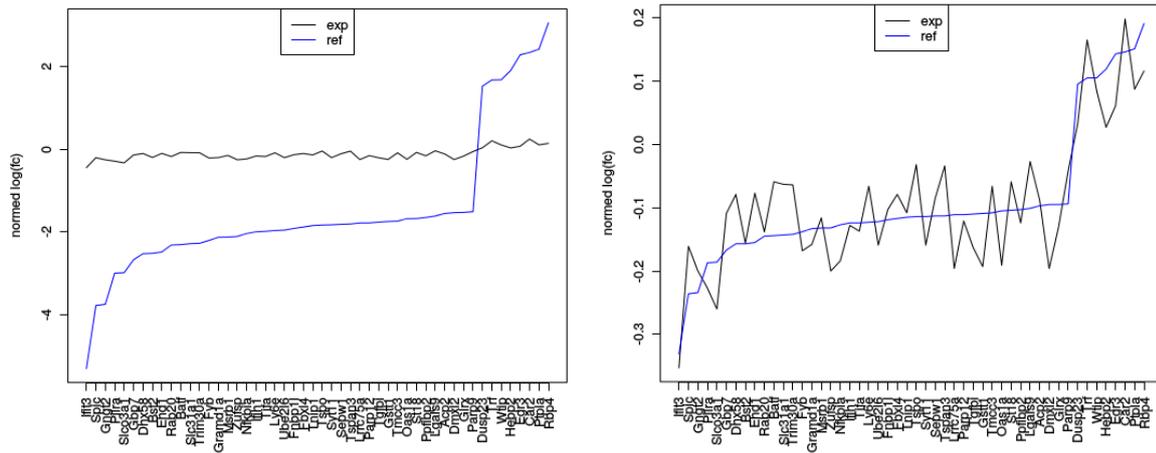
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247
Online Figure VII: Resolvin D2 and Maresin 1 treatment induces an M2 macrophage polarization gene expression signature in aortas. Plot shows the correlation of fold changes computed for the genes in the Jablonski et al.[4] data (blue line) and in our data set (black). Shown are absolute fold changes (left) and relative fold changes (right), *i.e.* actual fold changes as fractions of the sum of the 50 fc values (the 50 gene fold change vector has been normed to 1). Most signature genes (43) are down-regulated in M2 macrophages (when compared to M1 macrophages) and RvD2/MaR1-treated aortas (compared to PBS treatment), whereas only 7 are up-regulated.

A

Mac2$^+$ cells (per section)

p<0.0001

4 weeks   4 months

B

iNOS$^+$ CD206$^+$

% of macrophages

p<0.0001

4 weeks   4 months   4 weeks   4 months

C

M1/M2 ratio

p=0.0055

4 weeks   4 months
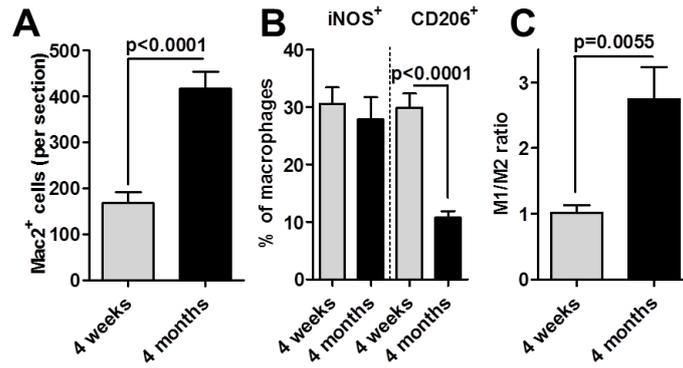
Online Figure VIII: During atherosclerosis progression macrophages accumulate in the lesion and an inflammatory macrophage phenotype is favored. *Apoe*$^{-/-}$ mice were fed a high fat diet for four weeks or four months. Atherosclerotic lesions in the artic root were analyzed for macrophage content (Mac2 staining) and phenotype (iNOS, M1, and CD206, M2). **(A)** Lesional macrophage content. **(B)** Relative numbers of iNOS$^+$ M1 macrophages and CD206$^+$ M2 macrophages. **(C)** Ratio of M1 and M2 macrophages after 4 weeks and 4 months of high fat diet. All values are displayed as mean±SEM. Unpaired t-test. n=9-15.
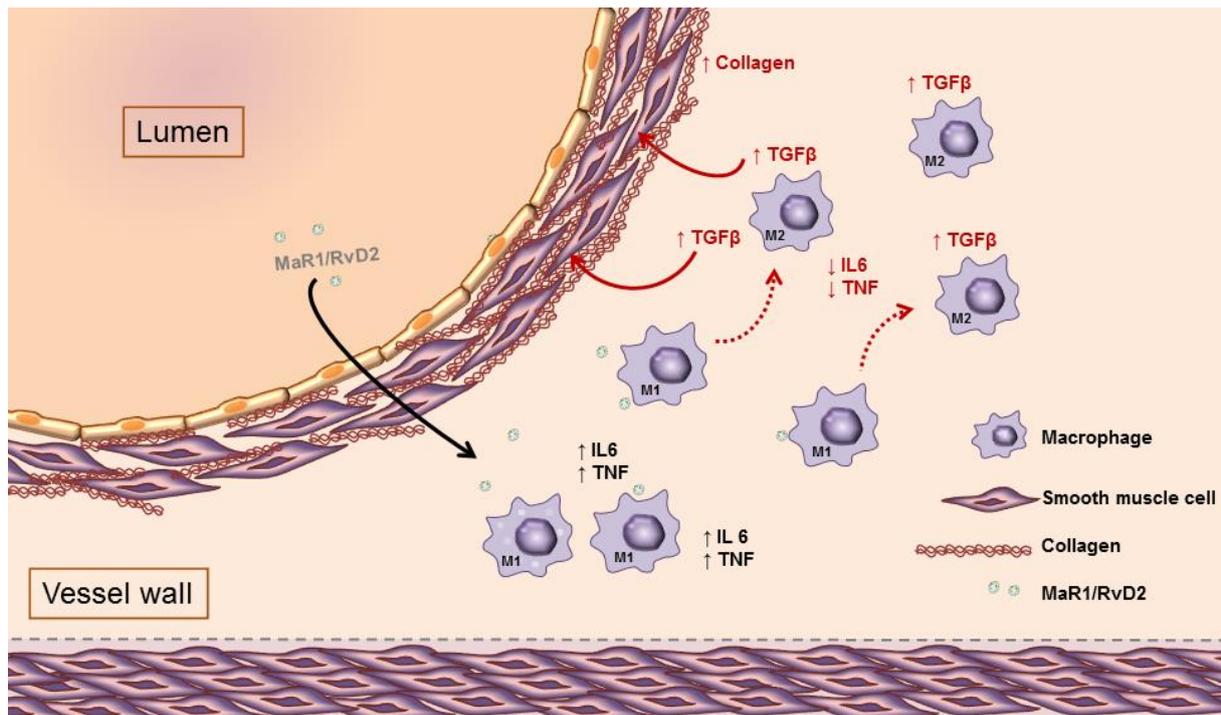
Online Figure IX. Suggested mechanism of action of MaR1/RvD2 in halting atheroprogression. Maresin 1 (MaR1) and Resolvin D2 (RvD2) act on lesional macrophages, inducing phenotype changes towards a reparative phenotype. These reparative macrophages reduce the inflammatory environment by enhanced secretion of TGFβ and decreasing the concentrations of secreted IL6 and TNF. The resolving milieu aids to decreased macrophage accumulation and smaller necrotic core sizes, and instructs smooth muscle cells to produce collagen, resulting in fibrous cap thickening and increased plaque stability.

	PGE2	PGD2	PGA1	8iso-PGA2	LTB4	15d-PGJ2	RvD2	MaR1
Lesion size	r=0.56 p=0.01	r=0.19 p=0.45	r=-0.12 p=0.61	r=0.21 p=0.39	r=0.58 p=0.01	r=-0.28 p=0.24	r=0.33 p=0.18	r=-0.02 p=0.93
macrophages	r=0.38 p=0.02	r=-0.15 p=0.55	r=-0.11 p=0.65	r=0.18 p=0.47	r=0.61 p<0.01	r=0.05 p=0.85	r=-0.14 p=0.59	r=-0.60 p<0.01
SMC	r=-0.43 p=0.05	r=0.28 p=0.7	r=0.12 p=0.87	r=0.21 p=0.92	r=-0.48 p=0.2	r=0.22 p=0.17	r=0.63 p<0.01	r=0.51 p=0.02
ICAM1$^+$ EC lining	r=0.0 p=1	r=-0.37 p=0.12	r=0.39 p=0.10	r=-0.35 p=0.12	r=0.30 p=0.21	r=-0.13 p=0.60	r=-0.24 p=0.36	r=-0.22 p=0.36
VCAM1$^+$ EC lining	r=0.10 p=0.67	r=-0.27 p=0.27	r=-0.36 p=0.13	r=0.03 p=0.89	r=0.05 p=0.83	r=-0.06 p=0.81	r=0.01 p=0.99	r=-0.66 p<0.01
Necrotic core size	r=-0.02 p=0.95	r=-0.28 p=0.24	r=-0.16 p=0.49	r=0.12 p=0.61	r=0.22 p=0.35	r=0.05 p=1	r=-0.46 p=0.04	r=-0.82 p<0.01
Collagen	r=-0.78 p<0.01	r=-0.42 p=0.12	r=-0.25 p=0.30	r=-0.16 p=0.53	r=-0.27 p=0.26	r=-0.11 p=0.65	r=0.48 p=0.04	r=0.55 p=0.03
Fibrous cap thickness	r=-0.43 p=0.06	r=0.19 p=0.46	r=-0.25 p=0.19	r=-0.32 p=0.21	r=-0.49 p=0.02	r=-0.32 p=0.22	r=0.53 p=0.04	r=0.61 p=0.01
Vulnerability Plaque index	r=0.63 p<0.01	r=0.39 p=0.21	r=0.33 p=0.14	r=0.56 p=0.53	r=0.48 p=0.01	r=0.17 p=0.38	r=-0.62 p<0.01	r=-0.72 p<0.01

272

Online Table I: Aortic lipid mediators correlate with signs of plaque stability. *Apoe*$^{-/-}$ mice were fed a high fat diet for 4 months and lipid mediators were quantified in aortic lipid extracts using liquid chromatography and tandem mass spectrometry. Assessed were Prostaglandin E2 (PGE2), Prostaglandin D2 (PGD2), Leukotriene B4 (LTB4), 15-deoxy-$\Delta^{12,14}$-Prostaglandin J2 (15d-PGJ2), 8iso-Prostaglandin A2 (8iso-PGA2), Prostaglandin A1 (PGA1), Resolvin D2 (RvD2), and Maresin 1 (MaR1). Lipid mediators were correlated with atherosclerotic lesion characteristics assessed in aortic root sections of the same mice. Measured parameters include: lesion size (HE staining, %), lesional macrophage number (Mac2 staining, cells/section), endothelial cell (EC) activation (endothelial ICAM1 and VCAM1 staining), necrotic core size (HE staining, % of intima), smooth muscle cells numbers (SMC, αSMA staining, cells/section), collagen amount (Sirius red staining, μm^2), and fibrous cap thickness (Sirius red staining, μm) in aortic root sections. Pearson correlation coefficient r (-1 indicates maximal negative correlation, 0 indicates maximal negative correlation, +1 maximal positive correlation) was calculated as a function of aortic lipid mediator and respective lesion criteria. Significant correlations (p≤0.05) are highlighted in gray. Data are calculated from 19 mice.

A

	CD45		Gr1		CD11b		CD115	
	PBS	MaR1/RvD2	PBS	MaR1/RvD2	PBS	MaR1/RvD2	PBS	MaR1/RvD2
Neutrophils								
Blood	10061±1680	10240±852	38360±2904	38370±2649	11799±3698	11496±1382		
Bone marrow	6868±639	6421±476	31785±2373	31674±2419	5969±1363	5386±888		
Spleen	5387±1021	5267±887	34464±1701	36287±2920	9864±1359	11835±2158		
Classical monocytes								
Blood	13430±2104	13456±1463	2514±2663	24666±1700	9121±2389	9818±1356	6410±1023	6745±1162
Bone marrow	5531±852	5659±566	19822±2954	19573±2766	10838±1217	11334±1498	9286±859	8957±1029
Spleen	11120±2020	10102±1878	20674±1402	19356±1534	7768±744	8469±1135	7783±648	6947±1176
Non classical monocytes								
Blood	14958±1438	15006±1002	189±29,8	205±35,8	7605±625	7269±879	7787±1277	7280±1216
Bone marrow	15030±1933	16797±2457	512±50,2	509±82,7	5154±666	5599±1197	8949±627	7886±975
Spleen	14041±2909	11997±2258	303±44,8	344±44,9	6677±731	6022±755	5681±277	5410±774

B

	CD45		CD3		CD4		CD8	
	PBS	MaR1/RvD2	PBS	MaR1/RvD2	PBS	MaR1/RvD2	PBS	MaR1/RvD2
CD4+CD8- T Cells								
Blood	18690±1737	18975±1380	6138±425	5835±354	1925±62,6	1991±87,5		
Bone marrow	20500±1435	19330±1553	6289±373	6081±583	2042±96,8	2023±135		
Spleen	16046±2931	12959±2084	6276±232	5772±281	1896±67,9	1851±79,1		
CD4-CD8+ T Cells								
Blood	26013±2678	25357±2075	4173±295	3731±260			4758±226	4490±241
Bone marrow	30364±1857	27417±2947	4459±266	4208±276			3607±316	3785±331
Spleen	18457±3685	14849±2898	4360±196	3996±190			3922±148	3608±115

287 **Online Table II: Blood, bone marrow and spleen show no differences in the expression of cell**
288 **surface markers in cell counts between untreated and treated groups.** *Apoe*^{-/-} mice were fed a high
289 fat diet for 4 months and during the last 4 weeks PBS or lipid mediators (MaR1 and RvD2) were
290 administered (each 100 ng/mouse, every second day, i.p.). Displayed is the MFI of phenotypic
291 markers of the indicated cell populations harvested from the blood, the bone marrow, or the spleen.
292 Data are calculated from 17 MaR1- and RvD2-treated mice and 16 vehicle-treated mice. All values are
293 displayed as mean±SD.