Genetic and Pharmacological Inhibition of TREM-1 Limits the Development of Experimental Atherosclerosis

Short title: TREM-1 inhibition reduces atherosclerosis

Jeremie Joffre, MD,[a,b] Stephane Potteaux, PhD,[a,b] Lynda Zeboudj, MSc,[a,b] Xavier Loyer, PhD,[a,b] Amir Boufenzer, PhD,[c] Ludivine Laurans, MSc,[a,b] Bruno Esposito, BSc,[a,b] Marie Vandestienne, MSc,[a,b] Saskia C.A. de Jager, MD, PhD,[d] Carole Hénique, PhD,[a,b] Ivana Zlatanova, MSc,[a,b] Soraya Taleb, PhD,[a,b] Patrick Bruneval, MD, PhD,[a,b,e] Alain Tedgui, PhD,[a,b] Ziad Mallat, MD, PhD,[a,b,f] Sebastien Gibot, MD, PhD,[g] Hafid Ait-Oufella, MD, PhD[a,b,h]

[a]Inserm U970, Paris Cardiovascular Research Center, Paris, France
[b]Université Paris Descartes, Sorbonne Paris Cité, Paris, France
[c]INOTREM SA, Nancy, France
[d]Laboratory for Experimental Cardiology, University Medical Center, Utrecht, The Netherlands
[e]Department of Anatomopathology, Hôpital Européen Georges Pompidou, AP-HP, Paris, France
[f]Department of Medicine, Division of Cardiovascular Medicine, University of Cambridge, Cambridge, United Kingdom
[g]Inserm UMR_S1116, Faculté de Médecine, Université de Lorraine. Medical Intensive Care Unit, Hôpital Central, Nancy, France
[h]Medical Intensive Care Unit, Hôpital Saint-Antoine, AP-HP, Université Pierre-et-Marie Curie, Paris, France

Correspondence to
Hafid Ait-Oufella
Inserm U970
Paris Cardiovascular Research Center
56, rue Leblan
Paris, France
Telephone: +33612011940
Fax: +33144631860
Email: hafid.aitoufella@inserm.fr

Competing Financial interests
Sébastien Gibot is co-founder of INOTREM, a company developing TREM-1 inhibitors. Amir Boufenzer, Sébastien Gibot, and Hafid Ait-Oufella applied a patent on the measurement of plasma sTREM-1 concentration to predict outcome following acute myocardial infarction and applied a patent on therapeutic strategies using inhibiting peptides derived from TREM-1.

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ABSTRACT

BACKGROUND Innate immune responses activated through myeloid cells contribute to the initiation, progression, and complications of atherosclerosis in experimental models. However, the critical upstream pathways that link innate immune activation to foam cell formation are still poorly identified.

OBJECTIVES We hypothesized that activation of the triggering receptor expressed on myeloid cells (TREM-1) plays a determinant role in macrophage atherogenic responses.

METHODS After genetically invalidating *Trem-1* in chimeric *Ldlr*$^{-/-}$ *Trem-1*$^{-/-}$ mice and double knockout *Apoe*$^{-/-}$ *Trem-1*$^{-/-}$ mice, we pharmacologically inhibited Trem-1 using LR12 peptide.

RESULTS *Ldlr*$^{-/-}$ mice reconstituted with bone marrow deficient for *Trem-1* (*Trem-1*$^{-/-}$) showed a strong reduction of atherosclerotic plaque size in both the aortic sinus and the thoraco-abdominal aorta, and were less inflammatory compared to plaques of *Trem-1*$^{+/+}$ chimeric mice. Genetic invalidation of *Trem-1* led to alteration of monocyte recruitment into atherosclerotic lesions and inhibited toll-like receptor (TLR) 4-initiated pro-inflammatory macrophage responses. We identified a critical role for Trem-1 in the upregulation of cluster of differentiation (CD) 36, thereby promoting the formation of inflammatory foam cells. Genetic invalidation of Trem-1 in *Apoe*$^{-/-}$*/Trem-1*$^{-/-}$ mice or pharmacological blockade of Trem-1 in *Apoe*$^{-/-}$ mice using LR-12 peptide also significantly reduced the development of atherosclerosis throughout the vascular tree, and lessened plaque inflammation. TREM-1 was expressed in human atherosclerotic lesions mainly in lipid-rich areas, with significantly higher levels of expression in atheromatous compared to fibrous plaques.

CONCLUSION We identify TREM-1 as a major upstream pro-atherogenic receptor. We propose that TREM-1 activation orchestrates monocyte/macrophage pro-inflammatory responses and foam cell formation through coordinated and combined activation of CD36 and TLR4. Blockade of TREM-1 signaling may constitute an attractive novel and double-hit approach for the treatment of atherosclerosis.

KEY WORDS apolipoprotein, foam cells, inflammation, macrophage, toll-like receptor

ABBREVIATIONS AND ACRONYMS

Apo = apolipoprotein
CD = cluster of differentiation
IL = interleukin
LDL = low density lipoprotein
SMC = smooth muscle cell
TLR = toll-like receptor
TREM = triggering receptors expressed on myeloid cells

Ischemic cardiovascular diseases, largely due to atherosclerosis, are expected to remain the main cause of death globally for the next 15 years, forcing us to revisit their basic mechanisms and consider new strategies for prevention and treatment (1-3). There is a large body of human and experimental evidence indicating that innate immune cells, particularly monocytes/macrophages, are involved in the initiation and progression of atherosclerosis and its complications, such as plaque rupture and consecutive acute myocardial infarction (AMI) (4,5). Activation of the vascular wall after subendothelial retention and modification of apolipoprotein (Apo) B containing low-density lipoproteins (LDL) promotes the recruitment of circulating monocytes into the intima. Accumulating monocytes significantly contribute to the pool of macrophages in lesions (6). Monocytes differentiate into macrophages and lipid-laden foam cells, and promote plaque development and vulnerability through cytokine, chemokine, and matrix metalloprotease production and through direct interactions with surrounding inflammatory and vascular cells. Macrophage differentiation, activation, and proliferation are necessary steps for atherosclerosis and are associated with upregulation of pattern recognition receptors for innate immunity, including scavenger receptors (SR-A, cluster of differentiation [CD] 36) and toll-like receptors (TLR) (7-9). Macrophages internalize and are activated by a broad range of molecules and particles bearing danger-associated molecular patterns (e.g., oxidized LDL, apoptotic debris) and ultimately are transformed into pro-inflammatory foam cells. However, the critical upstream pathways that link inflammatory cell activation and foam cell formation and lead to the generation of pro-inflammatory lipid-laden macrophages are still poorly defined. Direct interactions between scavenger receptors involved in lipid body formation (e.g., CD36), and TLR involved in the induction of inflammatory responses (e.g., TLR2, TLR4, TLR6) have been identified (10,11) and proposed to play a role in this process (11). However,

the independent in vivo impact of such pathways on lesion development and the accumulation of pro-inflammatory foamy macrophages remain modest. For example, deletion of CD36 does not limit lesion development in low-density lipoprotein receptor ($Ldlr$)$^{-/-}$ mice (12) and reduced atherosclerosis only in the descending aorta of $Apoe^{-/-}$ mice (13). Moreover, lesion development was not significantly altered in $Ldlr^{-/-}$ mice despite combined deficiency of TLR2 and TLR4 in macrophages (10). Thus, the critical receptor, or combination of receptors, that play nonredundant roles in orchestrating the development of pro-inflammatory foamy macrophages are still to be defined.

Triggering receptors expressed on myeloid cells (TREM) proteins are a family of cell surface receptors, members of the immunoglobulin family, discovered 15 years ago by Bouchon (14,15). TREM-1 expression is constitutive on neutrophils and monocytes/macrophages and increases in response to lipopolysaccharide or other microbial products (15). Engagement of TREMs, after association with the adapter protein DAP12, has been shown to stimulate the production of pro-inflammatory cytokines and chemokines, such as interleukin (IL)-8, CCL-2, and CCL-7, as well as stimulating rapid neutrophil degranulation. Activation of TREM-1 in the presence of TLR2 or TLR4 ligands amplifies the production of pro-inflammatory cytokines (tumor necrosis factor [TNF]-α, IL-1β) while inhibiting the release of anti-inflammatory IL-10 (16). TREM-1 also regulates monocyte and neutrophil migration to inflammatory sites (15,17). TREM-1 has been mainly studied during septic shock, but it also is critical during aseptic inflammation, both acute (pancreatitis) (18) and chronic (rheumatoid arthritis) (19). A recently designed dodecapeptide named LR12 (LQEEDAGEYGCM), derived from TREM-1-like transcript 1, strongly inhibits TREM-1 engagement by competing with its still unknown endogenous ligand. LR12 modulates the innate immune response following bacterial aggression

in rodents (20), pigs (21), and primates (22), translating into an attenuation of organ dysfunction and improved outcome (20,21). We recently reported that TREM-1 is involved is post-ischemic myocardial remodeling by orchestrating leukocyte recruitment to the ischemic heart and subsequent inflammatory responses (23). Additionally, in a multicenter cohort of patients with acute coronary syndromes, we showed that plasma TREM-1 level was an independent predictor of major adverse cardiovascular events (23).

Here, we evaluated the effects of TREM-1 in murine monocytes/macrophages on inflammatory cell activation, trafficking, and foam cell formation to assess TREM-1 as an upstream target in atherosclerosis.

Methods

Human Carotid Plaques

The processing and examination of the dissected atherosclerotic plaques have been previously described (24). All stainings were examined microscopically and plaque characteristics scored semi-quantitatively (24). Semi-quantitative analysis of atherosclerotic plaque histology is well reproducible, both intra- and interobserver (25). Plaque classification was based on the size of the lipid core and cell population dominance (macrophages or smooth muscle cells [SMC]). Fibrous plaques were characterized by a small lipid core (<10%) with a dominance of SMCs whereas atheromatous plaques had an important lipid core (>40%) dominated by macrophages. For these human plaques, immunostaining studies were performed on vascular tissues (normal aorta, atherosclerotic plaques from carotid artery) obtained post-mortem at autopsy. We used a TREM-1 goat polyclonal antibody on formalin-fixed-paraffin-embedded tissues after antigen retrieval by heating in citrate buffer and ABC peroxidase technique as previously described (26). Polyclonal goat anti-P57 antibody was used as a

nonrelevant antibody for negative control procedure. Additionally, we stained macrophages (anti-CD68), SMCs (α-actin$^+$ SMCs), CD3$^+$ T cells, and neutrophils (myeloperoxidase).

Total proteins were extracted from fresh human atherosclerotic carotid plaques after endarterectomy and TREM-1 protein levels were determined using Luminex xMAP Technology (Thermo Fisher Scientific, Inc., Waltham, Massachusetts). The plaques were obtained from the Athero-Express study, a longitudinal vascular biobank study that was approved by the Medical Ethics Committee of the University Medical Center Utrecht, the Netherlands, and in which participants provided written informed consent.

Animals

Experiments were conducted according to the guidelines formulated by the European Community for experimental animal use (L358-86/609EEC) and approved by the Ethical Committee of INSERM and the French Ministry of Agriculture (agreement A75-15-32). *Trem-1*$^{-/-}$ mice (null for the *Trem-1* gene) were generated by GenOway (Lyon, France) (23) and backcrossed for more than 10 generations into a C57BL/6J background. Ten-week old male C57BL/6J *Ldlr*$^{-/-}$ mice were subjected to medullar aplasia by lethal total body irradiation (9.5 grays). The mice were repopulated with an intravenous injection of bone marrow cells isolated from femurs and tibias of sex-matched C57BL/6J *Trem-1*$^{-/-}$ mice or *Trem-1*$^{+/+}$ littermates. After 4 weeks of recovery, mice were fed a pro-atherogenic diet containing 15% fat, 1.25% cholesterol, and 0% cholate for 4, 8, or 14 weeks. Eight-week old male *Apoe*$^{-/-}$ mice were treated daily by intraperitoneal injection of the Trem-1 inhibitor LR12 (100 µg/day) or the peptide scramble during 4 weeks and were put either on a chow or high-fat diet (15% fat, 1.25% cholesterol).

LR12 (LQEEDAGEYGCM) and LR12-scramble (the inactive control peptide) were chemically synthesized as COOH terminally amidated peptides. The correct peptides were obtained with >99% yields and were homogeneous after preparative purification, as confirmed by mass spectrometry and analytic reversed-phase high-performance liquid chromatography. These peptides were free of endotoxin. Animals were blindly randomized to receive 5 mg/kg LR12 or LR12-scramble peptides intraperitoneally once a day for 28 days. We used the same dose of peptide as in studies on septic shock (21,22) and AMI (23).

Extent and Composition of Atherosclerotic Lesions

Plasma cholesterol was measured using a commercial cholesterol kit. Quantification of lesion size was performed as described previously (27). In brief, the basal half of the ventricles and ascending aorta were perfusion-fixed in situ with 4% paraformaldehyde. Afterwards, they were removed, transferred to a phosphate buffered saline (PBS)/30% sucrose solution, embedded in frozen optimal cutting temperature (OCT) and stored at −70° C. Serial 10 μm sections of the aortic sinus with valves (80 per mouse) were cut on a cryostat, as previously described (28). One of every 5 sections was kept for plaque size quantification after Oil Red O staining. Thus, 16 sections, spanning an 800 μm stretch of the aortic root, were used to determine mean lesion area for each mouse. Oil Red O positive lipid contents were quantified by a blinded operator using HistoLab software (Microvisions Instruments, Paris France), which was also used for morphometric studies (29). En face quantification was used for atherosclerotic plaques along the thoraco-abdominal aorta. The aorta was flushed with PBS via the left ventricle and the aorta was removed from the root to the iliac bifurcation. Then, the aorta was fixed with 10% neutral-buffered formalin. After a thorough washing, adventitial tissue was removed and the aorta opened longitudinally to expose the luminal surface. Afterward, the aorta was stained with oil

Red O for visualizing with the atherosclerotic lesions quantified, as just noted, by a blinded operator. Collagen was detected using Sirius red staining and necrotic core was quantified after Masson's Trichrome staining. Macrophage presence was determined using specific antibodies, as previously described (29). At least 4 sections per mouse were examined for each immunostaining and appropriate negative controls were used. For immunostaining on mouse atherosclerotic plaques, we used antibodies against Trem-1 (Bs 4886R), macrophage + monocyte antibody (MOMA-2, specifically MAB1852), Ly6G (1A8), and CD3 (A0452). Terminal dUTP nick end-labeling (TUNEL) staining was performed using histochemistry and fluorescent staining. Total proteins were extracted from human atherosclerotic plaque and TREM-1 protein level was quantified by Luminex.

Cells were cultured in RPMI 1640 supplemented with Glutamax, 10% fetal calf serum (FCS), 0.02 mM β-mercaptoethanol, and antibiotics. For cytokine measurements, splenocytes were stimulated with lipopolysaccharide (LPS) (10 μg/ml) and interferon (Ifn)-γ (100 UI/ml) for 24 or 48 hours. Il-10, Il-12 and Tnf-α production in the supernatants were measured using specific enzyme-linked immunoadsorbent assays (ELISA).

Primary macrophages were derived from mouse bone marrow-derived cells (BMDM). Tibias and femurs of *C57Bl6/J* male mice were dissected and their marrow flushed out. Cells were grown for 7 days at 37° C in RPMI 1640 medium, 20% neonatal calf serum, and 20% macrophage–colony-stimulating factor (M-CSF)-rich L929-conditioned medium. To analyze oxidized LDL (oxLDL) uptake, BMDMs were exposed to human oxLDL (25 μg/ml) during 24 and 48 h. Cells were washed, fixed, and stained using Red Oil. Foam cells were quantified blindly on 6 to 8 fields and the mean was recorded. To analyze macrophage phenotype, BMDMs were stimulated with LPS (10 μg/ml) and Ifn-γ (100 UI/ml) for 24 h. Il-10, Il-12, Il-1β, and Tnf-

α production in the supernatant was measured using specific ELISAs. To analyze apoptosis susceptibility, macrophages were incubated with Tnf-α (10 ng/ml) and cycloheximide (10 μmol/l) for 6 h or etoposide (50 μmol/l) for 12 h, or in an FCS-free medium. Apoptosis was determined by independent experiments using Annexin V (FITC) apoptosis detection kit with 7-AAD (APC) (BD Biosciences, San Jose, California) according to the manufacturer's instructions.

Human monocytes were isolated using anti-CD14 microbeads from healthy donors. Cells were cultured with M-CSF (50 ng/ml) during 7 days to induce mature macrophages.

Nonclassical monocytes were labeled in vivo by retro-orbital intravenous injection of 1 μm fluorescent microsphere diluted one-quarter in sterile PBS. Chimeric *Ldlr*$^{-/-}$ mice were sacrified 48 h later and cell labeling was checked by flow cytometry (30). Beads that reflect monocyte recruitment were quantified in 8 aortic sinus sections per mouse. Additional information regarding methods used is in the **Online Appendix**.

Statistical Analysis

Values are expressed as mean ± SE of the mean. Differences between values were examined using the nonparametric Mann-Whitney *U* test and were considered significant at $p < 0.05$.

Results

To address the role of myeloid Trem-1 in the development of atherosclerosis, we performed bone marrow transplantation experiments using either *Trem-1*$^{+/+}$ or *Trem-1*$^{-/-}$ bone marrow to repopulate lethally irradiated *Ldlr*$^{-/-}$ mice. In the chimeric *Ldlr*$^{-/-}$/*Trem-1*$^{+/+}$ control group, we confirmed that Trem-1 was expressed by circulating nonclassical monocytes (**Figures 1A and 1B**) and also within the thoraco-abdominal aorta (**Figure 1C**). Trem-1 expression was almost abolished in circulating monocytes and atherosclerotic aorta of chimeric *Ldlr*$^{-/-}$/*Trem-1*$^{-/-}$

(**Figures 1A through 1C**). Leucocyte populations were not different between groups in the spleen, blood, or bone marrow except for a slight increase of $Cd4^+$ T cells in chimeric *Ldlr*$^{-/-}$/*Trem-1*$^{-/-}$ mice (**Online Figure 1**). As shown in **Figure 1**, *Trem-1* deficiency in the bone marrow was associated with a significant decrease in lesion development compared with controls; 42% decrease after 8 weeks of a fat diet ($p < 0.01$) (**Figures 1D through 1F**) and 60% decrease after 14 weeks of a fat diet ($p < 0.01$) (**Figures 1G and 1H**). Additionally, *Trem-1* deletion induced a less inflammatory plaque phenotype with significant reductions in both macrophage accumulation (**Figures 1I through IK**) and necrotic core size (**Figures 1L through 1N**) but no difference regarding collagen content (**Online Figure 2**). We observed no difference in body weights or serum cholesterol levels (**Online Figure 2**) between the 2 groups of mice.

TREM-1 Deficiency Effects

In order to gain insight into the mechanisms of reduction of macrophage and necrotic core content in *Ldlr*$^{-/-}$ mice reconstituted with *Trem-1*$^{-/-}$ bone marrow, we first evaluated apoptosis. In vitro, apoptosis susceptibility of *Trem-1*$^{+/+}$ *and Trem-1*$^{-/-}$ macrophages was comparable between the 2 genetic backgrounds after serum deprivation or exposure to etoposide. However, apoptosis was slightly reduced in *Trem-1*$^{-/-}$ macrophages after stimulation with Tnf-α/cycloheximide stimulation (**Online Figure 3**). In vivo, we found that *Trem-1* deficiency led to a significant reduction in TUNEL+ cells in the atherosclerotic plaques (**Figures 2A through 2C**). This observation might explain the decrease of necrotic core size but did not reconcile the apparent decrease in macrophages, suggesting that other mechanisms prevail. We then tested the possibility that Trem-1 controls monocyte infiltration into the lesions. We quantified monocyte recruitment to atherosclerotic plaque using a validated bead-labeling technique (30). After 4 weeks of fat diet, chimeric *Ldlr*$^{-/-}$ mice were intravenously injected with fluorescent beads and

sacrificed 48 h later. We confirmed the reduction of atherosclerosis development at early stages (data not shown) in *Ldlr*$^{-/-}$/*Trem-1*$^{-/-}$ mice and observed a significant reduction of the recruitment of *Trem-1*$^{-/-}$ bead-positive monocytes within the lesions in comparison with *Trem-1*$^{+/+}$ monocytes (**Figures 2D through 2F**). In parallel, we evaluated the peritoneal recruitment of nonclassical monocytes following a septic and a nonseptic injury. After intraperitoneal injection of LPS or thioglycollate, nonclassical monocyte recruitment was significantly reduced within the peritoneal cavity in Trem-1 deficient animals (**Online Figure 4**). These findings showed that *Trem-1*$^{-/-}$ circulating monocytes have reduced ability to migrate into inflammatory sites and colonize plaques, corroborating a previously identified role of Trem-1 in promoting neutrophil migration during acute inflammation (31).

Because Trem-1 is reported to amplify cytokine production, particularly in response to Tlr agonists (32,33), we next investigated the immuno-inflammatory response in chimeric *Ldlr*$^{-/-}$ mice. LPS/Ifn-γ-stimulated splenocytes showed a deviation toward an anti-inflammatory profile in *Ldlr*$^{-/-}$/*Trem-1*$^{-/-}$ chimeric mice with a reduction of Tnf-α/Il-10 and Il-12p70/Il-10 ratios (**Figure 2G**). The expression of *Il-10, Il-12p70, Il1*β, and *Il-6* messenger ribonucleic acids (mRNA) was also significantly reduced in the spleens of *Ldlr*$^{-/-}$/*Trem-1*$^{-/-}$ mice (**Figures 2H through 2K**). Furthermore, *Trem-1*$^{-/-}$ BMDMs were less prone to polarize toward a pro-inflammatory phenotype after LPS/Ifn-γ, as they produced less Tnf-α, Il-12p70, and Il-1β and more Il-10 compared to *Trem-1*$^{+/+}$ BMDMs (**Figure 2L**). However, expressions of *Nos2* and *Arg1* in the abdominal aorta (as markers of M1 and M2 macrophages, respectively) were not different between chimeric *Ldlr*$^{-/-}$/*Trem-1*$^{-/-}$ and *Ldlr*$^{-/-}$/*Trem-1*$^{+/+}$ mice after 6 weeks of fat diet (**Figures 2M and 2N**). Finally, we investigated macrophage metabolism including mitochondrial

respiration and glycolysis; those functions were very similar between *Trem-1*$^{+/+}$ and *Trem-1*$^{-/-}$ macrophages (**Online Figure 5**).

We speculated that the marked reduction of Oil Red O+ lesions in *Ldlr*$^{-/-}$/*Trem-1*$^{-/-}$ mice could be due, at least in part, to reduced foam cell formation. To explore this hypothesis, we performed in vitro experiments to examine the uptake of oxLDL by BMDMs and their ability to accumulate intracellular lipids. Interestingly, foam cell formation was significantly reduced in *Trem-1*$^{-/-}$ cultured macrophages compared to *Trem-1*$^{+/+}$ macrophages after 24 and 48 h of incubation with oxLDL (**Figures 3A through 3F**). In contrast, phagocytosis of apoptotic cells or Zymosan beads was not altered by *Trem-1* deficiency (**Online Figure 6**), suggesting a selective role in lipid uptake. We found no difference in *Sr-b1, Abca1*, or *Abcg1* mRNA expression and only a slight and delayed reduction of *Sr-a* mRNA expression in *Trem-1*$^{-/-}$ cells (**Figures 3G through 3I**; data not shown). However, we observed a marked reduction of *Cd36* mRNA expression in *Trem-1*$^{-/-}$ cultured macrophages at baseline and after stimulation with oxLDL (**Figures 3G through 3I**). A reduction of Cd36 protein was further confirmed by flow cytometry (**Figures 3J and 3K**). We also quantified Cd36 protein expression in the plaques of chimeric *Ldlr*$^{-/-}$ mice and found a profound decrease of Cd36 staining in the absence of bone marrow-derived Trem-1 (**Figures 3L though 3N**). To start addressing the mechanisms of Trem-1-dependent upregulation of Cd36, we investigated signaling pathways downstream of Trem-1 that may control Cd36 expression. BMDMs co-incubated with oxLDL were treated with different pharmacological inhibitors and Cd36 expression was quantified 16 h later using flow cytometry. Pharmacological blockade of Erk1/2 (PD98059) or Nf-κb (PDTC) had no effect on Cd36 expression (data not shown). However, incubation with Wortmaninn significantly reduced oxLDL-induced Cd36 expression on *Trem-1*$^{+/+}$ BMDMs down to levels seen in *Trem-1*$^{-/-}$

BMDMs (**Figure 3O**), suggesting a role for phosphatidylinositol 3 kinase (PI3K) activation in TREM-1-dependent upregulation of Cd36 following exposure to oxLDL.

Genetic Invalidation of TREM-1

We next addressed the role of TREM-1 in *Apoe*$^{-/-}$ mice. We found an elevated expression of *Trem-1* mRNA expression in the aorta of *Apoe*$^{-/-}$ mice on fat diet and, to a lesser extent, in *Apoe*$^{-/-}$ mice on chow diet, compared to healthy *C57Bl6* aortas, which showed no expression of *Trem-1* (**Online Figure 7A**). *Trem-1* expression levels within the aorta significantly correlated with plaque size ($r = 0.71$; $p = 0.0003$) and macrophage infiltration ($r = 0.87$; $p < 0.0001$) in the aortic sinus (**Online Figure 7B**). Immunostaining confirmed that TREM-1 was expressed in atherosclerotic lesions of *Apoe*$^{-/-}$ mice and co-localized mainly with MOMA^{+} macrophages (**Figure 4A**). We also detected a few TREM-1^{+} Ly6G^{+} neutrophils in atherosclerotic lesions at that stage of lesion development (**Figure 4B**). To investigate the role of TREM-1 in this model, we generated *Apoe*$^{-/-}$*/Trem-1*$^{-/-}$ mice. Trem-1 deficiency in myeloid population was confirmed by flow cytometry (**Figures 4C and 4D**) and by immunostaining in atherosclerotic plaques (**Figures 4E and 4F**). Experiments in chimeric *Ldlr*$^{-/-}$ mice suggested that TREM-1 modulates nonclassical recruitment into atherosclerotic lesions. We therefore explored the expression of Cx3 chemokine receptor 1 (Cx3cr1), a major chemokine receptor of non-classical monocytes (34). Using flow cytometry on *Apoe*$^{-/-}$ mice, we found that Cx3cr1 expression was restricted to TREM-1^{+} nonclassical monocytes (**Figures 4G through 4I**). After 2 weeks of fat diet, Cx3cr1 expression (mean fluorescence intensity [MFI]) on TREM-1^{+} nonclassical monocytes was 14-fold higher than on TREM-1^{-} nonclassical monocytes. Then we compared Cx3cr1 expression on nonclassical monocytes of *Apoe*$^{-/-}$*/Trem-1*$^{+/+}$ *and Apoe*$^{-/-}$*/Trem-1*$^{-/-}$ mice. As shown in **Figures 4J and 4K**, we found a strong reduction of Cx3cr1 expression (MFI) on *Apoe*$^{-/-}$*/Trem-1*$^{-/-}$

nonclassical monocytes in comparison to control *Apoe*$^{-/-}$*/Trem-1*$^{+/+}$ nonclassical monocytes. Cx3cr1 expression (MFI) on classical monocytes was not different between *Apoe*$^{-/-}$*/Trem-1*$^{+/+}$ and *Apoe*$^{-/-}$*/Trem-1*$^{-/-}$ mice (data not shown). Finally, we analyzed the phenotype of mature peritoneal macrophages isolated from *Apoe*$^{-/-}$*/Trem-1*$^{+/+}$ and *Apoe*$^{-/-}$*/Trem-1*$^{-/-}$ mice by quantitative polymerase chain reaction after LPS/Ifn-γ stimulation. We found a significant increase of *Il-10* mRNA expression and a significant decrease of *Il-12p70* and *Arginase 1* by *Apoe*$^{-/-}$*/Trem-1*$^{-/-}$ macrophages (**Figures 4L through 4P**).

There was no significant difference in plasma cholesterol levels between *Apoe*$^{-/-}$*/Trem-1*$^{+/+}$ *and Apoe*$^{-/-}$*/Trem-1*$^{-/-}$ groups (**Figure 5A**). At 10 weeks of age, animals were put on a fat diet for 6 weeks to accelerate plaque formation. *Apoe*$^{-/-}$*/Trem-1*$^{-/-}$ showed a significant reduction of atherosclerotic lesion formation in the aortic sinus (-41%; $p < 0.05$) (**Figures 5B through 5D**) and along the thoraco-abdominal aorta (-56%; $p = 0.005$) (**Figures 5E through 5G**). Additionally, *Trem-1* deletion in *Apoe*$^{-/-}$ background induced a switch toward a less inflammatory plaque phenotype with significant reductions in macrophage accumulation (**Figures 5H through 5K**), necrotic core size (**Figures 5L though 5O**), and TUNEL^{+} area (only absolute area) (**Figures 5P through 5S**). We did not observe any difference in T cell accumulation (**Online Figure 8A**) and plaque collagen content (**Online Figure 8B**) between the 2 groups of mice.

Pharmacologic Blockade of TREM-1

To evaluate a new therapeutic approach in atherosclerosis, we used a dodecapeptide LR12, a validated inhibitor of TREM-1 activation (21,23). LR12 reduced oxLDL uptake by BMDMs (**Figures 6A through 6C**) and down-regulated Cd36 expression (**Figure 6D**). Both results were confirmed in human monocyte-derived macrophages (**Online Figure 9**). Using

Apoe$^{-/-}$ peritoneal macrophages, we confirmed that LR12 treatment reduced *Cd36* mRNA *(***Figure 6E**) and induced a deviation of the immune response toward an anti-inflammatory profile with an increase of Il-10 and a reduction of Il-12p70 and Tnf-α production following LPS/Ifn-γ stimulation (**Figure 6F**). Using human monocytes, LR12 also down-regulated *IL-12, TNF-α,* and *CD36* mRNA expression (**Online Figure 10**). Next, we investigated the consequences of TREM-1 pharmacological inhibition on monocyte trafficking. *Apoe*$^{-/-}$ mice fed a fat diet and treated with daily intraperitoneal injections of scramble or LR12 peptide during 2 weeks were intravenously injected with fluorescent beads 48 h before sacrifice. We observed a significant reduction of the recruitment of bead-positive monocytes within the lesions of LR12-treated *Apoe*$^{-/-}$ mice (**Figures 6G through 6I**).

To evaluate the effects of TREM-1 inhibition on atherosclerosis, *Apoe*$^{-/-}$ male mice were treated during 4 weeks with daily intraperitoneal injections of LR12 or a scramble control peptide. LR12 treatment had no effect on cholesterolemia (**Online Figures 11A and 11B**) but significantly reduced atherosclerosis growth on a chow diet (56,600 ± 22,300 μm^2 vs. 98,900 ± 30,900 μm^2; $p < 0.05$) (**Figures 6J through 6L**), and on a high-fat diet both in the aortic sinus (103,300 ± 21 100 μm^2 vs. 148,700 ± 15,200 μm^2; $p < 0.05$) (**Figures 6M through 6O**) and the thoraco-abdominal aorta (6.0 ± 3.0% vs. 2.5 ± 0.9%; $p < 0.05$) (**Figures 6p through 6R**). TREM-1 pharmacological inhibition also reduced macrophage accumulation (**Figures 6S through 6U**) and TUNEL$^+$ cells/debris (**Online Figure 11C**) in the lesions, but had no effect on T cell infiltration (**Online Figure 11D**) or collagen content (**Online Figure 11E**). To investigate the role of neutrophils in the atheroprotection induced by TREM-1 blockage, neutrophil-depleted *Apoe*$^{-/-}$ mice were treated with daily intraperitoneal injections of scramble or LR12 peptide for 4 weeks. Administration of anti-Ly6G depleting antibody every 3 days led to 70% depletion of

circulating neutrophils. As shown in **Online Figure 12**, the atheroprotection induced by pharmacological inhibition of TREM-1 was preserved in neutrophil-depleted animals.

TREM-1 EXPRESSED IN ATHEROSCLEROTIC HUMAN LESIONS. To evaluate the clinical relevance of our results, we examined TREM-1 expression in human atherosclerotic plaques from carotid arteries. TREM-1 was not detected in normal aorta (**Figures 7A and 7B**). However, TREM-1 was expressed in fatty streak lesions (**Figures 7C and 7D**) and in advanced atherosclerotic plaques (**Figure 7E** and **7F**), in areas surrounding the necrotic core (**Figure 6G**). Immunohistochemistry showed that TREM-1 staining localized to the membrane of giant lipid-laden foam cells (**Figure 7H**). Fluorescent staining confirmed that TREM-1 expression was mostly confined to $CD68^+$ intimal macrophages (**Figure 7I**) and a few neutrophils (**Online Figure 13A**), but was not detected in α-$actin^+$ SMCs (**Figure 7J**) or $CD3^+$ T cells (**Online Figure 13B**). We quantified TREM-1 protein levels in human plaque extracts obtained after endarterectomy (**Online Figure 14**), and found TREM-1 expression was significantly higher in atheromatous lesions compared to fibrous lesions ($p = 0.002$) (**Figure 7K**).

Discussion

Using 3 complementary approaches, we found that TREM-1 deficiency/inhibition significantly reduced atherosclerosis growth in mice and induced a less inflammatory plaque phenotype characterized by reduced macrophage infiltration and necrotic core size. It is interesting to note that TREM-1 deletion or blockade was associated with significant and profound (up to 60%) reduction of the development of both early and advanced atherosclerosis and the protective effect was observed throughout the aorta (aortic sinus, ascending and descending aorta). This contrasted with the relatively modest (and mostly nonsignificant) impact of many other innate immune pathways on lesion development in this model, including deletion

of *Cd36* (12), *Tlr2/Tlr4* (10), *Il-1* (35), or *Tnfr* signaling (36) in bone marrow-derived cells. We believe that the marked nonredundant effect of TREM-1 on atherosclerosis is due to the major role of this innate receptor in coordinating several complementary pro-atherogenic processes. Here, we identified a few atheroprotective mechanisms induced by TREM-1 deletion/blockade: reduction of nonclassical monocyte infiltration in plaques; reduction of macrophage pro-inflammatory response; and reduced foam cell formation (**Central Illustration**). We have also shown that TREM-1 was expressed in advanced human atherosclerotic lesions, mostly close to cholesterol-rich and necrotic-rich areas and its expression was significantly higher in atheromatous lesions.

TREM-1 is highly conserved during evolution (37) and mainly expressed by bone marrow-derived leukocytes (15). In our study, TREM-1 was highly expressed in circulating neutrophils and $Gr1^{lo}$, but not $Gr1^{hi}$, monocytes. Deletion of TREM-1 in bone marrow-derived cells did not significantly alter leukocyte number in blood but limited $Gr1^{lo}$ monocyte recruitment into developing atherosclerotic lesions. TREM-1 was mainly expressed by macrophages in atherosclerotic lesions and colocalized with a few infiltrating neutrophils. We believe that the atheroprotection induced by TREM-1 invalidation/blockage was not mediated by neutrophil modulation. First, TREM-1 inhibition reduced both atherosclerosis formation and progression whereas neutrophils are mostly involved in the initiation of atherosclerosis in experimental models (38). Second, the reduction of atherosclerosis induced with TREM-1 pharmacological inhibition was preserved despite marked (70%) neutrophil depletion. However, we cannot exclude a small contribution of neutrophils to the pro-atherogenic role of TREM-1.

It is plausible that reduced recruitment of nonclassical monocytes has contributed to the reduction of lesion development after TREM-1 deletion/blockade. We identified a sub-

population of nonclassical monocytes that co-express TREM-1 and Cx3cr1. Interestingly, the reduction of Cx3cr1 expression on this monocyte subset in *Trem-1*-deficient animals suggested an important role for TREM-1 in the regulation of Cx3cr1 expression under conditions of high-fat feeding. However, downstream signaling pathways remain unknown. Another potential important consequence of Cx3cr1 modulation by TREM-1 could be reduced interaction of TREM-1-deficient macrophages with intimal smooth muscle cells that express high levels of Cxc3cr1 ligand, Cx3cl1. Collectively, our data were consistent with the role of Cx3cl1/Cxc3cr1 signaling in experimental atherosclerosis (39-42).

Whether differentiating from $Gr1^{lo}$ or $Gr1^{hi}$ monocytes, most macrophages upregulate TREM-1 expression upon activation. Our study extended previous findings by highlighting the amplifier effect of TREM-1 engagement on the inflammatory response of macrophages in the context of atherosclerosis. Genetic invalidation of *Trem-1* in BMDMs modulated the systemic innate inflammatory response as illustrated by the reduction of Tnf-α/Il-10 and Il-12p70/Il-10 ratios in the spleens of $Ldlr^{-/-}/Trem\text{-}1^{-/-}$ mice. $Trem\text{-}1^{-/-}$ macrophages displayed a more anti-inflammatory phenotype with less production of pro-atherogenic Il-12, Il-1β, and Tnf-α (3,8) and increased production of anti-atherogenic Il-10 (43,44). A similar anti-inflammatory phenotype was observed after pharmacological inhibition of TREM-1 in both human monocytes and mouse macrophages. The regulation of Il-1β and Tnf-α production by TREM-1 has previously been reported in vitro using LPS-stimulated (human and animal) neutrophils, LPS-stimulated monocytes (45), and cultured foam cells (46). Similar regulation of innate immune responses by TREM-1 were described in other aseptic contexts, as during experimental colitis (47,48) or, more recently, by our group during AMI (23). Furthermore, in a model of ureteral obstruction–induced nephritis, *Trem-1* deficiency reduced M1 macrophage polarization within

the kidney, as illustrated by a decrease of *Nos2* and an increase of *Arg1* expression (49). We observed no major differences in those 2 markers in the aorta of *Ldlr*$^{-/-}$/*Trem-1*$^{+/+}$ and *Ldlr*$^{-/-}$/*Trem-1*$^{-/-}$ mice. The discrepancy could be related to the disease setting (different types of sterile inflammation, acute versus chronic context) or to differences in local environmental cues, which are known to impact macrophage phenotype (50).

Necrotic core size is another major predictor of plaque vulnerability (51). The formation and progression of the necrotic core is influenced by a variety of pro-atherogenic processes, including lipid accumulation, inflammatory cell activation, and the rates of apoptotic cell death and efferocytosis within the lesions. The TREM-1/DAP12 signaling pathway involves extracellular signal-regulated kinase and PI3K/AKT, 2 cascades regulating cell survival (52,53). However, the effect of Trem-1 stimulation on monocyte/macrophage survival is not well-defined. So far, available studies have reported contradictory results suggesting that the role of TREM-1 on cell survival depends on the cell type and the experimental conditions. For example, Radsak et al. found that TREM-1 engagement on neutrophils had no effect on apoptosis susceptibility, but promoted apoptosis in the presence of TLR ligands (54). In contrast, Cai et al. observed that TREM-1 overexpression on human monocytes promoted cell survival in the presence of staurosporine (55). In our study, we used 3 different apoptotic challenges and observed a slight reduction of apoptosis in *Trem-1*$^{-/-}$ BMDM only after Tnf-α/cycloheximide stimulation. In vivo, we observed a reduction of TUNEL^{+} area in atherosclerotic plaques of *Ldlr*$^{-/-}$/*Trem-1*$^{-/-}$ chimeric mice, A*poe*$^{-/-}$*Trem-1*$^{-/-}$ mice, and LR12-treated *Apoe*$^{-/-}$mice compared to their respective controls, consistent with the reduced susceptibility of *Trem-1*$^{-/-}$ macrophages to apoptosis. However, the reduction of plaque apoptosis and necrotic core in the absence of TREM-1 could also be secondary to reduced macrophage accumulation and activation.

Foam cell formation is a major determinant of plaque development and progression. Given the drastic reduction of the area of aortic Oil Red O staining in the absence of TREM-1, we investigated the role of TREM-1 in macrophage foam cell formation and found a marked reduction of oxLDL uptake and lipid accumulation in *Trem-1*$^{-/-}$ macrophages. Among the major receptors that govern lipid body formation in macrophages (i.e., Sr-a, Sr-b1, Cd36, Abca1, Abcg1), TREM-1 deletion selectively altered the expression of Cd36 both at the gene and cell surface protein expression levels, and both in vitro and within atherosclerotic plaque macrophages in vivo. We found that signaling through PI3K, which is activated downstream of TREM-1 (33,56) was required for TREM-1-dependent upregulation of Cd36 in response to oxLDL, consistent with the role of PI3K activation in the induction of Cd36 (57,58). The profound reduction of atherosclerosis observed in chimeric *Ldlr*$^{-/-}$/*Trem-1*$^{-/-}$ mice was independent of the genetic background (observed under A*poe*$^{-/-}$ and *Ldlr*$^{-/-}$ background), and the high-fat diet (observed under chow-fed A*poe*$^{-/-}$ mice and high-fat diet-fed *Ldlr*$^{-/-}$ mice). Thus, it is difficult to entirely attribute this marked impact on atherosclerosis to the reduction of CD36 expression, given the variable impact of CD36 on the development of atherosclerosis in mice, which depends on the Apoe/Ldlr background and the presence or absence of cholesterol-enriched high-fat diet (13,59-61). Previous studies have shown that interactions between Cd36 and several Tlrs were required for optimal induction of sterile inflammation (and macrophage apoptosis) by atherogenic lipids (10,11,62). However, in vivo evidence for an additive or synergistic effect of combined blockade of Cd36 and Tlr pathways was still lacking. TREM-1 signaling is a major amplifier of Tlr-dependent responses (63), and we demonstrated here that it is also critical for both basal and oxLDL-induced Cd36 expression. Therefore, we believe that the combined reduction of inflammatory signaling and Cd36 expression after TREM-1 deletion promoted a

synergistic effect and was ultimately responsible for the marked reduction in inflammatory foam cell formation and plaque development. Our data supported the combinatorial concept of synergistic Cd36-Tlr signaling and showed that blockade of a single cell surface receptor, here TREM-1, might be equivalent to a double-hit approach, simultaneously impacting 2 major and interdependent pro-atherogenic processes.

Despite the crucial role of the immune response in atherosclerosis development (1,3), therapeutic strategies targeting innate immune pathways have mostly failed (4,64), although some continue clinical testing (65). We believe that a double-hit approach through TREM-1blockade, as is the case with the use of the inhibitory LR12 dodecapeptide, may provide extended benefit and will be worthy of clinical testing. The relevance of such an approach is further supported by our clinical findings of increased levels of TREM-1 in atheromatous human atherosclerotic lesions and the very significant association between high circulating levels of soluble TREM-1 and major adverse cardiovascular events in patients with AMI (23). A first clinical trial to assess the safety, tolerability, and pharmacokinetics of TREM-1 blockade using LR12 peptide in healthy volunteers is ongoing. We speculate that acute coronary disease patients represent the population of choice that could benefit from LR12 treatment because TREM-1 inhibition would prevent myeloid cell recruitment to the ischemic heart (23) and the atherosclerotic plaques, limiting deleterious post-ischemic cardiac remodeling, the acceleration of atherosclerosis after ischemic injury (66) and the recurrence of atherothrombotic events (67).

Study Limitations

In this study, we found that TREM-1 inhibition protected against atherosclerosis development through several anti-inflammatory and anti-atherogenic mechanisms. However, we

did not specifically modulate these pathways (i.e., Cx3cr1 or Cd36) to decipher their direct roles and relative importance in mediating TREM-1 pro-atherogenic effects.

Conclusions

Altogether, our studies identified TREM-1 as a crucial player in the development and complications of atherosclerosis, both in experimental models and in humans. This was achieved through the unique role of TREM-1 in connecting and coordinating signaling through several pattern recognition receptors (e.g., CD36 and TLR4), which led to the activation of synergistic pro-atherogenic pathways, namely innate inflammatory activation of macrophages and foam cell formation. Targeting TREM-1 may constitute a promising new therapeutic approach to combat cardiovascular diseases.

Perspectives

COMPETENCY IN MEDICAL KNOWLEDGE: TREM-1 is an important factor in the progression of atherosclerosis in both experimental and clinical models.

TRANSLATIONAL OUTLOOK: Further studies are needed to assess the efficacy of pharmacological inhibition of TREM-1 to prevent complications of atherosclerosis.

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Figure Legends

Central Illustration: Proatherogenic Effects of TREM-1 Engagement. Triggering receptors expressed on myeloid cells (TREM-1) activation orchestrates monocyte recruitment, macrophage pro-inflammatory responses, and foam cell formation. Pharmacological blockade of TREM-1 signaling using LR12 peptide may constitute an attractive novel and double-hit approach for the treatment of atherosclerosis. CD = cluster of differentiation; Cx3cr1 = Cx3 chemokine receptor 1; LDL = low-density lipoprotein.

Figure 1 *Trem-1* Deficiency in Reduces Atherosclerosis. In low-density lipoprotein receptor (*Ldlr*)$^{-/-}$ mice, triggering receptors expressed on myeloid cells (*Trem-1*) deficiency in myeloid cells reduced development of atherosclerosis and induced a stable plaque phenotype. As seen in flow cytometry on blood leucocytes gated on cluster of differentiation (CD)11b+CD115high monocytes, TREM-1 was expressed by nonclassical Gr1^{low} monocytes in the control (*Ldlr*$^{-/-}$/*Trem-1*$^{+/+}$) group **(A)** but was absent in *Ldlr*$^{-/-}$/*Trem-1*$^{-/-}$ mice **(B)**.**(C)** Using quantitative polymerase chain reaction on aortas of chimeric *Ldlr*$^{-/-}$ mice after 6 weeks of fat diet, *Trem-1* messenger ribonucleic acid (mRNA) was detected in the control group but its expression was almost abolished in the *Ldlr*$^{-/-}$/*Trem-1*$^{-/-}$ group. Representative photomicrographs of controls **(D)** and Trem-1-deficient mice **(E)** and quantitative analysis **(F)** of atherosclerotic lesions of chimeric *Ldlr*$^{-/-}$ mice after 8 weeks of fat diet. Quantitative analysis of atherosclerotic lesions of chimeric *Ldlr*$^{-/-}$ mice after 14 weeks of fat diet in the **(G)** left aortic sinus and **(H)** thoraco-abdominal aorta. E, representative photomicrographs of controls **(I)** and Trem-1-deficient mice **(J)** and quantitative analysis **(K)** of macrophage accumulation (macrophage + monocyte antibody [MOMA] staining) in atherosclerotic lesions of chimeric *Ldlr*$^{-/-}$ mice. Representative

photomicrographs of controls **(L)** and Trem-1-deficient mice **(M)** and quantitative analysis **(N)** of acellular area (Masson's Trichrome) of chimeric *Ldlr*$^{-/-}$ mice. *p < 0.05; **p < 0.01; ***p < 0.001 Scale bar = 200 μm.

Figure 2: Monocyte Infiltration in Atherosclerotic Plaques. TREM-1 deficiency reduced monocyte migration into atherosclerotic lesions and limited macrophage inflammatory response. Representative photomicrographs of controls **(A)** and Trem-1-deficient mice **(B)** and quantitative analysis **(C)** of terminal dUTP nick end-labeling (TUNEL)+ apoptotic area (Red/Brown, arrows) within atherosclerotic lesions of chimeric *Ldlr*$^{-/-}$ mice after 8 weeks of fat diet. Representative photomicrographs of controls **(D)** and Trem-1-deficient mice **(E)** and quantitative analysis **(F)** of beads within atherosclerotic lesions of chimeric *Ldlr*$^{-/-}$ mice after 4 weeks of fat diet. **(G)** Cytokine production by lipopolysaccharide (LPS)/interferon (Ifn)-γ-stimulated splenocytes isolated from chimeric *Ldlr*$^{-/-}$ mice. Quantification of interleukin (Il) **(H)** *Il-10,* **(I)** *Il-12p70,* **(J)** *Il-1β*, and **(K)** *Il-6* mRNA expression in the spleens of chimeric *Ldlr*$^{-/-}$ mice. **(L)** Cytokine production by LPS/Ifnγ-stimulated bone marrow-derived macrophages (BMDM) isolated from *Ldlr*$^{-/-}$ chimeric mice, 6 weeks after transplantation. Quantification of **(M)** *iNOS* and **(N)** *Arginase 1* mRNA expression in abdominal aortas from chimeric *Ldlr*$^{-/-}$ mice. *p < 0.05; **p < 0.01; ***p < 0.001 Scale bar = 200 μm. Data are expressed as median and percentiles (5^{th}-95^{th}). Abbreviations as in Figure 1.

Figure 3: Pro-inflammatory Macrophage Responses and Lipid Uptake. Trem-1 promoted phosphatidylinositol 3 kinase (PI3K)-dependent expression of Cd36 in macrophages and induced foam cell formation. Quantitative analysis **(A)** and representative photomicrographs of controls

(B) and Trem-1-deficient mice **(C)** at 24 h and at 48 h **(D-F)** after incubation of BMDMs from *Trem-1*$^{+/+}$ and *Trem-1*$^{-/-}$ mice with oxidized low-density lipoprotein (oxLDL). Quantification of **(G)** *Sr-a,* **(H)** *Sr-b1*, and **(I)** *Cd36* mRNAs in BMDMs from *Trem-1*$^{+/+}$ and *Trem-1*$^{-/-}$ mice at baseline and after stimulation with oxLDL. **(J)** Flow cytometry quantification of Cd36 expression by BMDMs from *Trem-1*$^{+/+}$ and *Trem-1*$^{-/-}$ mice at baseline and after stimulation with oxLDL. **(K)** Histograms depict specific MFI of *Trem-1*$^{+/+}$ and *Trem-1*$^{-/-}$ macrophages exposed to oxLDL and stained with an antibody to Cd36. Quantification **(L)** and representative photomicrographs of Cd36 expression in plaques from chimeric **(M)** *Ldlr*$^{-/-}$/*Trem-1*$^{+/+}$ and **(N)** *Ldlr*$^{-/-}$/*Trem-1*$^{-/-}$ mice. **(O)** Effects of PI3K inhibition on Cd36 expression by BMDMs (flow cytometry). * P<0.05. ** P<0.01. Data are expressed as median and percentiles (5th-95th). MFI = mean fluorescence intensity; other abbreviations as in Figures 1 and 2.

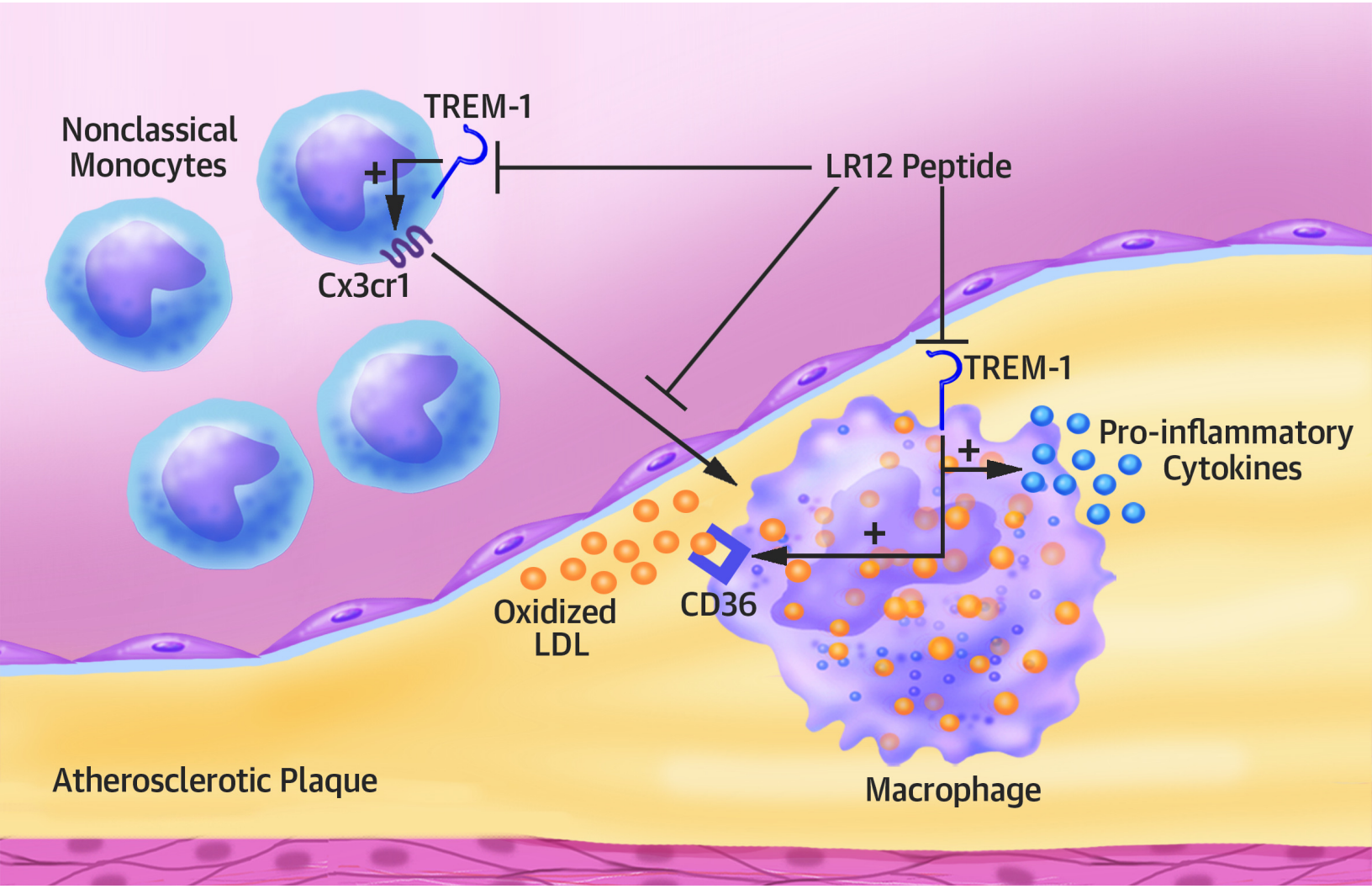
Figure 4: **TREM-1 in Atherosclerotic Plaque and Expression of Cx3cr1**. **(A)** TREM-1 (green) is expressed in atherosclerotic plaques of apoliprotein E-deficient (*Apoe*$^{-/-}$) tissue and colocalizes (yellow) with MOMA^{+} macrophages (red). **(B)** Few Ly6G+ neutrophils (red) were detected in plaque of *Apoe*$^{-/-}$ mice and colocalized (yellow) with TREM-1. **(C)** Flow cytometry analysis confirmed that TREM-1 was expressed on circulating CD11b^{+} myeloid cells from **(C)** *Apoe*$^{-/-}$*/Trem-1*$^{+/+}$ but not **(D)** *Apoe*$^{-/-}$*/Trem-1*$^{-/-}$ mice. TREM-1 (green) is expressed in **(E)** *Apoe*$^{-/-}$*/Trem-1*$^{+/+}$ but was not detected in **(F)** *Apoe*$^{-/-}$*/Trem-1*$^{-/-}$ plaques. **(G-I)** TREM-1^{+} Cd11b^{+}Ly6G^{-} LyC^{low} nonclassical monocytes express Cx3 chemokine receptor 1 (Cx3cr1). **(J and K)** Comparison of Cx3Cr1 expression by Cd11b^{+}Ly6G^{-}LyC^{low} nonclassical monocytes of *Apoe*$^{-/-}$*/Trem-1*$^{+/+}$ and *Apoe*$^{-/-}$*/Trem-1*$^{-/-}$ mice. Quantification of **(L)** *Il-10,* **(M)** *Il-12,* **(N)** *Nos2,* **(O)** *Arginase 1*, and **(P)** *Ym1 mRNAs* expression by peritoneal macrophages of *Apoe*$^{-/-}$*/Trem-1*$^{+/+}$ and

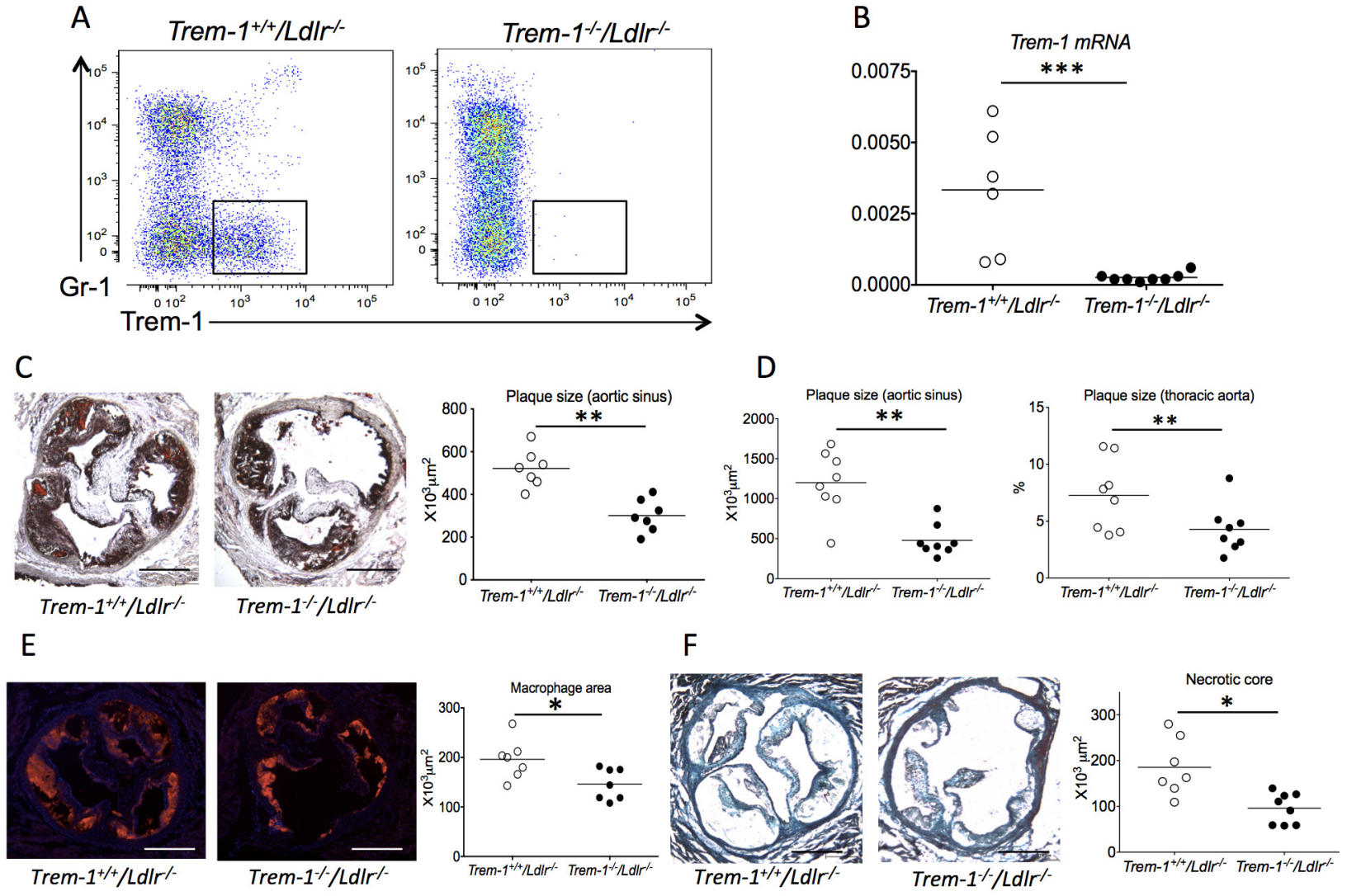
Apoe$^{-/-}$*/Trem-1*$^{-/-}$ mice. *p < 0.05; **p < 0.01. Scale bar = 100 μm. Data are expressed as median and percentiles (5th-95th).

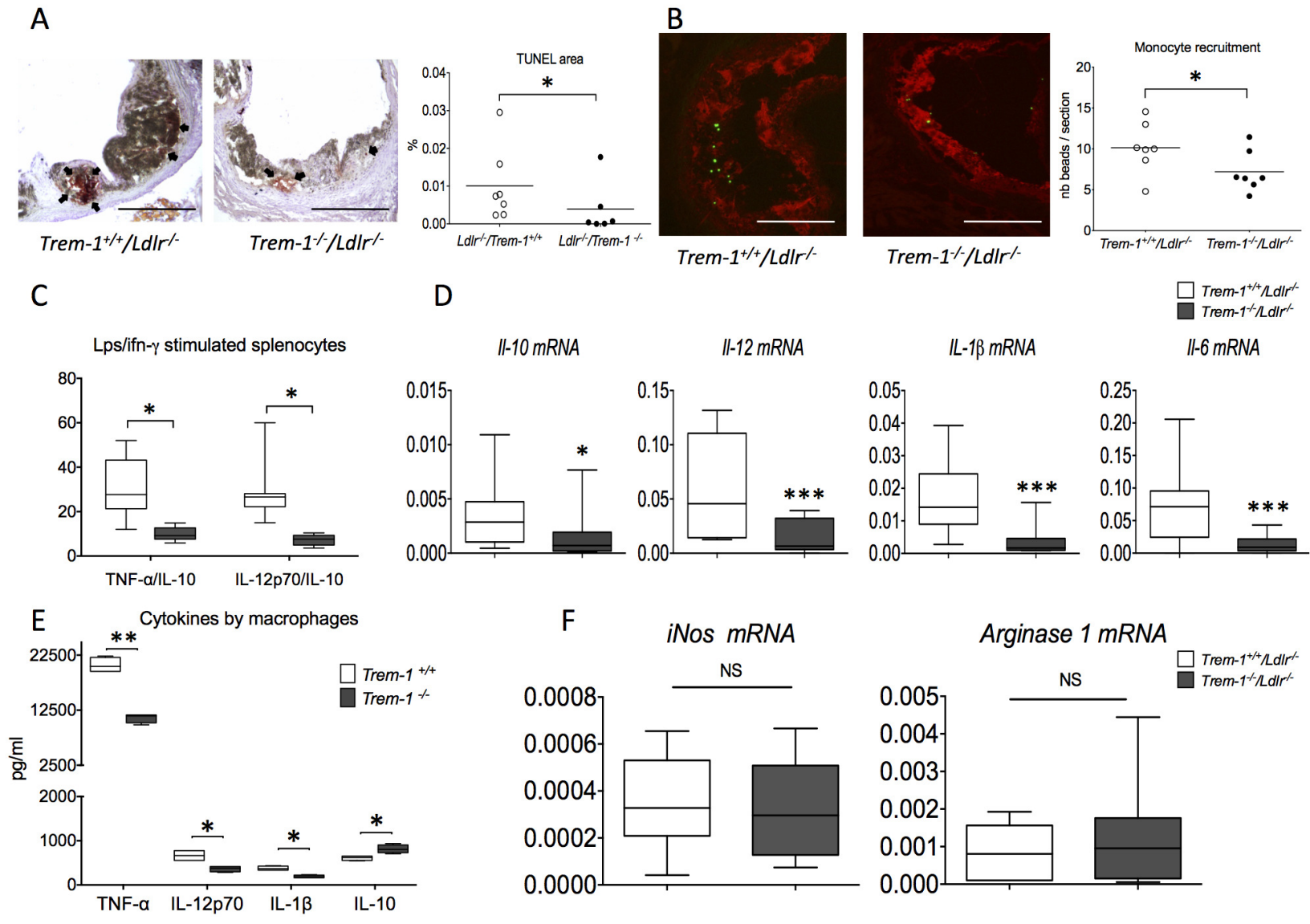
Figure 5: Genetic Invalidation of *Trem-1*: Atherosclerosis Reduction. After 6 weeks of a fat diet, various parameters differed in mouse groups: **(A)** plasma cholesterol levels; atherosclerotic lesions in **(B-D)** the aortic sinus and **(E-G)** in the thoraco-abdominal aorta. Variations are seen in **(H-K)** macrophage accumulation, **(L-O)** acellular area (after Masson's Trichrome), and **(P-S)** TUNEL+ in *ApoE*$^{-/-}$*/Trem-1*$^{+/+}$ *and ApoE*$^{-/-}$*/Trem-1*$^{-/-}$ mice. *p < 0.05; **p < 0.01. Scale bar = 200 μm (C and D) and 100 μm (J, K, N, O, R, S). Abbreviations as in Figures 1, 2, 4, and 5.

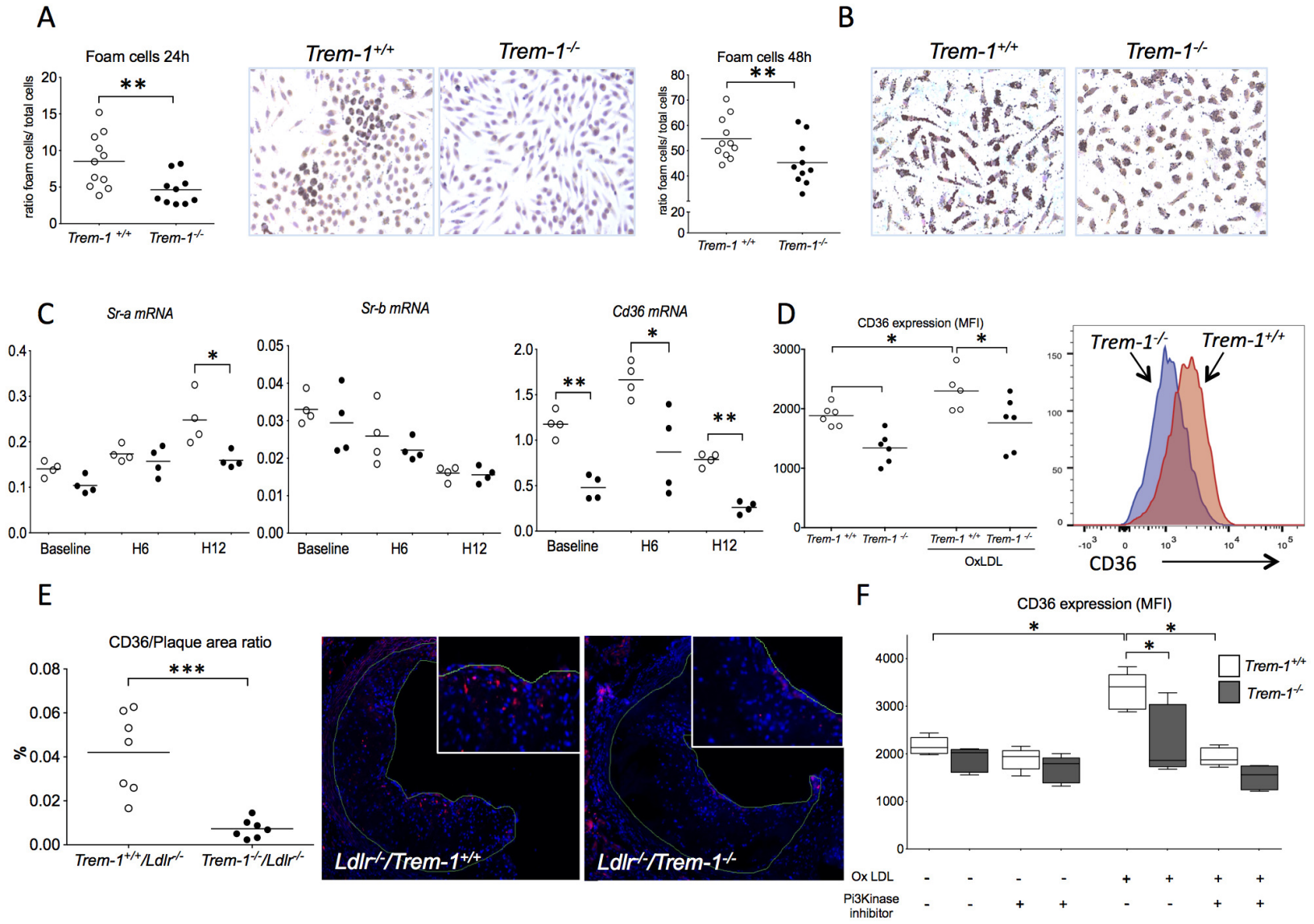
Figure 6: Monocyte/macrophage Function Modulations. Pharmacological blockade of TREM-1, though injections of scramble or LR12 peptide, modulated monocyte and macrophage functions reducing atherosclerosis development as seen via **(A-C)** foam cell formation and **(D)** Cd36 expression after co-incubation of BMDMs with oxLDL and peptide during 24 h; **(E)** *Cd36* mRNA expression in peritoneal macrophages from *ApoE*$^{-/-}$ mice treated with daily peptide injections during 3 days prior to cell isolation; **(F)** cytokine production by LPS/Ifn-γ-stimulated peritoneal macrophages; **(G-I)** beads within atherosclerotic lesions of treated *ApoE*$^{-/-}$ mice after 2 weeks of fat diet; atherosclerotic lesion size after 4 weeks of **(J-L)** chow diet or fat diet as seen in the **(M-O)** aortic sinus and **(P-R)** thoracic aorta; and **(S-U)** macrophage infiltration in atherosclerotic lesions after 4 weeks of fat diet. *p < 0.05. Scale bar = 200 μm. Data are expressed as median and percentiles (5th-95th). Abbreviations as in Figures 1, 2, and 3.

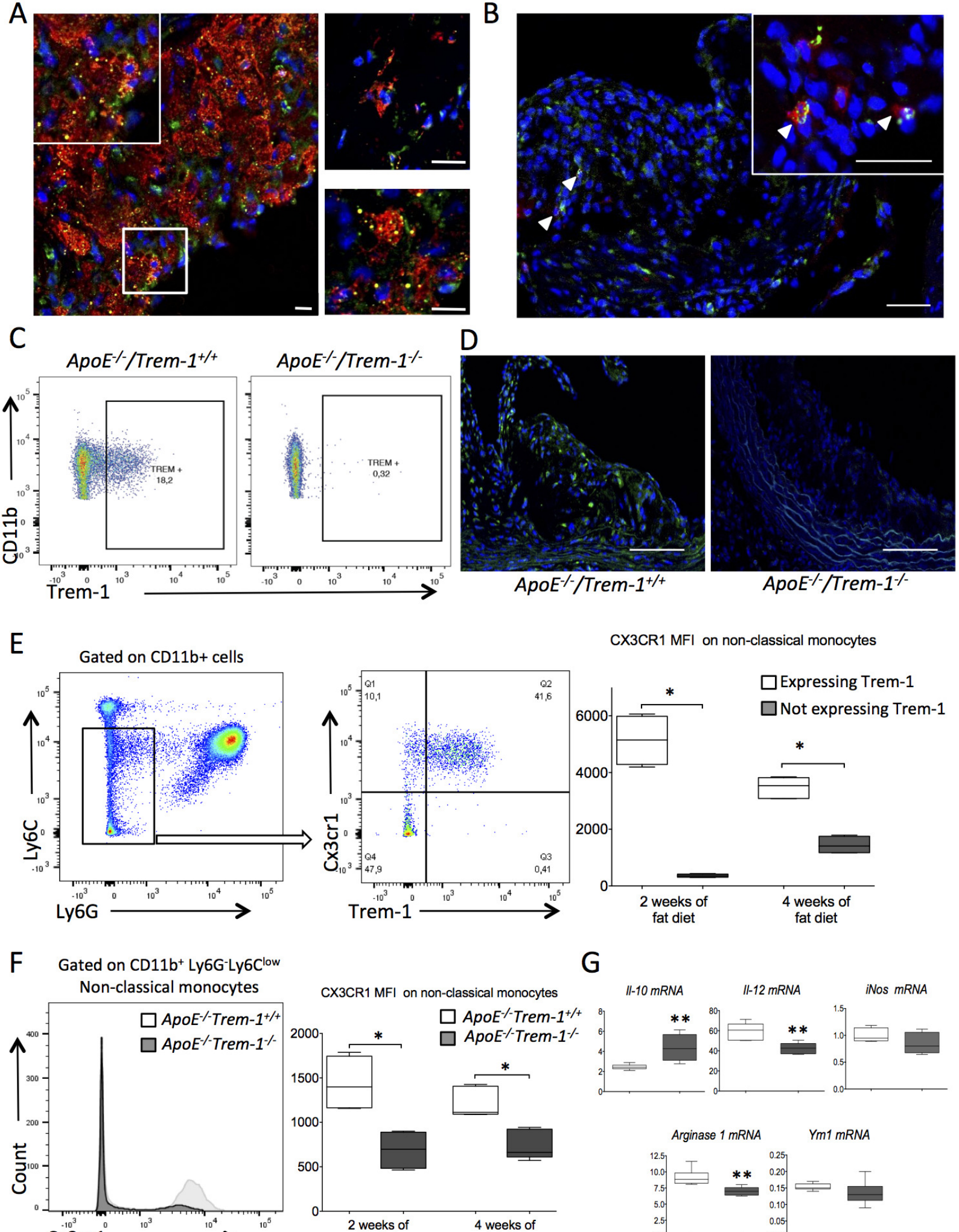
Figure 7: TREM-1 protein expression in human atherosclerotic plaques. TREM-1 is not expressed in normal aorta **(A, B)** but is expressed in fatty streak lesions of the aorta **(C, D)** and in advanced carotid artery plaques **(E, F)** mainly around the necrotic core **(G)**. TREM-1 was strongly expressed by cells that engulf lipids and cholesterol crystals and on the membrane of giant lipid-laden foam cells **(H)**. Fluorescent staining confirmed that TREM-1 was expressed by $CD68^+$ macrophages **(I)** but not by α-actin+ smooth muscle cells **(J)**. TREM-1 expression was significantly higher in atheromatous lesions in comparison with fibrous plaques **(K)**. Magnitude: X 20 (A-F, I, J); X 2.5 (G); X 40 (H). Lum = lumen; other abbreviations as in Figures 1 and 2.

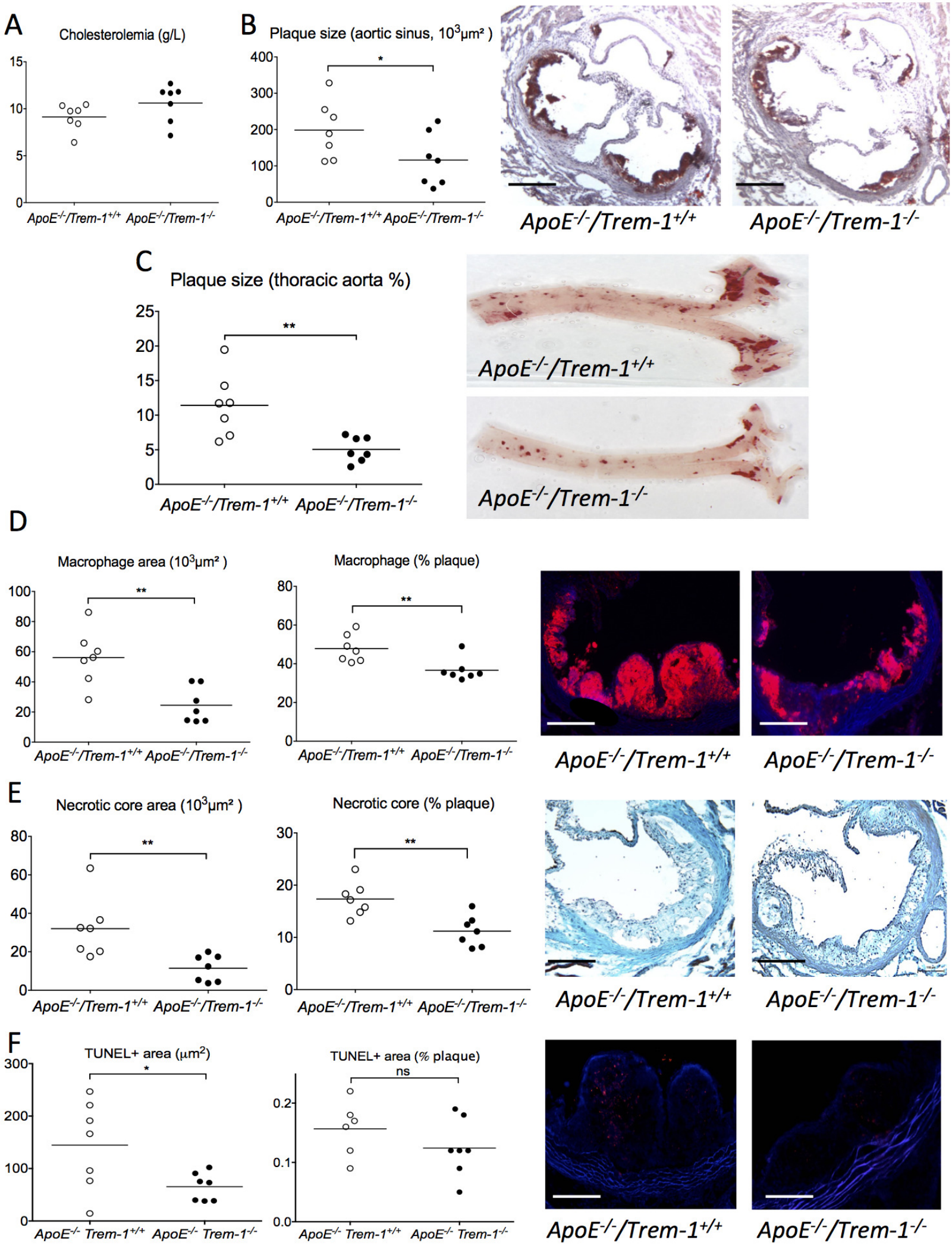

Nonclassical
Monocytes
TREM-1
+
LR12 Peptide
Cx3cr1
TREM-1
Pro-inflammatory
Cytokines
+
+
Oxidized
LDL
CD36
Atherosclerotic Plaque
Macrophage

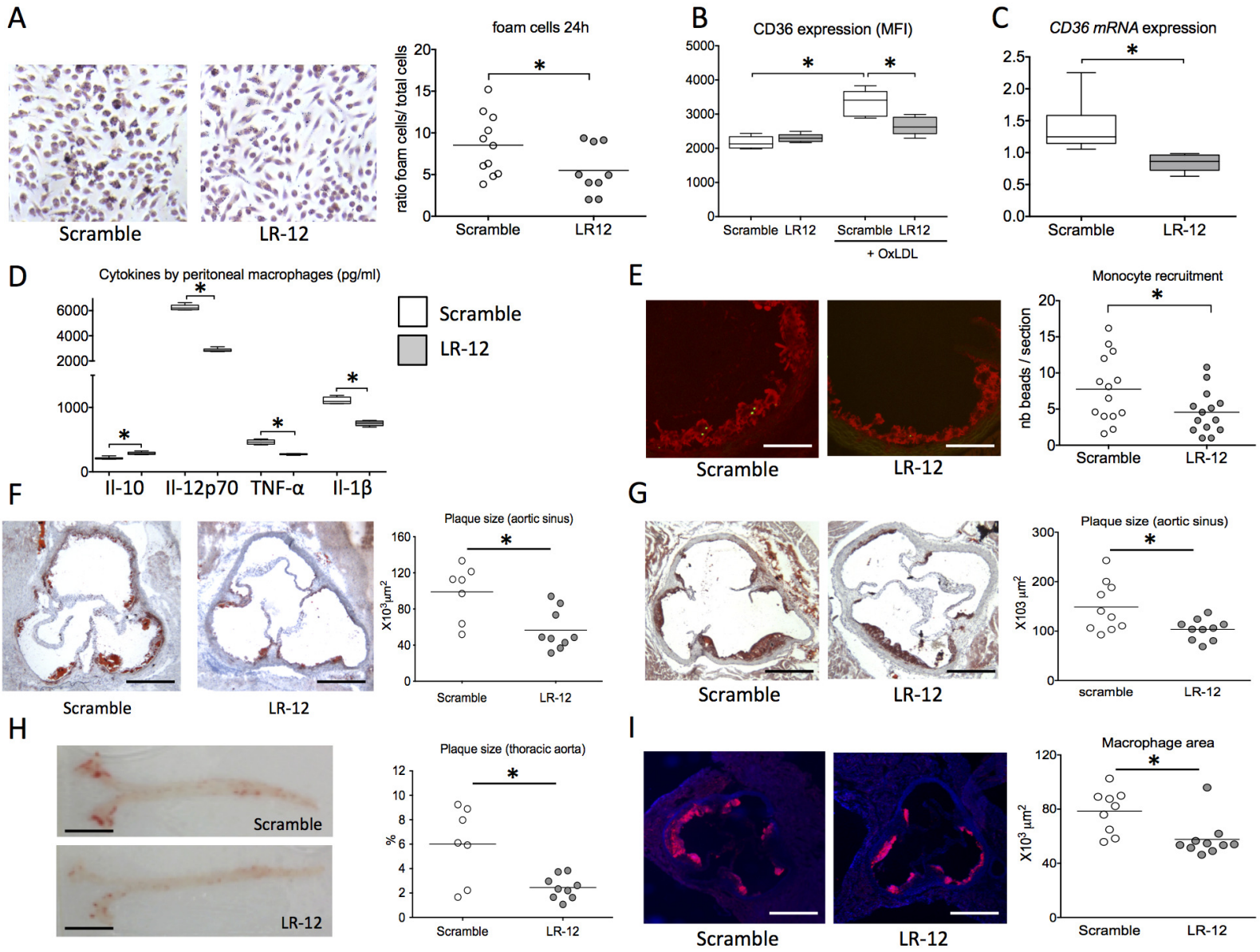

A
Trem-1+/+/Ldlr-/-
Trem-1-/-/Ldlr-/-
Gr-1
Trem-1
B
Trem-1 mRNA
***
0.0075
0.0050
0.0025
0.0000
Trem-1+/+/Ldlr-/-
Trem-1-/-/Ldlr-/-
C
Trem-1+/+/Ldlr-/-
Trem-1-/-/Ldlr-/-
Plaque size (aortic sinus)
**
X10³µm²
D
Plaque size (aortic sinus)
**
Plaque size (thoracic aorta)
**
%
E
Trem-1+/+/Ldlr-/-
Trem-1-/-/Ldlr-/-
Macrophage area
*
F
Trem-1+/+/Ldlr-/-
Trem-1-/-/Ldlr-/-
Necrotic core
*

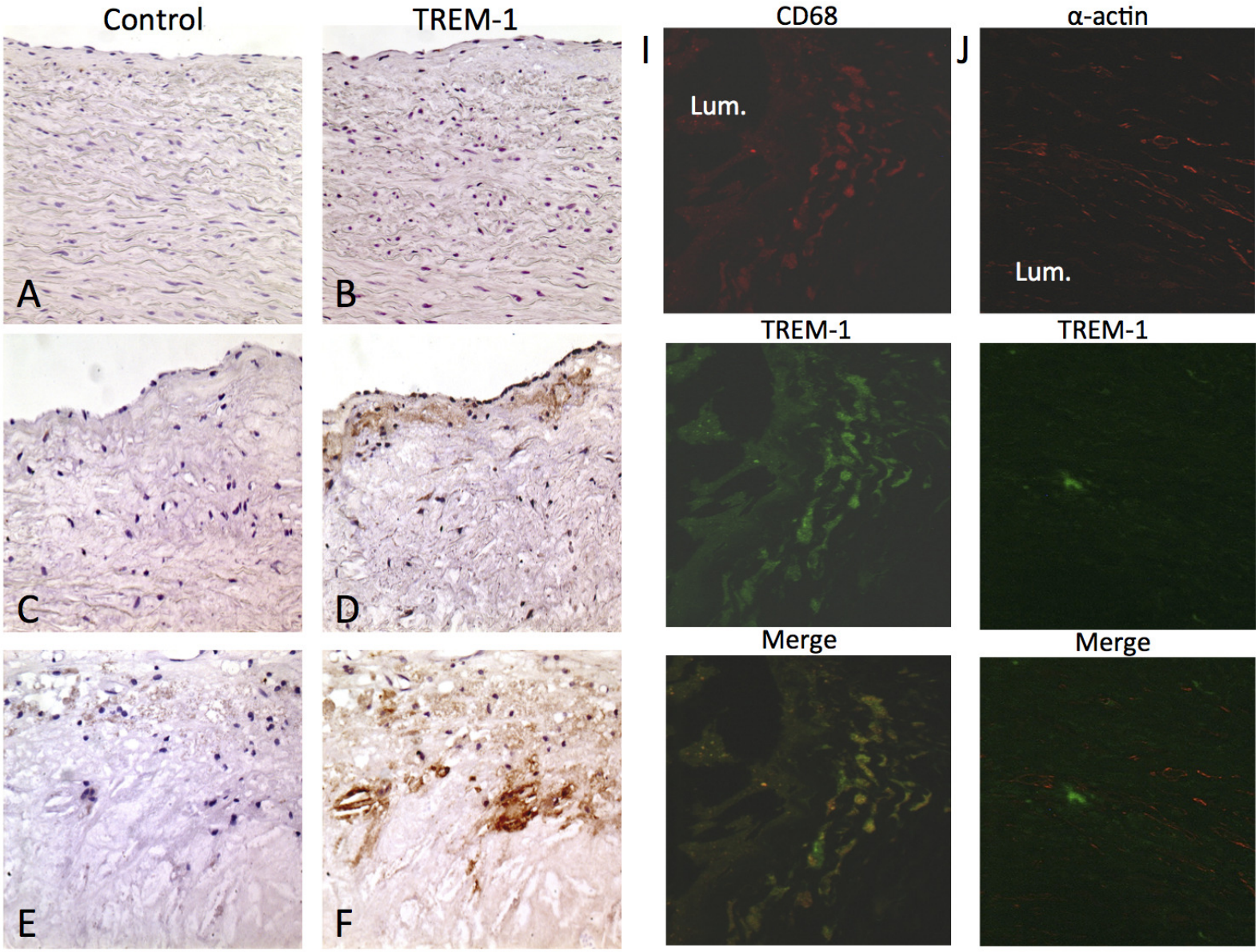

A
Trem-1+/+/Ldlr-/-
Trem-1-/-/Ldlr-/-
TUNEL area
%
0.04
0.03
0.02
0.01
0.00
*
Ldlr-/-/Trem-1+/+
Ldlr-/-/Trem-1-/-
B
Trem-1+/+/Ldlr-/-
Trem-1-/-/Ldlr-/-
Monocyte recruitment
nb beads / section
20
15
10
5
0
*
Trem-1+/+/Ldlr-/-
Trem-1-/-/Ldlr-/-
C
Lps/ifn-γ stimulated splenocytes
80
60
40
20
0
*
*
TNF-α/IL-10
IL-12p70/IL-10
D
Trem-1+/+/Ldlr-/-
Trem-1-/-/Ldlr-/-
Il-10 mRNA
0.015
0.010
0.005
0.000
*
Il-12 mRNA
0.15
0.10
0.05
0.00
***
IL-1β mRNA
0.05
0.04
0.03
0.02
0.01
0.00
***
Il-6 mRNA
0.25
0.20
0.15
0.10
0.05
0.00
***
E
Cytokines by macrophages
pg/ml
22500
12500
2500
2000
1000
0
**
*
*
*
Trem-1 +/+
Trem-1 -/-
TNF-α
IL-12p70
IL-1β
IL-10
F
iNos mRNA
NS
0.0008
0.0006
0.0004
0.0002
0.0000
Arginase 1 mRNA
NS
0.005
0.004
0.003
0.002
0.001
0.000
Trem-1+/+/Ldlr-/-
Trem-1-/-/Ldlr-/-

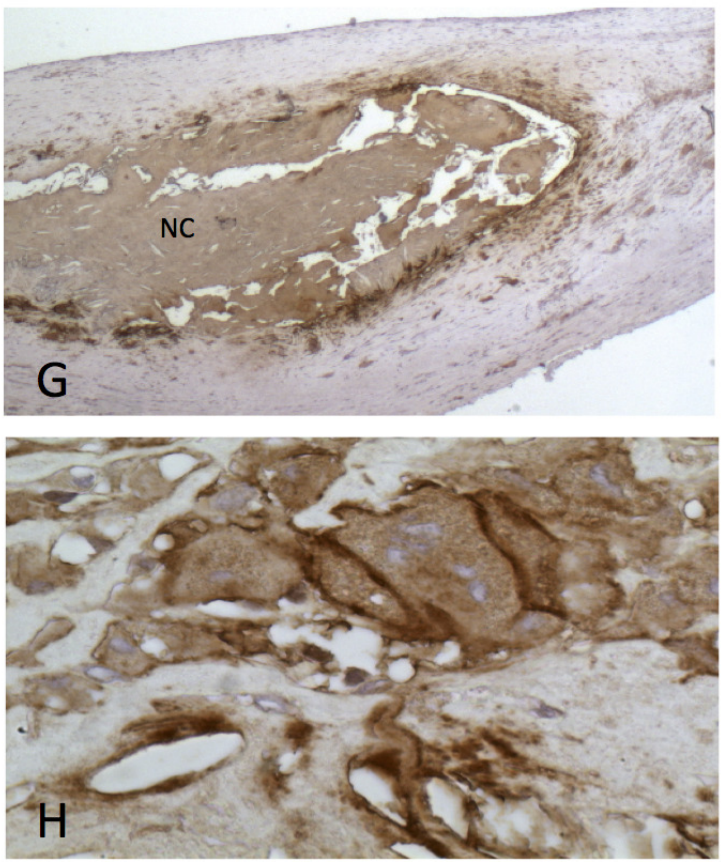

A
Foam cells 24h
ratio foam cells/ total cells
Trem-1 +/+
Trem-1 -/-
Trem-1+/+
Trem-1-/-
Foam cells 48h
B
C
Sr-a mRNA
Sr-b mRNA
Cd36 mRNA
Baseline
H6
H12
D
CD36 expression (MFI)
OxLDL
CD36
E
CD36/Plaque area ratio
Trem-1+/+/Ldlr-/-
Trem-1-/-/Ldlr-/-
Ldlr-/-/Trem-1+/+
Ldlr-/-/Trem-1-/-
F
CD36 expression (MFI)
Ox LDL
Pi3Kinase inhibitor

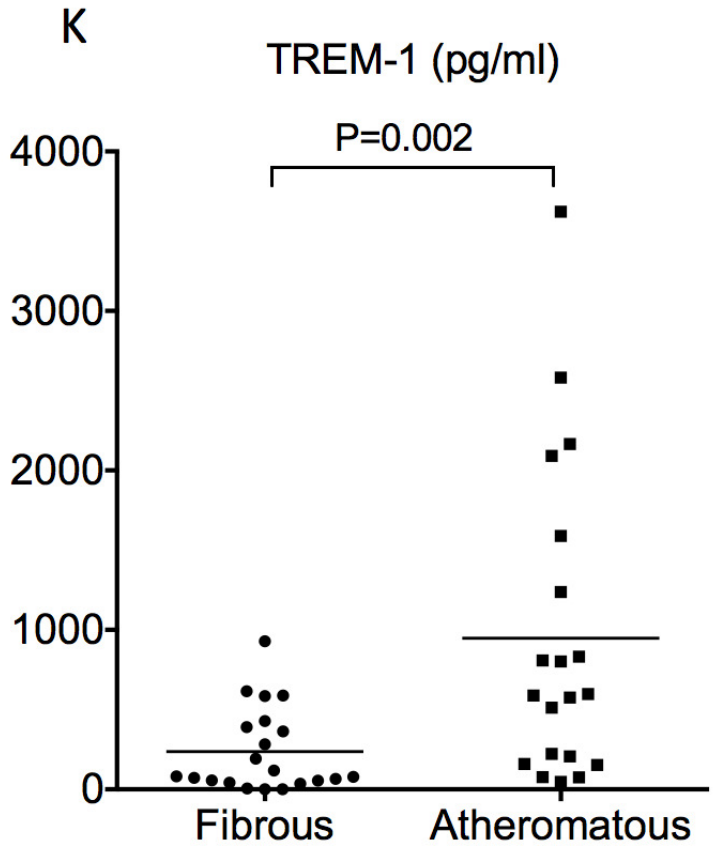

A
B
C
ApoE-/-/Trem-1+/+
ApoE-/-/Trem-1-/-
TREM +
18,2
TREM +
0,32
CD11b
Trem-1
D
ApoE-/-/Trem-1+/+
ApoE-/-/Trem-1-/-
E
Gated on CD11b+ cells
Ly6C
Ly6G
Q1
10,1
Q2
41,6
Q4
47,9
Q3
0,41
Cx3cr1
Trem-1
CX3CR1 MFI on non-classical monocytes
Expressing Trem-1
Not expressing Trem-1
6000
4000
2000
0
2 weeks of
fat diet
4 weeks of
fat diet
F
Gated on CD11b+ Ly6G-Ly6Clow
Non-classical monocytes
ApoE-/-Trem-1+/+
ApoE-/-Trem-1-/-
Count
Cx3cr1
CX3CR1 MFI on non-classical monocytes
2000
1500
1000
500
0
2 weeks of
fat diet
4 weeks of
fat diet
G
Il-10 mRNA
Il-12 mRNA
iNos mRNA
Arginase 1 mRNA
Ym1 mRNA


A
Cholesterolemia (g/L)
15
10
5
0
ApoE-/-/Trem-1+/+ ApoE-/-/Trem-1-/-
B
Plaque size (aortic sinus, 10³µm²)
400
300
200
100
0
*
ApoE-/-/Trem-1+/+ ApoE-/-/Trem-1-/-
ApoE-/-/Trem-1+/+
ApoE-/-/Trem-1-/-
C
Plaque size (thoracic aorta %)
25
20
15
10
5
0
**
ApoE-/-/Trem-1+/+ ApoE-/-/Trem-1-/-
ApoE-/-/Trem-1+/+
ApoE-/-/Trem-1-/-
D
Macrophage area (10³µm²)
100
80
60
40
20
0
**
ApoE-/-/Trem-1+/+ ApoE-/-/Trem-1-/-
Macrophage (% plaque)
80
60
40
20
0
**
ApoE-/-/Trem-1+/+ ApoE-/-/Trem-1-/-
ApoE-/-/Trem-1+/+
ApoE-/-/Trem-1-/-
E
Necrotic core area (10³µm²)
80
60
40
20
0
**
ApoE-/-/Trem-1+/+ ApoE-/-/Trem-1-/-
Necrotic core (% plaque)
30
20
10
0
**
ApoE-/-/Trem-1+/+ ApoE-/-/Trem-1-/-
ApoE-/-/Trem-1+/+
ApoE-/-/Trem-1-/-
F
TUNEL+ area (µm²)
300
200
100
0
*
ApoE-/- Trem-1+/+ ApoE-/- Trem-1-/-
TUNEL+ area (% plaque)
0.2
0.1
0.0
ns
ApoE-/- Trem-1+/+ ApoE-/- Trem-1-/-
ApoE-/-/Trem-1+/+
ApoE-/-/Trem-1-/-


A
Scramble
LR-12
foam cells 24h
ratio foam cells/ total cells
Scramble
LR12
B
CD36 expression (MFI)
Scramble LR12
Scramble LR12
+ OxLDL
C
CD36 mRNA expression
Scramble
LR-12
D
Cytokines by peritoneal macrophages (pg/ml)
Scramble
LR-12
Il-10
Il-12p70
TNF-α
Il-1β
E
Monocyte recruitment
nb beads / section
Scramble
LR-12
F
Scramble
LR-12
Plaque size (aortic sinus)
Scramble
LR-12
G
Scramble
LR-12
Plaque size (aortic sinus)
scramble
LR-12
H
Scramble
LR-12
Plaque size (thoracic aorta)
Scramble
LR-12
I
Scramble
LR-12
Macrophage area
Scramble
LR-12


Control
TREM-1
A
B
C
D
E
F
CD68
α-actin
I
J
Lum.
Lum.
TREM-1
TREM-1
Merge
Merge


NC
G
H


K
TREM-1 (pg/ml)
P=0.002
4000
3000
2000
1000
0
Fibrous
Atheromatous

ONLINE APPENDIX

From the Methods Section

FLOW CYTOMETRY AND PCR. Blood samples were collected at the sacrifice for analysis of leukocyte subsets. Myeloid cells were identified as CD11b+. Monocytes were identified as CD11b+Ly6G- or CD11b+CD115high. Among them, classical monocytes were Gr1high (or Ly6Chigh) and non-classical monocytes were Gr1low (or Ly6Clow). Neutrophils were identified as CD11b+CD115low or CD11b+Ly6G+. CD4+ and CD8+ lymphocyte subsets were also analyzed. Stainings included the following antibodies: PerCP-conjugated anti-CD45, FITC-conjugated anti-CD11b, PE-conjugated anti-CD115, Anti-Gr1 (Ly6C and G)-PERCP-Cy5, anti-Cx3cr1-PERCP-Cy7, anti-F4/80-PE, anti-CD4-V-450 anti-CD3ε-APC, anti-B220-V-500, anti-CD8a-AF-700, anti-MHCII-FITC, and anti-CD19 AF-700.

Forward scatter (FSC) and side scatter (SSC) were used to gate live cells excluding red blood cells, debris, and cell aggregates in total blood cells and splenocytes. Cells were acquired using a flow cytometer and analyzed with FlowJo software (FloJo, LLC, Ashland, Oregon).

Quantitative real-time polymerase chain reaction (PCR) was performed, after Trizol ribonucleic acid (RNA) extraction and real-time PCR, on an ABI Prism 7700 Sequence Detection System (Thermo Fisher Scientific, Inc.) in duplicate for each sample. Expression of all genes has been normalized using 3 housekeeping genes (Gapdh, Hprt and Ppia). Relative expression was calculated using the 2-delta-delta computed tomography (CT) method followed by geometric average, as recommended (1,2). The following primer sequences were used: *Il-10* (F:5' –AAG TGA TGC CCC AGG CA- 3'; R:5' –TCT CAC CCA GGG AAT TCA AA- 3'), *Trem-1* (F:5'- TGC GGT TGT TCC CTC TCC TGG TCT TG-3'; R:5' - TGT GAA ATA GAC ACC GCT GAA GGT CAC T-3'); *Nos2* (F:5'- TTG GGC CTG GTA CGG GCA TTG-3'; R:5' - CAA GCT CAT GCG GCC TCC TTT-3'), *Arg1* (F:5'- TCT GCA TGG GCA ACC TGT GTC-3'; R:5' –TGC AGG GAG TCA CCC AGG AGA-3'), *Ym1* (F :5'-

GAA GCC CTC CTA AGG ACA AAC-3' ; R :5'-GCA GCC TTG GAA TGT CTT TCT-3'), *Tnf* (F:5'- GAT GGG GGG CTT CCA GAA CT-3'; R:5' –CGT GGG CTA CAG GCT TGT CAC-3'), *Il-6* (F:5'- AAA GAC AAA GCC AGA GTC CTT CAG AGA GAT-3'; R:5'– GGT CTT GGT CCT TAG CCA CTC CTT CTG T-3'), *Il-12a* (F:5'- TCA CAC GGG ACC AAA CCA GCA CAT -T-3'; R:5' –TGT GGG GGC AGG CAG CTC CCT CTT -3'), *Il-1b* (F:5'- GAA GAG CCC ATC CTC TGT GA – 3'; R:5'- GGG TGT GCC GTC TTT CAT TA – 3'), *Sra* (F:5'- TGG ATG CAA TCT CCA AGT CCT- 3'; R:(5': ACG TGC CTT GTT CTT CTT T -3') , *Srb* (F:5'- GCT CCG GAA CAA GGC AAA TA – 3'; R: 5'- GGC CAG ATC CAC GAC AGT 6 – 3'), *Cd36* (F:5'- TGG CCA AGC TAT TGC GAC ATG ATT -A-3'; R:5' – CGG GGA TTC CTT TAA GGT CGA TTT-C-3'), *Abcg1* (F:5'- GGG GCC CTG GTC GAA GAA GAA AGC A-3'; R:5' –TCC TTT CTT CTT CCA CCA GGG CCC C-3'), and *Abca1* (F:5'- TAA ATT CCA CAT CTC ATC TCC CGA CCC AG-3'; R:5' –CTG GGT CGG GAG ATG AGA TGT GGA ATT TA-3').

References

1. Bustin SA, Benes V, Garson JA, et al. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin Chem 2009;55:611-22.

2. Vandesompele J, De Preter K, Pattyn F, et al. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol 2002;3(7):RESEARCH0034.

Online Figure Legends

Online Figure 1. No difference of blood leukocyte populations between chimeric *Ldlr$^{-/-}$ /Trem-1$^{+/+}$ and chimeric Ldlr$^{-/-}$ /Trem-1$^{-/-}$ mice.* Flow cytometry characterization of leucocyte subsets in chimeric *Ldlr$^{-/-}$* mice in the blood (A) and the bone marrow (B). *P<0.05

Online Figure 2. No difference of cholesterolemia and collagen content between chimeric *Ldlr$^{-/-}$ /Trem-1$^{+/+}$ and chimeric Ldlr$^{-/-}$ /Trem-1$^{-/-}$ mice.* Cholesterolemia from *Ldlr$^{-/-}$* chimeric mice after 8 weeks (A) and 14 weeks of high fat diet (B). Representative photomicrographs and quantitative analysis of collagen content within atherosclerotic lesions of chimeric *Ldlr$^{-/-}$* mice after 8 weeks of fat diet (Sirius red staining) (C).

Online Figure 3. Trem-1 deficiency did not alter macrophage apoptosis susceptibility. Bone marrow-derived macrophages isolated from *Trem-1 $^{+/+}$* (opened circles) or *Trem-1 $^{-/-}$* mice (filled circles) were challenged *in vitro* during 24 hours and apoptosis was evaluated by flow cytometry. Apoptotic cells were defined as annexin V^{pos} 7-AAD^{neg} cells. * P<0.05, NS, non significant.

Online Figure 4. Trem-1 deficiency impaired non-classical monocyte recruitment into inflammatory sites. Decrease of non-classical monocyte recruitment within the peritoneal cavity following septic or non-septic injury. Twelve-week old male Apoe$^{-/-}$Trem-1$^{+/+}$ and Apoe$^{-/-}$Trem-1$^{-/-}$ received LPS (2.5mg/kg) (B) or Thioglycollate (C) intraperitoneally and were sacrified 3 hours (LPS) or 12 hours (Thioglycollate) later. Peritoneal cells were harvested and analysed by flow cytometry (A). Neutrophils were identified as CD45+CD11b+Ly6G+, classical monocytes were CD45+CD11b+Ly6G-Ly6Chigh and non-classical monocytes were CD45+CD11b+Ly6G-Ly6Clow. **, P<0.01

Online Figure 5. Trem-1 deficiency did not impair macrophage metabolism Exploration of BMDMs metabolism including mitochondrial respiration and glycolysis (Seahorse XF Technology). Extracellular acidification rates (ECAR, left) and real-time oxygen consumption rates (OCR, right) of *Trem-1*$^{+/+}$ *and Trem-1*$^{-/-}$ macrophages were determined using the Seahorse Extracellular Flux (XFe96) analyzer (Seahorse Bioscience, Paris, France). Macrophages were cultured into XFe96 well culture plates and incubated at 37°C in a 5% CO_2 humidified atmosphere. At day 7, macrophages were stimulated with LPS (1 μg/ml) during 24 hours. Then cells were washed in XF assay media (or for OCR measurement, XF assay media supplemented with 10 mM glucose, 1 mM Pyruvate, 2 mM L-glutamine and adjusted at 7.4 pH), which were pre-warmed to 37°C. Macrophages cells were then maintained in 140 μL/well of XF assay media at 37°C, in a non-CO2 incubator for 1 h. During incubation, we loaded 16 μL of 10 mM glucose, 1 μM oligomycin, 50 mM 2-deoxyglucose (for ECAR measurement) and 1 μM oligomycin, 1 μM FCCP, 0.5 μM rotenone, 0.5 μM antimycin A (for OCR measurement), in XF assay media into the injection ports in the XFe96 sensor cartridge. Data set was analyzed by XFe96 software and GraphPad Prism software, using one-way ANOVA calculations. All experiments were performed in triplicate, three times.

Online Figure 6. Trem-1 deficiency did not impair efferocytosis. A, phagocytosis of CFSE-labeled apoptotic thymocytes by bone marrow-derived macrophages (gated on CD11b+F4/80+ cells). Flow cytometry after 1 hour of co-incubation (N=6/group). Phagocytosis of Zymosan beads by bone marrow-derived macrophages. Quantification using Imagestream technology after 1 hour of co-incubation (N=4/group).

Online Figure 7. Trem-1 is expressed in mouse atherosclerotic plaques. A, *Trem-1 mRNA expression* is not detectable in aorta of *C57Bl6* mice but is expressed in atherosclerotic aorta of *apoE*$^{-/-}$ mice fed a chow (CD) or fat diet (FD). B, in *apoE*$^{-/-}$ mice, *Trem-1 mRNA* expression in thoracic aorta was strongly correlated to macrophage infiltration in atherosclerotic plaques in the aortic sinus. Expression of Trem-1 has been normalised using three housekeeping genes (Gapdh, Hprt and Ppia). Relative expression was calculated using the 2-delta-delta CT method followed by geometric average. N=6-7/group. * **P<0.001.

Online Figure 8. No difference of T cell infiltration and collagen content in plaques from *apoE*$^{-/-}$ *Trem*$^{+/+}$ *and apoE*$^{-/-}$*Trem*$^{-/-}$ mice. Representative photomicrographs and quantitative analysis of CD3+ T cell infiltration (fluorescent staining, A) and collagen content (Sirius Red, B) within atherosclerotic lesions of *apoE*$^{-/-}$ *Trem*$^{+/+}$ *and apoE*$^{-/-}$*Trem*$^{-/-}$ mice after 6 weeks of fat diet. N=7/group. Scale bar 100 µm.

Online Figure 9. LR-12 peptide limited lipid uptake by human macrophages and reduces CD36 expression. A, B. Human peripheral blood mononuclear cells were isolated from the whole blood of healthy volunteers using CD14 microbeads isolation kit (Stemcell^{TM}). After 7 days of incubation with M-CSF (50ng/mL), the cells were used as human monocyte-derived macrophages. Cells were incubated with oxLDL (25µg/mL) and treated with Scramble or LR-12 peptide (50 ng/mL) during 24H and 48 hours. Foam cells were quantified after red oil staining. C, Human monocytes (Monomac6) were stimulated for 6 hours by oxLDL (25 µg/mL) in presence of Scramble or LR12 peptide (50 ng/mL). CD36 expression was analyzed by flow cytometry * P<0.05, ** P<0.01.

Online Figure 10. LR-12 down regulates *IL-12, TNF-α and CD36 mRNA* expression by human monocytes. Isolated blood human monocytes were stimulated in vitro with LPS (1μg/ml) and co-incubated with Scramble or LR-12 peptide during 6 hours. Expression of all genes has been normalised using three housekeeping genes (Gapdh, Hprt and Ppia). Relative expression was calculated using the 2-delta-delta CT method followed by geometric average. N=3/group. * P<0.05.

Online Figure 11. LR-12 peptide reduced apoptosis within atherosclerotic plaques. Cholesterolemia from *apoE*$^{-/-}$ mice treated with daily intraperitoneal injection of LR-12 or scramble peptide during 4 weeks on a chow (A) and a high fat diet (B). Representative photomicrographs and quantitative analysis of TUNEL+ area (Brown, C), CD3+ T cell infiltration (D) and collagen content (E) within atherosclerotic lesions of *apoE*$^{-/-}$ mice treated with daily injection of LR-12 or scramble peptide during 4 weeks on a high fat diet. ** P<0.01. scale bar 100 μm.

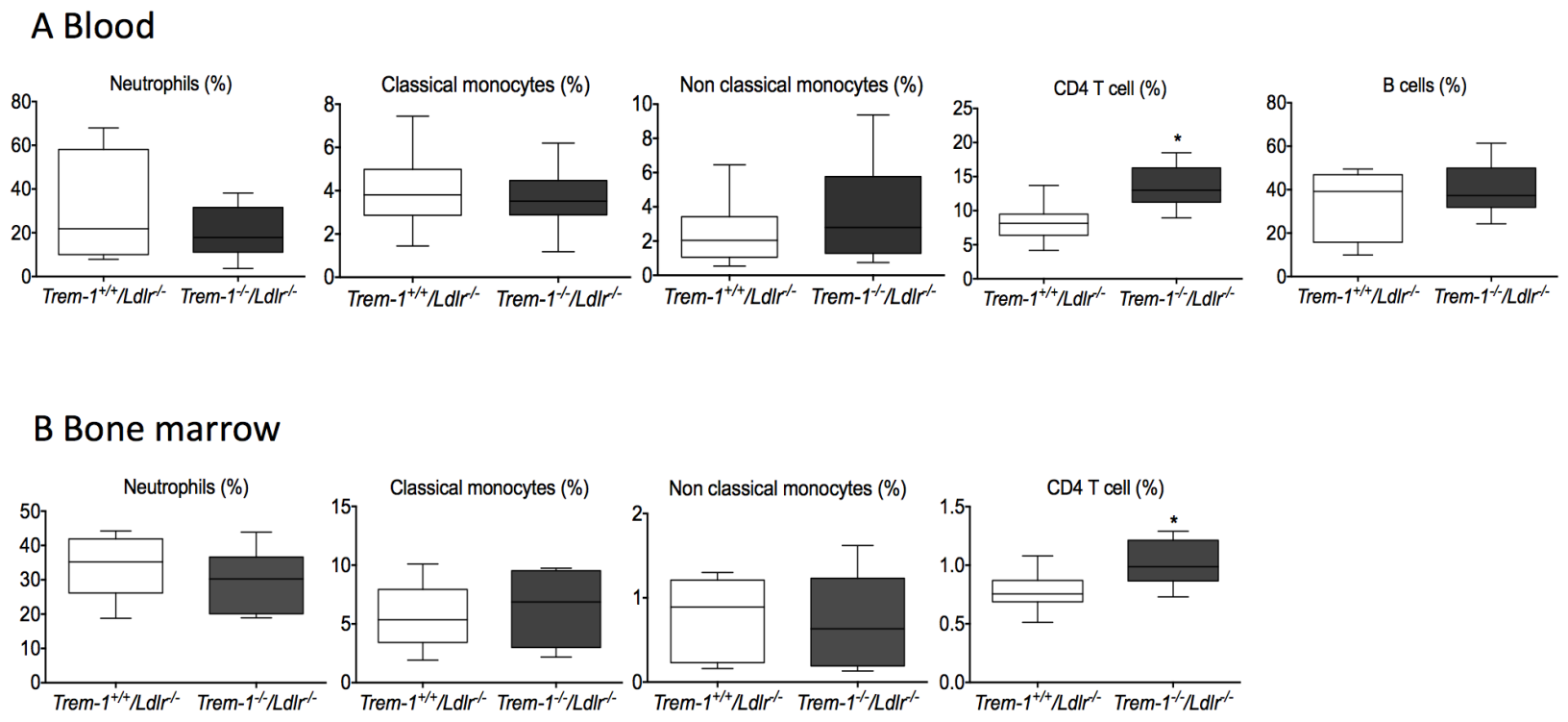
Online Figure 12. Atheroprotection induced by LR-12 peptide was preserved in neutrophil-depleted *apoE*$^{-/-}$ mice. A, intraperitoneal injection of Anti-Ly6G depleting antibody (1A8) or Isotype in *apoE*$^{-/-}$ mice lead to a 65% reduction of circulating neutrophils at day 2. B, experimental protocol of neutrophil depletion in Scramble or LR-12-treated *apoE*$^{-/-}$ mice fed a fat diet. C, Blood neutrophil count at the sacrifice. D, Representative photomicrographs and quantification of atherosclerotic plaque size (aortic sinus) after 4 weeks of fat diet. * P<0.05, ** P<0.01.

Online Figure 13. TREM-1 expression by neutrophils in human atherosclerotic plaques. Immunohistochemistry. A, Few Myeloperoxydase+ neutrophils (Brown, arrow) are detected

in atherosclerotic plaques and they colocalize with TREM-1 (Red). B, CD3+ T cells (Brown, arrow) do not colocalize with TREM-1 (Red).

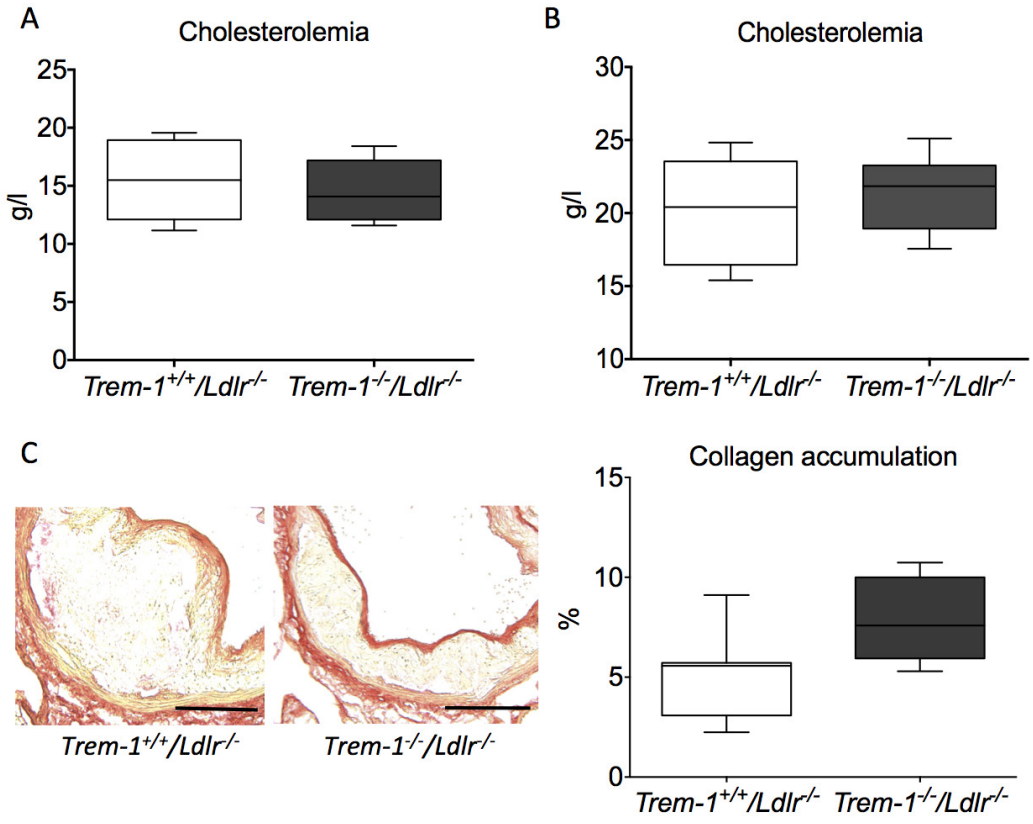
Online Figure 14. Clinical characteristics of human plaque samples. NS, Non significant

Supplemental figure 1



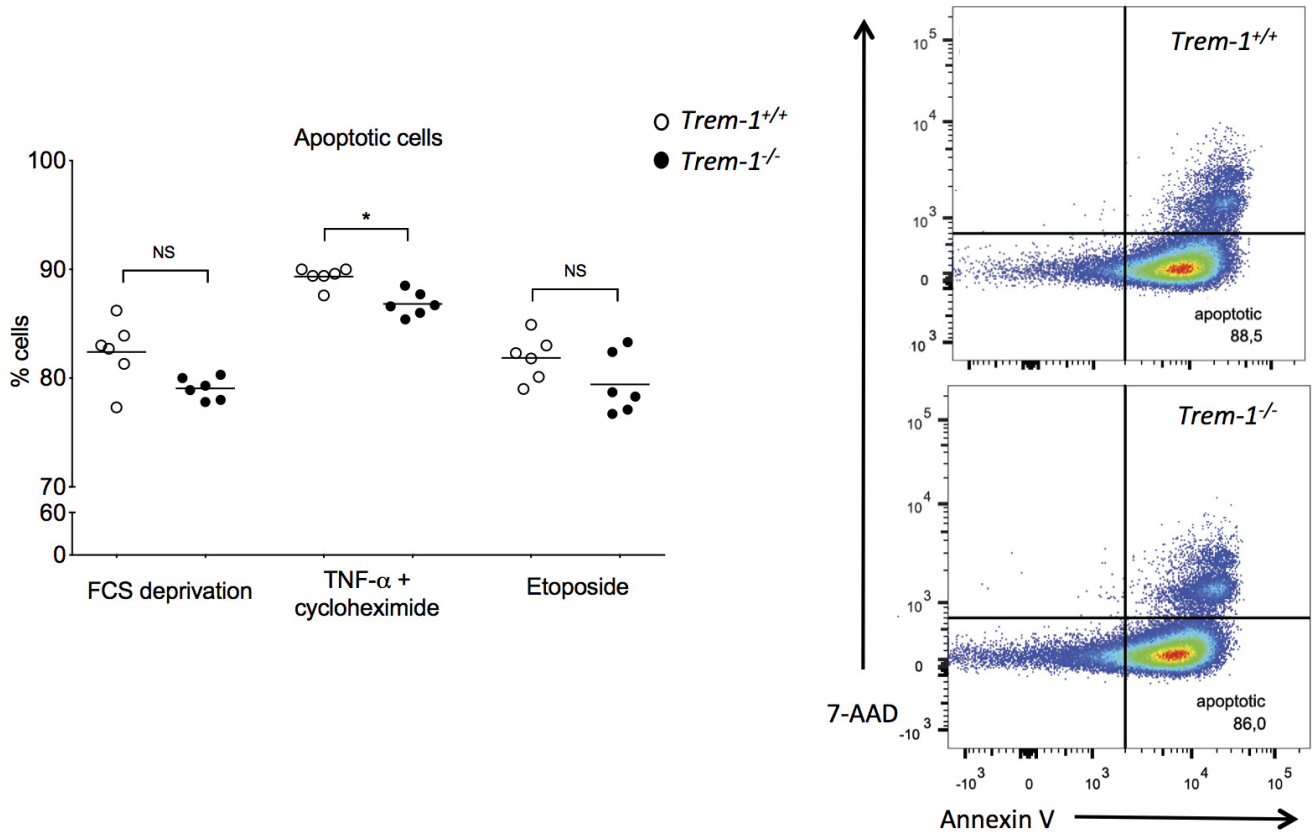
Flow cytometry characterization of leucocyte subsets in chimeric *Ldlr*$^{-/-}$ mice in the blood (A) and the bone marrow (B).
*P<0.05

Supplemental figure 2



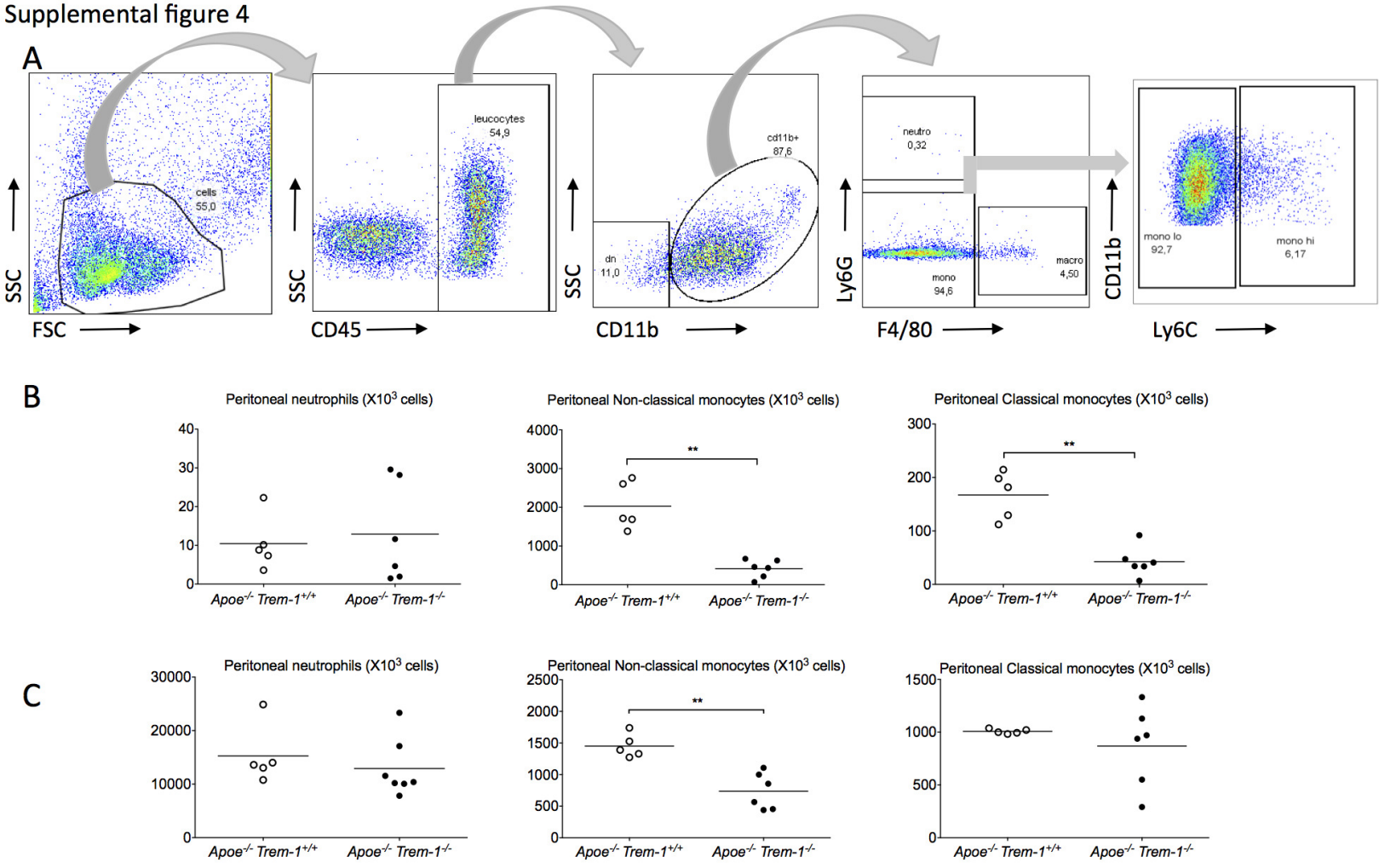
Cholesterolemia from *Ldlr*$^{-/-}$ chimeric mice after 8 weeks (A) and 14 weeks of high fat diet (B). Representative photomicrographs and quantitative analysis of collagen content within atherosclerotic lesions of chimeric *Ldlr*$^{-/-}$ mice after 8 weeks of fat diet (Sirius red staining) (C)

Supplemental figure 3



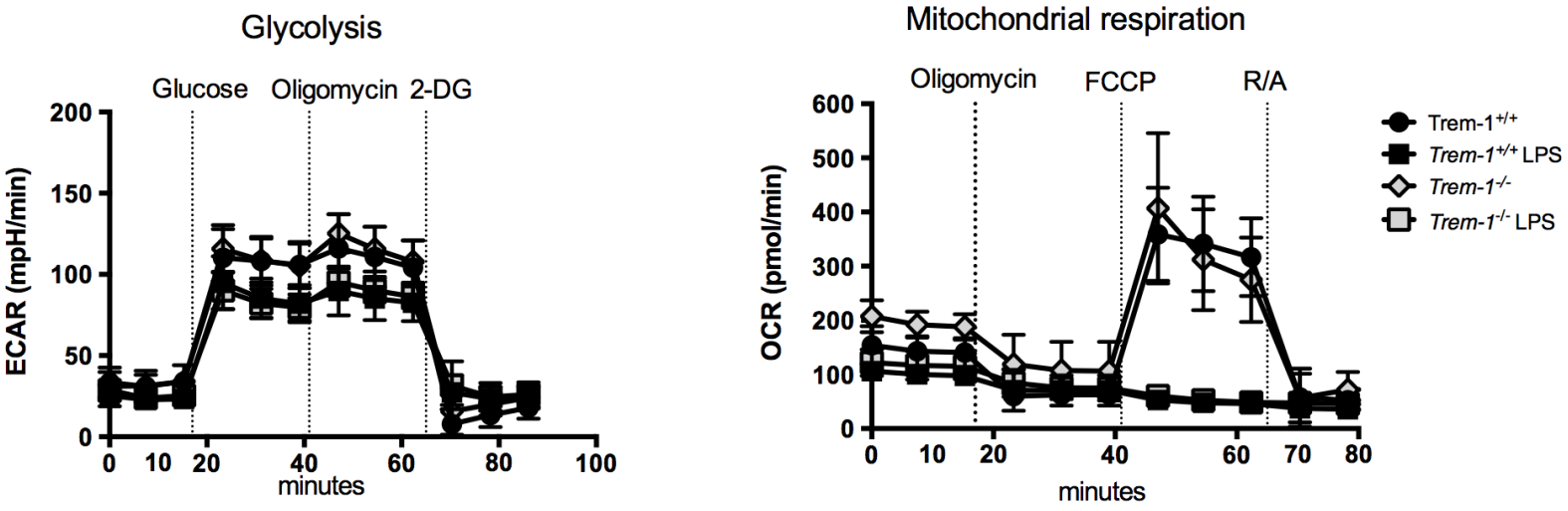
Bone marrow-derived macrophages isolated from *Trem-1* $^{+/+}$ (opened circles) or *Trem-1* $^{-/-}$ mice (filled circles) were challenged *in vitro* during 24 hours and apoptosis was evaluated by flow cytometry. Apoptotic cells were defined as annexin V^{pos} 7-AAD^{neg} cells. * P<0.05, NS, non significant.

Supplemental figure 4



Decrease of non-classical monocyte recruitment within the peritoneal cavity following septic or non-septic injury. Twelve-week old male Apoe$^{-/-}$Trem-1$^{+/+}$ and Apoe$^{-/-}$Trem-1$^{-/-}$ received LPS (2.5mg/kg) (B) or Thioglycollate (C) intraperitoneally and were sacrified 3 hours (LPS) or 12 hours (Thioglycollate) later. Peritoneal cells were harvested and analysed by flow cytometry (A). Neutrophils were identified as CD45+CD11b+Ly6G+, classical monocytes were CD45+CD11b+Ly6G-Ly6Chigh and non-classical monocytes were CD45+CD11b+Ly6G-Ly6Clow.**, P<0.01

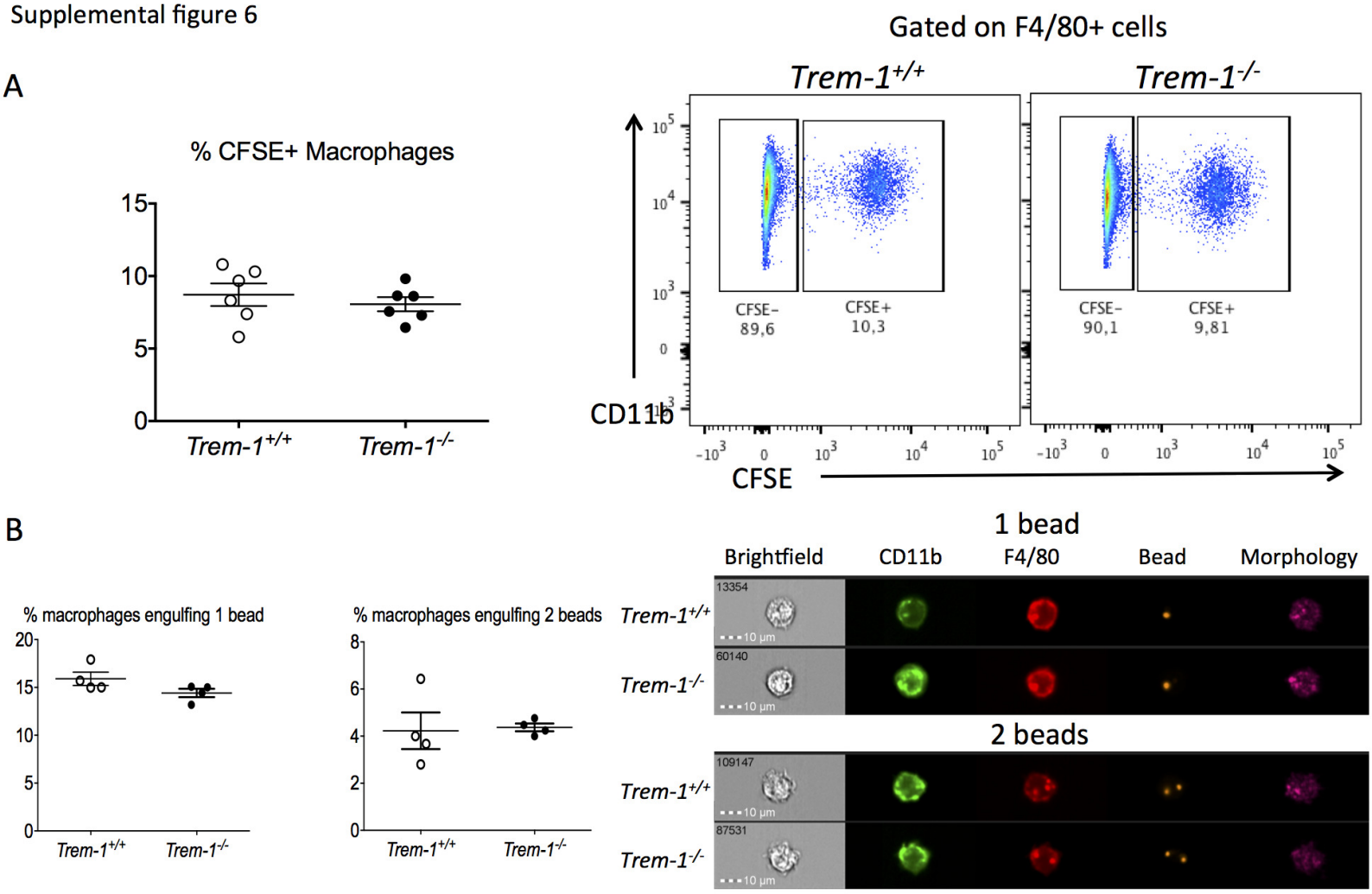
Supplemental figure 5



Exploration of BMDMs metabolism including mitochondrial respiration and glycolysis (Seahorse XF Technology). No difference between *Trem-1*$^{+/+}$ and *Trem-1*$^{-/-}$ macrophage metabolism.

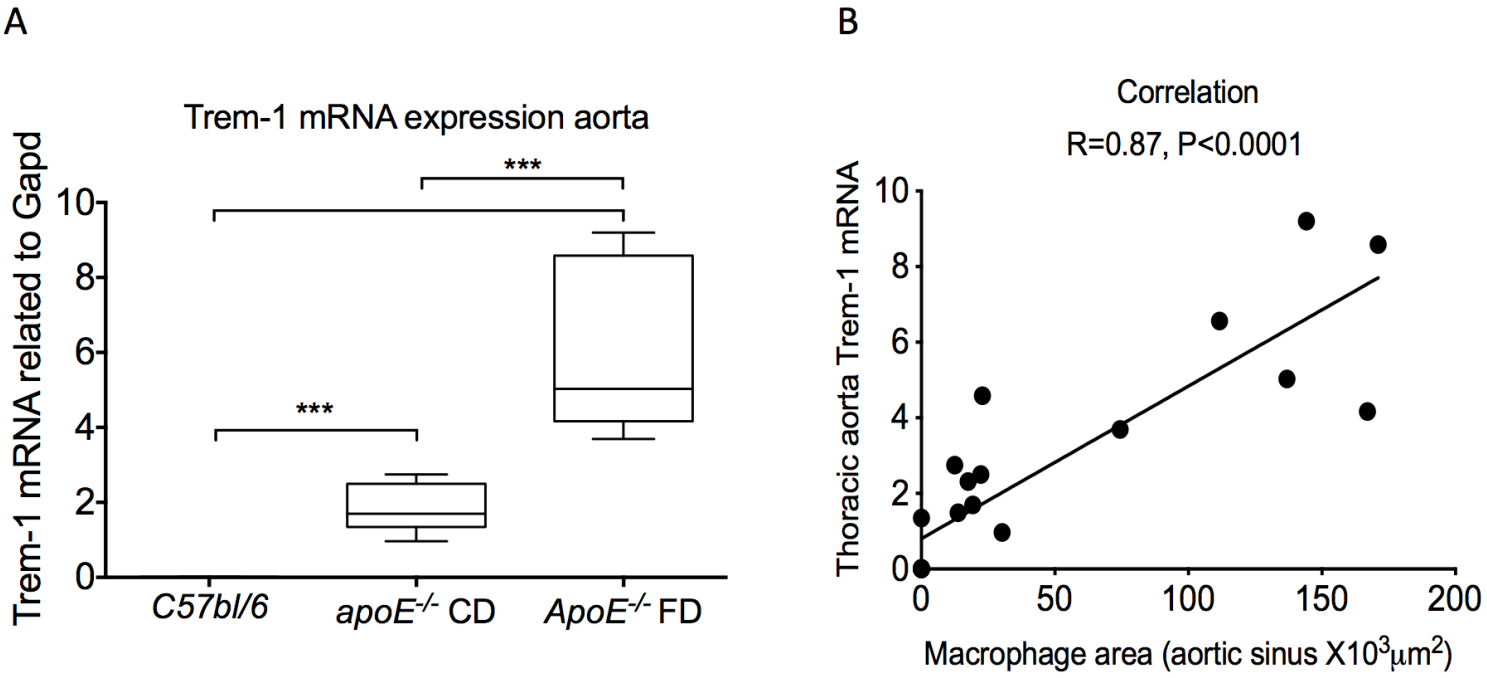
Extracellular acidification rates (ECAR, left) and real-time oxygen consumption rates (OCR, right) of *Trem-1*$^{+/+}$ *and Trem-1*$^{-/-}$ macrophages were determined using the Seahorse Extracellular Flux (XFe96) analyzer (Seahorse Bioscience, Paris, France). Macrophages were cultured into XFe96 well culture plates and incubated at 37°C in a 5% CO_2 humidified atmosphere. At day 7, macrophages were stimulated with LPS (1 µg/ml) during 24 hours. Then cells were washed in XF assay media (or for OCR measurement, XF assay media supplemented with 10 mM glucose, 1 mM Pyruvate, 2 mM L-glutamine and adjusted at 7.4 pH), which were pre-warmed to 37°C. Macrophages cells were then maintained in 140 µL/well of XF assay media at 37°C, in a non-CO2 incubator for 1 h. During incubation, we loaded 16 µL of 10 mM glucose, 1 µM oligomycin, 50 mM 2-deoxyglucose (for ECAR measurement) and 1 µM oligomycin, 1 µM FCCP, 0.5 µM rotenone, 0.5 µM antimycin A (for OCR measurement), in XF assay media into the injection ports in the XFe96 sensor cartridge. Data set was analyzed by XFe96 software and GraphPad Prism software, using one-way ANOVA calculations. All experiments were performed in triplicate, three times.

Supplemental figure 6



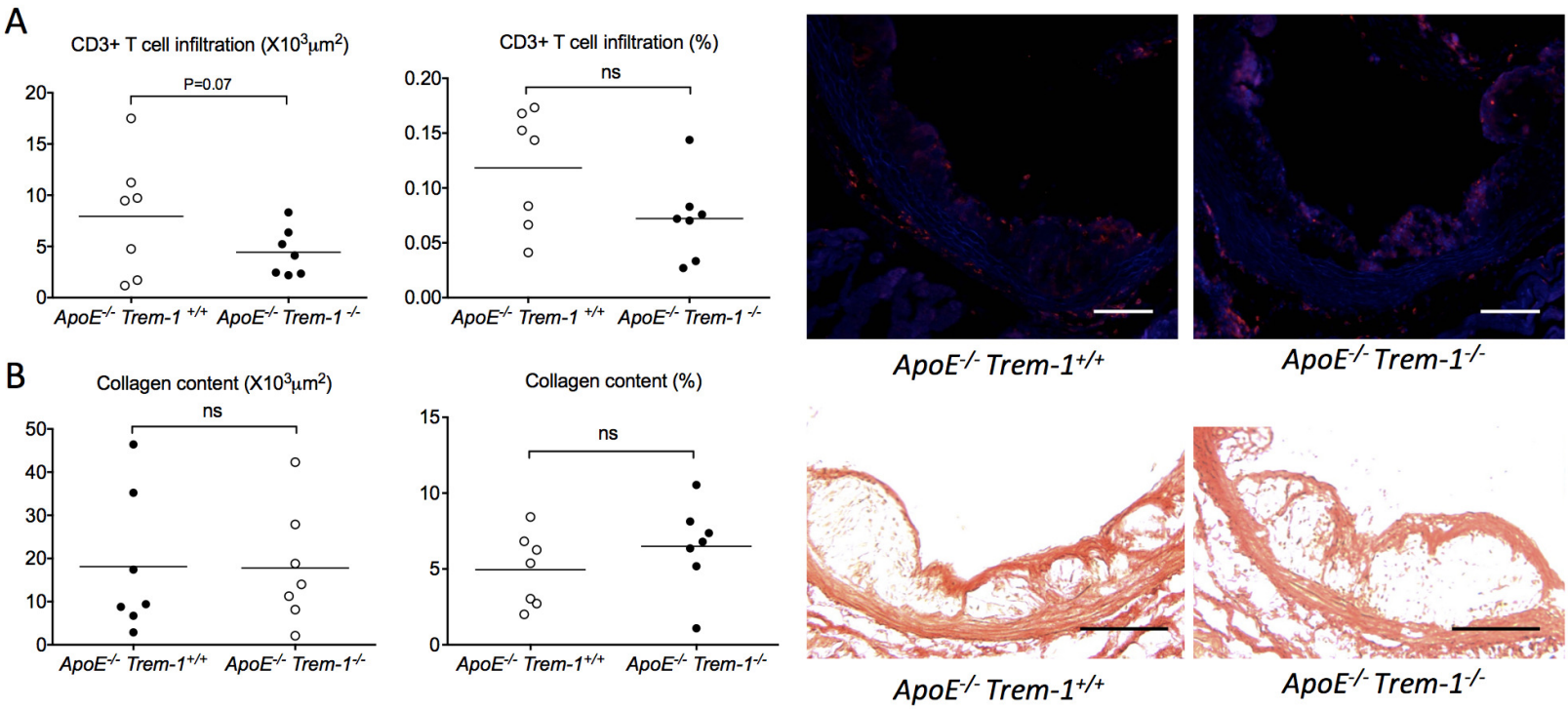
A, phagocytosis of CFSE-labeled apoptotic thymocytes by bone marrow-derived macrophages (gated on CD11b+F4/80+ cells). Flow cytometry after 1 hour of co-incubation (N=6/group). Phagocytosis of Zymosan beads by bone marrow-derived macrophages. Quantification using Imagestream technology after 1 hour of co-incubation (N=4/group)

Supplemental figure 7



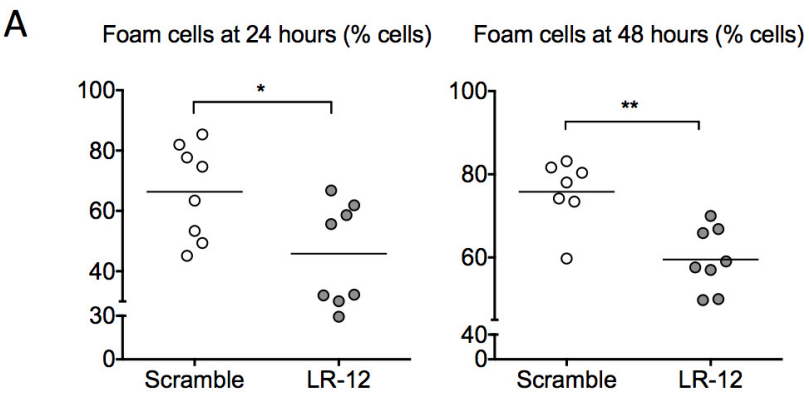
A, *Trem-1 mRNA expression* is not detectable in aorta of *C57Bl6* mice but is expressed in atherosclerotic aorta of *apoE*$^{-/-}$ mice fed a chow (CD) or fat diet (FD). B, in *apoE*$^{-/-}$ mice, *Trem-1 mRNA* expression in thoracic aorta was strongly correlated to macrophage infiltration in atherosclerotic plaques in the aortic sinus. Expression of Trem-1 has been normalised using three housekeeping genes (Gapdh, Hprt and Ppia). Relative expression was calculated using the 2-delta-delta CT method followed by geometric average. N=6-7/group. * **P<0.001

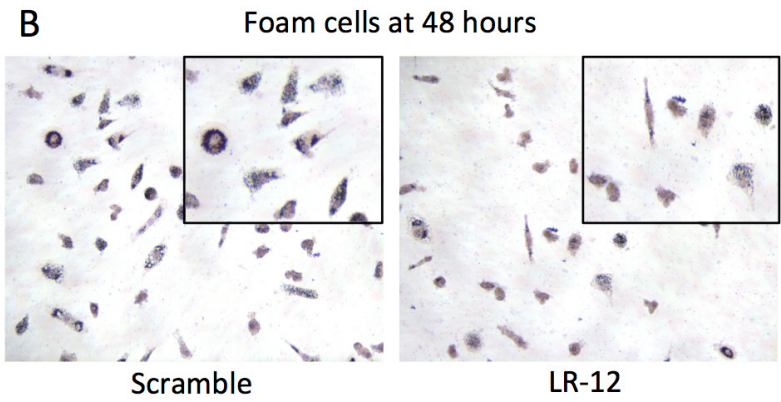
Supplemental figure 8

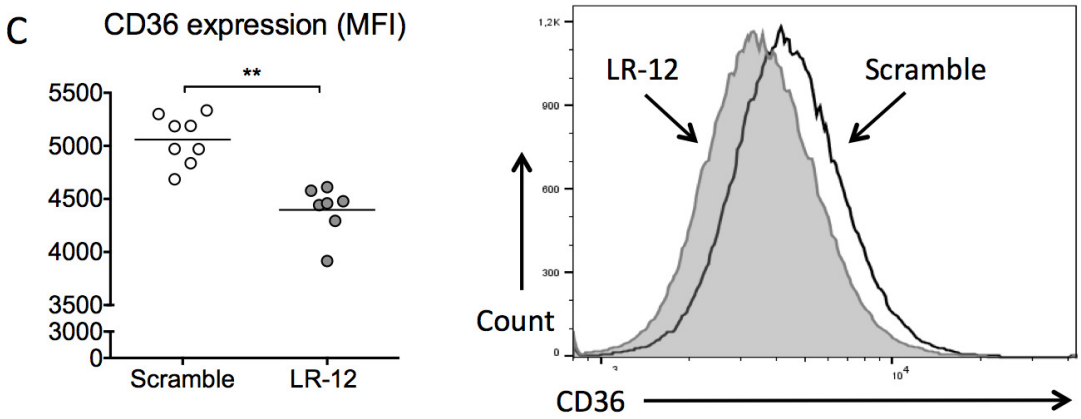


Representative photomicrographs and quantitative analysis of CD3+ T cell infiltration (fluorescent staining, A) and collagen content (Sirius Red, B) within atherosclerotic lesions of *apoE$^{-/-}$ Trem$^{+/+}$ and apoE$^{-/-}$Trem$^{-/-}$* mice after 6 weeks of fat diet. N=7/group. Scale bar 100 µm.

Supplemental figure 9

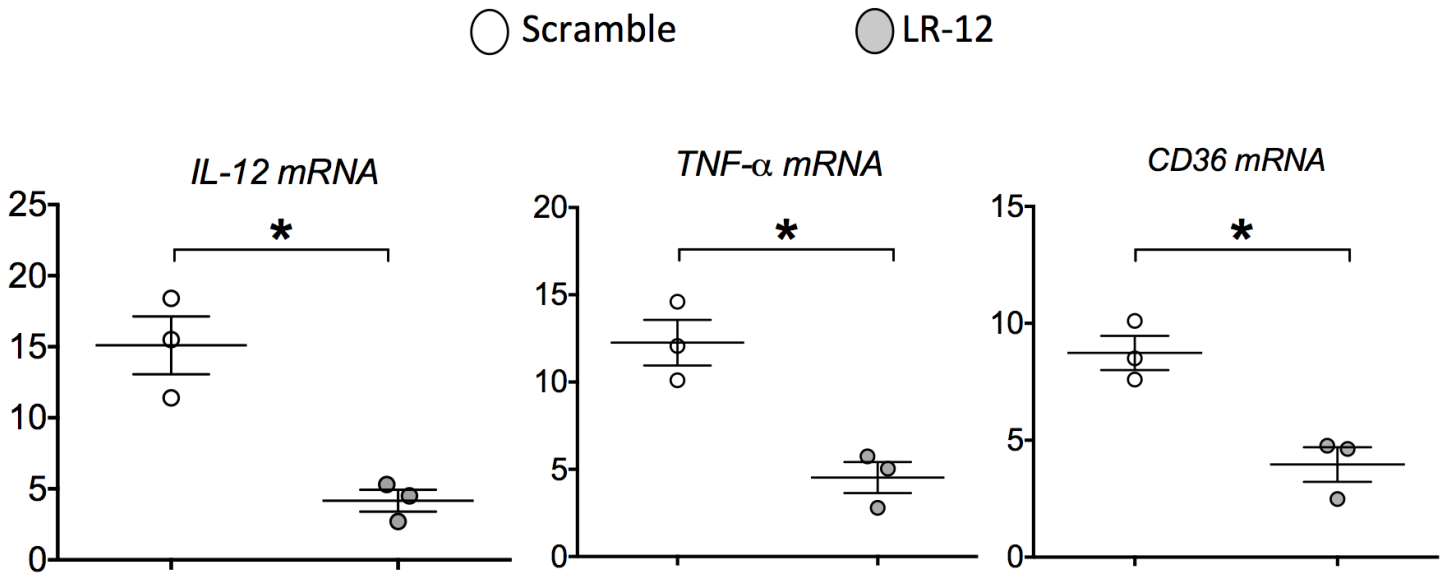






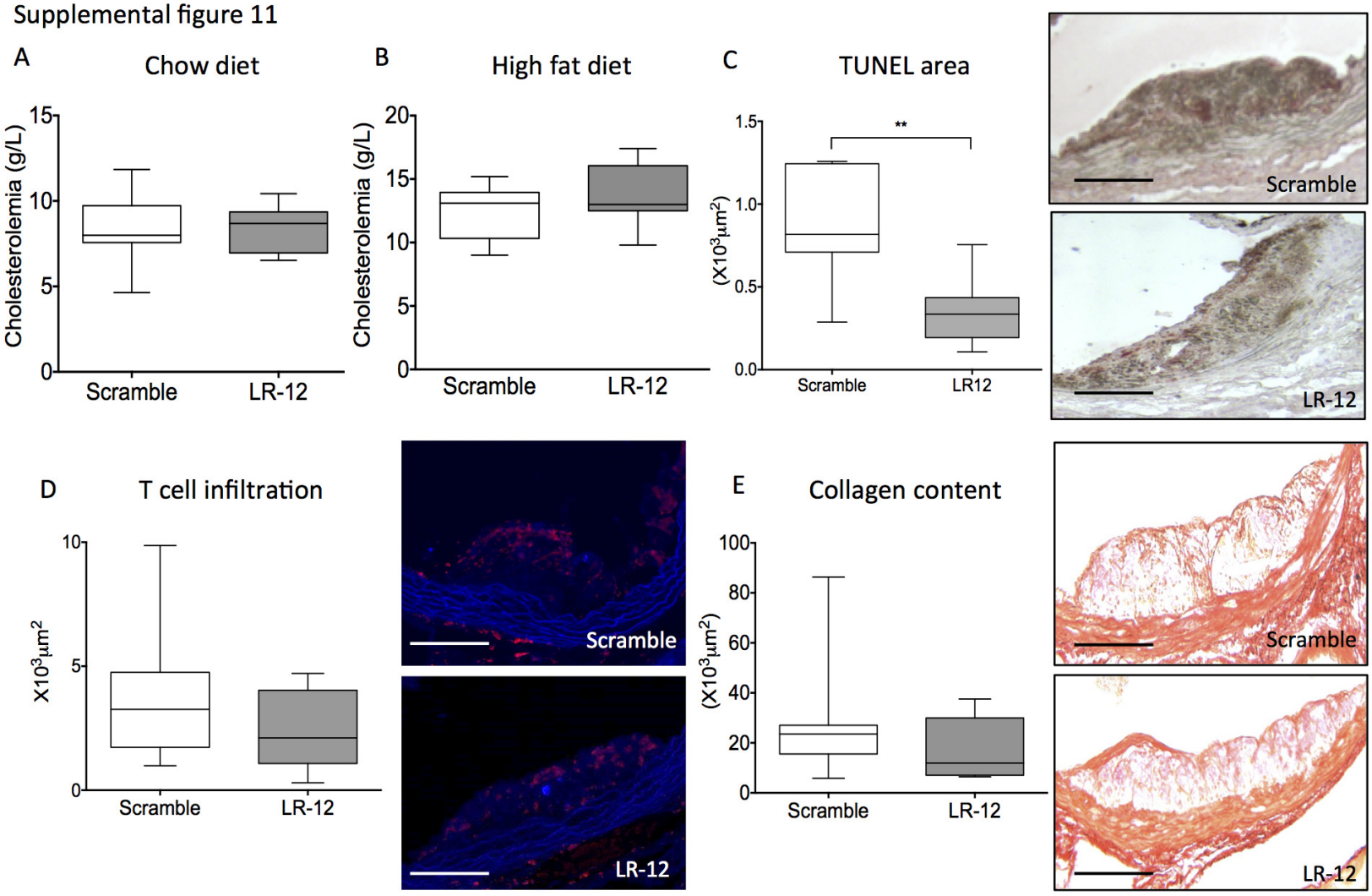
LR-12 limits lipid uptake by human macrophages and reduces CD36 expression. A, B. Human peripheral blood mononuclear cells were isolated from the whole blood of healthy volunteers using CD14 microbeads isolation kit (Stemcell™). After 7 days of incubation with M-CSF (50ng/mL), the cells were used as human monocyte-derived macrophages. Cells were incubated with oxLDL (25µg/mL) and treated with Scramble or LR-12 peptide (50 ng/mL) during 24H and 48 hours. Foam cells were quantified after red oil staining.
C, Human monocytes (Monomac6) were stimulated for 6 hours by oxLDL (25 µg/mL) in presence of Scramble or LR12 peptide (50 ng/mL). CD36 expression was analyzed by flow cytometry * P<0.05, ** P<0.01.

Supplemental figure 10



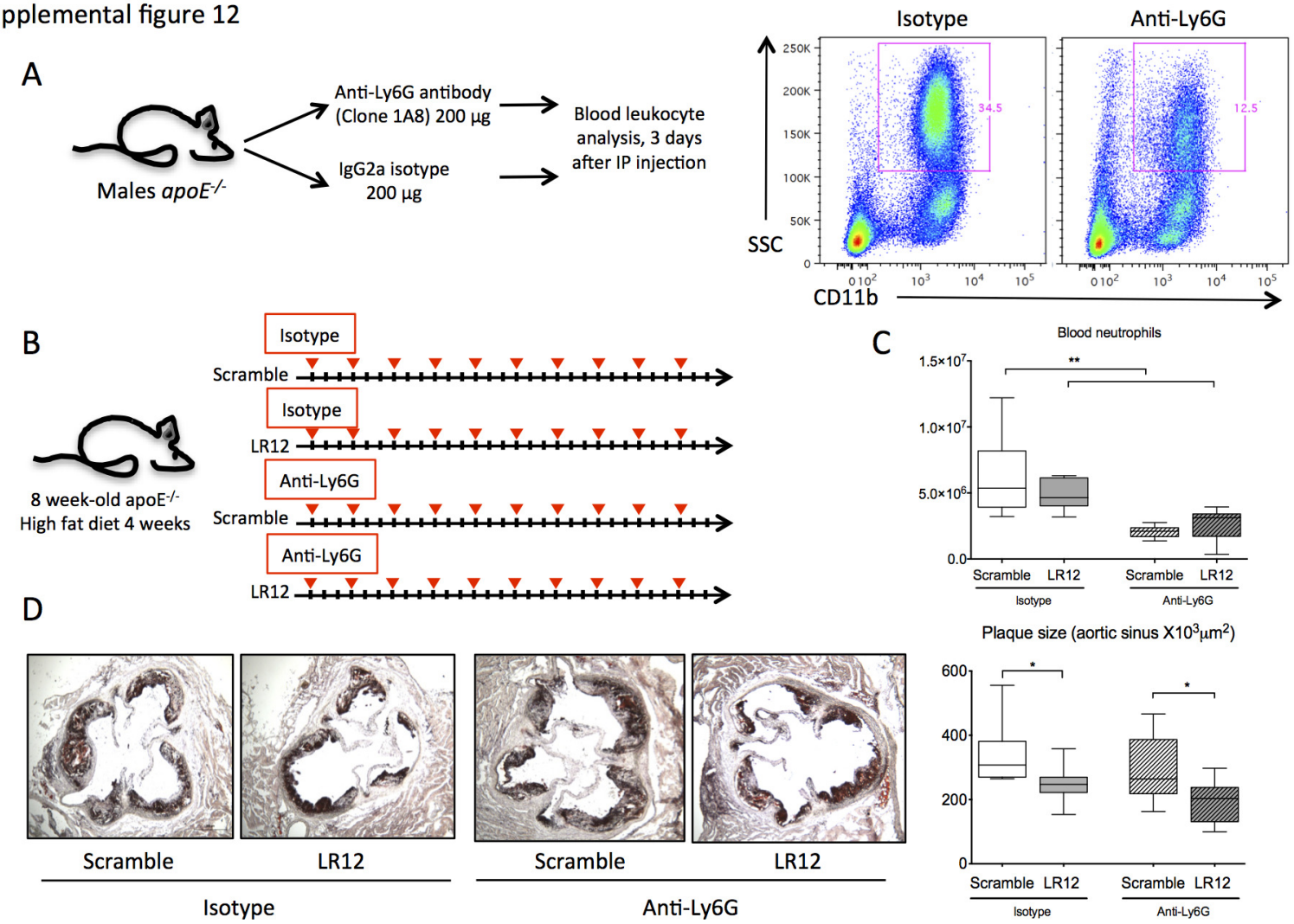
LR-12 down regulates *IL-12, TNF-α and CD36 mRNA* expression by human monocytes. Isolated blood human monocytes were stimulated in vitro with LPS (1µg/ml) and co-incubated with Scramble or LR-12 peptide during 6 hours. Expression of all genes has been normalised using three housekeeping genes (Gapdh, Hprt and Ppia). Relative expression was calculated using the 2-delta-delta CT method followed by geometric average. N=3/group. * P<0.05

Supplemental figure 11



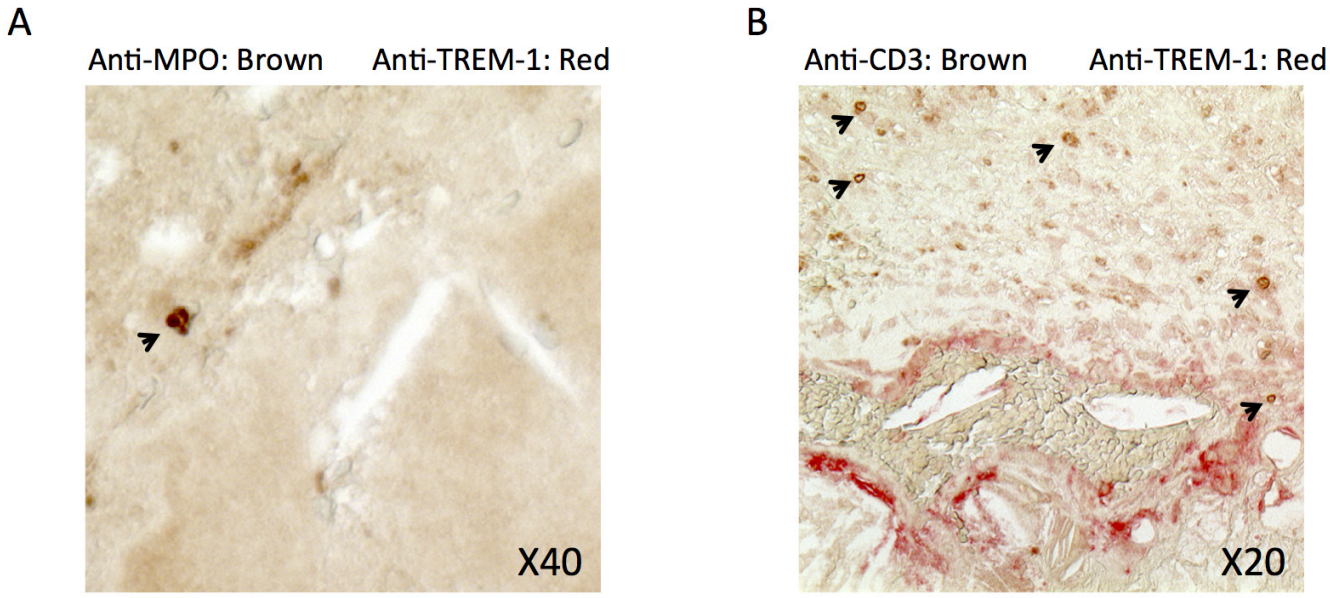
Cholesterolemia from *apoE*$^{-/-}$ mice treated with daily intraperitoneal injection of LR-12 or scramble peptide during 4 weeks on a chow (A) and a high fat diet (B). Representative photomicrographs and quantitative analysis of TUNEL+ area (Brown, C), CD3+ T cell infiltration (D) and collagen content (E) within atherosclerotic lesions of *apoE*$^{-/-}$ mice treated with daily injection of LR-12 or scramble peptide during 4 weeks on a high fat diet. ** P<0.01. scale bar 100 µm

Supplemental figure 12



A, intraperitoneal injection of Anti-Ly6G depleting antibody (1A8) or Isotype in *apoE*$^{-/-}$ mice lead to a 65% reduction of circulating neutrophils at day 2. B, experimental protocol of neutrophil depletion in Scramble or LR-12-treated *apoE*$^{-/-}$ mice fed a fat diet. C, Blood neutrophil count at the sacrifice. D, Representative photomicrographs and quantification of atherosclerotic plaque size (aortic sinus) after 4 weeks of fat diet. * P<0.05, ** P<0.01

Supplemental figure 13



Immunohistochemistry in human atherosclerotic plaques. A, Few Myeloperoxydase+ neutrophils (Brown, arrow) are detected in atherosclerotic plaques and they colocalize with TREM-1 (Red). B, CD3+ T cells (Brown, arrow) do not colocalize with TREM-1 (Red).

Supplemental figure 14

	Fibrous N=21	Atheromatous N=20	P
Male gender (%)	100 %	100 %	NS
Age, years (mean ± SD)	69 ± 9	70 ± 10	NS
Hypertension (%)	71 %	76 %	NS
Smoker (%)	42%	25 %	NS
Body Mass Index (mean ± SD)	27 ± 4	26 ± 2	NS
Statin (%)	80 %	80 %	NS

Clinical characteristics of human plaque samples. NS, Non significant

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Article entitled: Genetic and pharmacologic inhibition of TREM-1 limits the development of experimental atherosclerosis

Manuscript number: JACC060216-2308

Corresponding Author: Prof. Ait Oufella

Corresponding author's printed name: Hafid Ait Oufella

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Corresponding author's printed name: Stephane Potteaux

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Corresponding author's printed name: Lynda Zeboudj

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Manuscript number: JACC060216-2308R

Corresponding Author: Prof. Ait Oufella

Corresponding author's printed name: Hafid Ait Oufella

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