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# Resistin-Like Molecule $\beta$ Is Abundantly Expressed in Foam Cells and Is Involved in Atherosclerosis Development

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**Objective**—Resistin-like molecule (RELM)  $\beta$  is a secretory protein homologous to resistin and reportedly contributes to local immune response regulation in gut and bronchial epithelial cells. However, we found that activated macrophages also express RELM $\beta$  and thus investigated the role of RELM $\beta$  in the development of atherosclerosis.

**Approach and Results**—It was demonstrated that foam cells in atherosclerotic lesions of the human coronary artery abundantly express RELM $\beta$ . RELM $\beta$  knockout ( $^{-/-}$ ) and wild-type mice were mated with apolipoprotein E-deficient background mice. RELM $\beta^{-/-}$  apolipoprotein E-deficient mice exhibited less lipid accumulation in the aortic root and wall than RELM $\beta^{+/+}$  apolipoprotein E-deficient mice, without significant changes in serum lipid parameters. In vitro, RELM $\beta^{-/-}$  primary cultured peritoneal macrophages (PCPMs) exhibited weaker lipopolysaccharide-induced nuclear factor- $\kappa$ B classical pathway activation and inflammatory cytokine secretion than RELM $\beta^{+/+}$ , whereas stimulation with RELM $\beta$  upregulated inflammatory cytokine expressions and increased expressions of many lipid transporters and scavenger receptors in PCPMs. Flow cytometric analysis revealed inflammatory stimulation-induced RELM $\beta$  in F4/80(+) CD11c(+) PCPMs. In contrast, the expressions of CD11c and tumor necrosis factor were lower in RELM $\beta^{-/-}$  PCPMs, but both were restored by stimulation with recombinant RELM $\beta$ .

**Conclusions**—RELM $\beta$  is abundantly expressed in foam cells within plaques and contributes to atherosclerosis development via lipid accumulation and inflammatory facilitation. (*Arterioscler Thromb Vasc Biol.* 2013;33:1986-1993.)

**Key Words:** atherosclerosis ■ inflammation ■ macrophage

Resistin was initially identified as an adipocyte-secreted factor that causes insulin resistance.<sup>1</sup> Subsequent studies have suggested its association with type 2 diabetes mellitus,<sup>2</sup> congestive heart failure,<sup>3</sup> and coronary artery disease.<sup>4,5</sup> After the discovery of resistin, 3 resistin-related proteins, termed resistin-like molecule (RELM)  $\alpha$ ,  $\beta$  (found in inflammatory zone 2, murine ten-cysteine protein 3, human ten-cysteine protein 2), and  $\gamma$  were identified in mice. These resistin family proteins share a cysteine-rich domain at their C terminus (Cx11Cx8Cx2Cx10Cx9CC), and the roles of RELM $\alpha$ , RELM $\beta$ , and RELM $\gamma$  have been extensively studied.<sup>6-10</sup> Unlike the situation in mice, resistin and RELM $\beta$ , but not RELM $\alpha$  and RELM $\gamma$ , are present in humans.<sup>9,11</sup>

To date, expression of RELM $\beta$  has been reported to be limited to the gut or bronchi.<sup>9,12</sup> RELM $\beta$  expression in the colon was reportedly induced by a high-fat diet,<sup>10,13</sup> and RELM $\beta$  expression in the human colonic cell line LS174T was upregulated by saturated free fatty acid (SFA) or tumor necrosis factor- $\alpha$  (TNF $\alpha$ ) stimulation.<sup>13</sup> Thus, RELM $\beta$  seems to contribute to local immune system function in the gut by acting against bacteria and nematodes,<sup>7,12,14</sup> and probably reflecting this function, RELM $\beta$  absence reportedly influences the microbiome composition.<sup>15</sup> It was also documented that RELM $\beta$  augments interferon  $\gamma$ -induced TNF $\alpha$  secretion in thioglycolate-isolated macrophages and infection-induced intestinal inflammation.<sup>16</sup>

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In terms of insulin resistance, the function of RELM $\beta$  might be similar to that of resistin. Peripheral infusion of RELM $\beta$  impaired insulin action in the liver.<sup>17</sup> Transgenic mice expressing RELM $\beta$  in the liver reportedly exhibited insulin resistance with hyperinsulinemia, hyperglycemia, hyperlipidemia, and fatty liver when consuming a high-fat diet. Furthermore, glucose absorption in the intestinal tract was suggested to involve RELM $\beta$ .<sup>18</sup> Based on these previous reports, we attempted to clarify the association of RELM $\beta$  expression levels in the intestinal tract with the pathophysiology of chronic inflammation in humans. During this investigation, unexpectedly, we found that considerable percentages of macrophages in gut connective tissue stained for the anti-RELM $\beta$  antibody, although staining intensities differed among individuals.

This finding led to the speculation that macrophages might possess the potential to express RELM $\beta$  when exposed to certain stimuli or conditions and raised the possibility that RELM $\beta$  contributes to atherosclerosis development. Herein, we present the first evidence showing the critical role of RELM $\beta$  in the development of atherosclerosis using human atherosclerotic coronary arteries and RELM $\beta$  knockout ( $-/-$ ) mice, as well as *in vitro* data, suggesting possible molecular mechanisms underlying the processes of atherosclerosis induction.

## Materials and Methods

Materials and Methods are available in the online-only Supplement.

## Results

### Expression of RELM $\beta$ in Atherosclerotic Lesions

The monoclonal antibodies against human RELM $\beta$  were generated by immunizing mice with amino acids 14 to 66 of RELM $\beta$  fused with glutathione S-transferase (Figure I in the online-only Data Supplement). As reported previously, epithelial cells, particularly goblet cells, were strongly stained in the intestine (Figure IIA in the online-only Data Supplement). However, some macrophages in the villi of the intestine contained considerable amounts of RELM $\beta$  (Figure IIB in the online-only Data Supplement).

Because foam cells in unstable plaques in the aorta would be derived from highly activated macrophages via many factors, an immunohistochemical analysis using 3A-6 anti-human RELM $\beta$  monoclonal antibody was performed on 10 autopsied human coronary arteries from subjects who had died of acute myocardial infarction (Figure 1). RELM $\beta$  was abundantly expressed in macrophages in atherosclerotic lesions of all 10 samples, without exception, and representative staining is shown in Figure 1A. The enlarged picture (Figure 1B) revealed RELM $\beta$  staining to be dot-like in the cytoplasm, likely reflecting the presence of RELM $\beta$  in secretory vesicles. The staining with anti-CD68 and anti-RELM $\beta$  antibodies was performed for tissues of atherosclerotic lesions, as well as tissues outside the aorta, using adjacent slides (Figure 1C). It was shown that enlarged macrophages in plaques are both CD68 and RELM $\beta$  positive, whereas RELM $\beta$  was not detected in the small CD68-positive cells in normal fat tissue around the artery (Figure 1C). In plaques, double staining by anti-CD68 (green) and anti-RELM $\beta$  (red) antibodies confirmed the RELM $\beta$ -expressing cells to be macrophages (Figure 1D).

### RELM $\beta^{-/-}$ ApoE $^{-/-}$ Mice Showed Amelioration of Atherosclerotic Change Without Changes in Serum Lipid Profiles Compared With RELM $\beta^{+/+}$ ApoE $^{-/-}$ Mice

We generated RELM $\beta^{-/-}$  mice with exon 2 to 4 deletions of the RELM $\beta$  gene using gene targeting technology (Figure III in the online-only Data Supplement). Then, we prepared RELM $\beta^{-/-}$  mice with the C57Bl6 background using marker-assisted breeding<sup>19</sup> and examined the expressions of RELM $\beta$  in the colons of RELM $\beta$   $+/+$ ,  $+/-$ , and  $-/-$  mice. RELM $\beta$  expression in RELM $\beta^{+/+}$  was very low under specific pathogen-free conditions but markedly increased under conventional conditions, whereas none was detected in RELM $\beta^{-/-}$  mice under either condition. The colonic expression level in RELM $\beta^{+/+}$  mice was significantly lower than that in RELM $\beta^{+/+}$  mice, but was still readily detectable (Figure IVA in the online-only Data Supplement). However, PCPMs collected from RELM $\beta^{+/+}$  expressed RELM $\beta$  abundantly when incubated with watanabe heritable hyperlipidemic (WHHL) rabbit serum but at a very low level when incubated with normal rabbit serum (Figure IVB in the online-only Data Supplement). The induction of RELM $\beta$  expression by WHHL rabbit serum was not detected by Western blotting of PCPMs from RELM $\beta^{+/+}$  mice, similar to the results from RELM $\beta^{-/-}$  mice, although the reason was unclear.

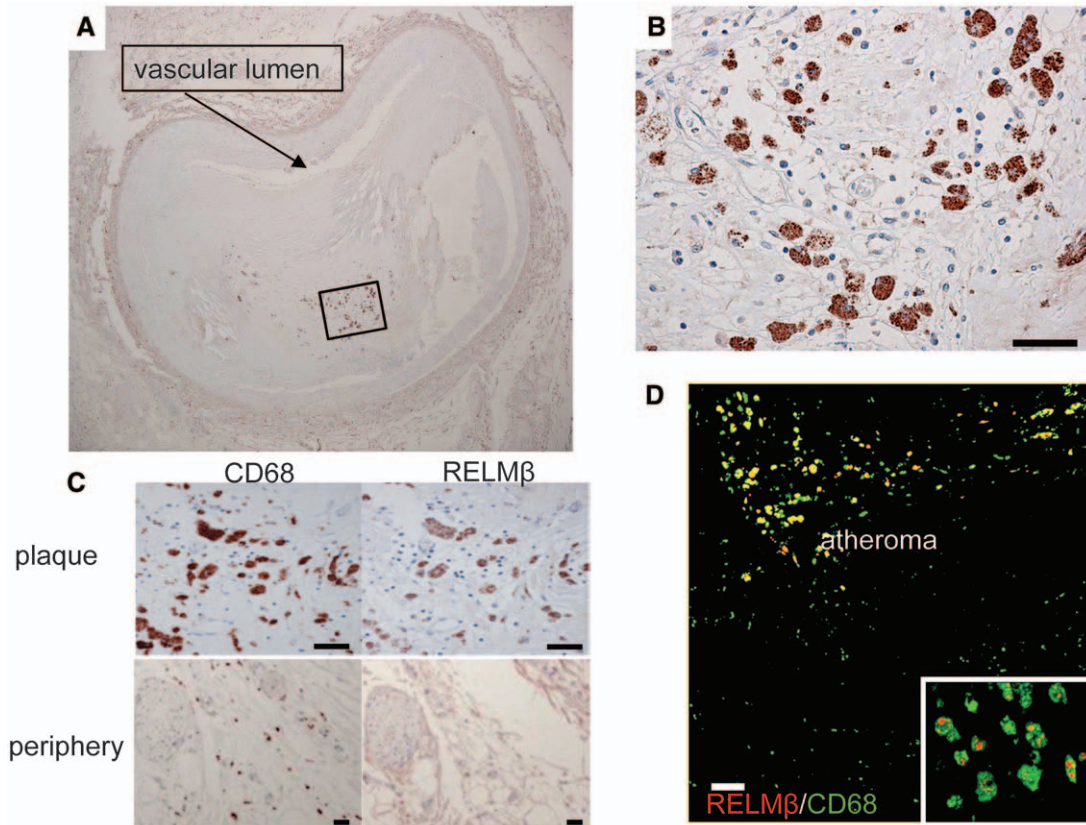
Then, RELM $\beta^{-/-}$  mice were crossed with apolipoprotein E-deficient (ApoE $^{-/-}$ ) mice to generate RELM $\beta^{-/-}$  with the ApoE $^{-/-}$  background. RELM $\beta$   $-/-$ ,  $+/-$ ,  $+/+$  with ApoE $^{-/-}$  background mice grew up without significant body weight gain. RELM $\beta$  was detected in atherosclerotic macrophages in the aortic roots of RELM $\beta^{+/+}$  ApoE $^{-/-}$  mice by immunostaining, whereas those of RELM $\beta^{-/-}$  ApoE $^{-/-}$  mice were unstained (Figure IVC and IVD in the online-only Data Supplement). There were no significant differences in lipid profiles (Figure IVE in the online-only Data Supplement) among the 3 RELM $\beta$  genotypes.

These mice were subjected to investigation of lipid accumulations in the aortic root (Figure 2A and 2C) at the ages of 3 and 6 months. Oil Red O staining of the aortic roots from RELM $\beta^{-/-}$  mice with the ApoE $^{-/-}$  background showed significantly reduced lipid accumulation compared with their littermates. The area immunohistochemically stained with anti-macrophage antibodies in the aortic roots from 6-month-old RELM $\beta^{-/-}$  ApoE $^{-/-}$  mice was also significantly reduced compared with their littermates. The change started from 3 months of age and was significant at 6 months.

Subsequently, the atherosclerotic lesions in the aortas of 12-month-old mice were subjected to Oil Red O staining (Figure 2B, 2D, and 2E). Quantification analysis revealed the ratios of areas with fat deposition to whole aortic wall areas to be significantly lower in RELM $\beta^{-/-}$  ApoE $^{-/-}$  or RELM $\beta^{+/+}$  ApoE $^{-/-}$  mice than in RELM $\beta^{+/+}$  ApoE $^{-/-}$  mice (Figure 2D). The numbers of these areas in whole aortic areas were also lower in RELM $\beta^{-/-}$  ApoE $^{-/-}$  mice (Figure 2E). RELM $\beta^{+/+}$  ApoE $^{-/-}$  mice showed a significant reduction in the Oil Red O-stained area compared with RELM $\beta^{+/+}$  ApoE $^{-/-}$  mice, but the reduction in the number of areas was not significant.

Furthermore, by crossing with low-density lipoprotein receptor (LDLR) $^{-/-}$  mice, RELM $\beta$   $-/-$ ,  $+/-$ , and  $+/+$  mice with the LDLR $^{-/-}$  background were generated. These mice were fed





**Figure 1.** Resistin-like molecule (RELM)  $\beta$  is abundantly expressed in the plaques of human atherosclerotic lesions. **A**, RELM $\beta$  expression by foam cells in coronary atherosclerotic lesions. Autopsied coronary arteries from 10 patients who died of acute myocardial infarction (AMI) were obtained and processed for immunohistochemistry using anti-hRELM $\beta$  antibody. Representative staining using 3A-6 antibody is shown. Nuclei were counterstained with hematoxylin. **B**, Magnified picture of the boxed-in area in **A** is shown. **C**, RELM $\beta$  and CD68 expressions in plaque and the coronary artery periphery. Another autopsied sample from an AMI patient was processed for immunohistochemistry using anti-hRELM $\beta$  and anti-CD68 antibodies, allowing comparison of the staining properties of these 2 antibodies in adjacent slides. Larger CD68 macrophages in plaque were readily stainable, whereas the smaller and more peripheral CD68-positive cells were not stained by anti-hRELM $\beta$  antibody. **D**, The double-stained area is indicated in yellow. Staining using anti-RELM $\beta$  antibody is indicated in red and anti-macrophage antibody in green. A subpopulation of CD68-positive cells expressed RELM $\beta$ . Scale bars, 100  $\mu$ m.

a high-cholesterol diet from the age of 3 months for 8 weeks. It was demonstrated that RELM $\beta^{-/-}$  LDLR $^{-/-}$  mice showed marked amelioration of atherosclerotic lesions compared with RELM $\beta^{+/+}$  LDLR $^{-/-}$  mice, which is similar to that of ApoE $^{-/-}$  mice (Figure 2F).

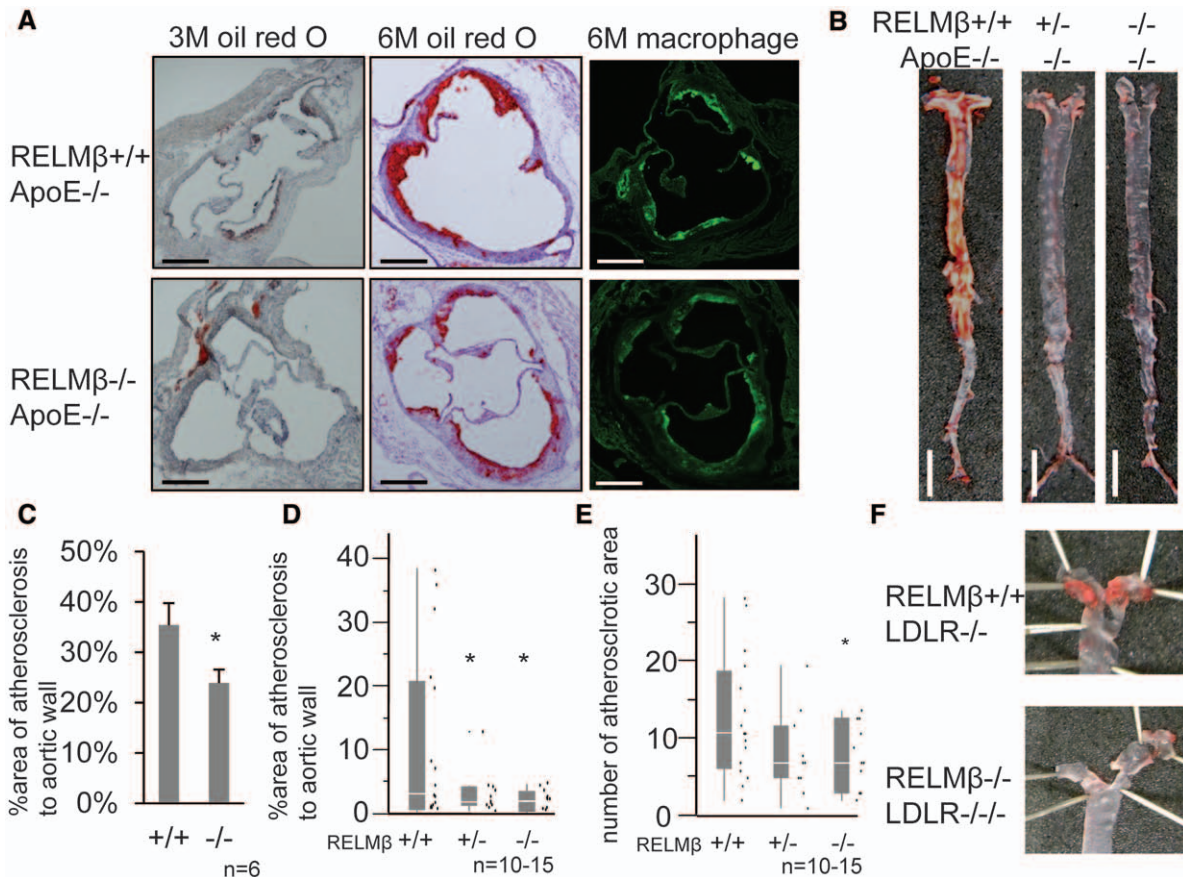
### RELM $\beta$ Promotes Macrophage Foam Cell Formation

The *in vitro* effect of RELM $\beta$  expression on macrophage foam cell formation was investigated (Figure 3A and 3B). PCPMs were obtained from RELM $\beta^{-/-}$  mice and their littermates 2 days after peritoneal injection of thioglycolate (TGC) and treated with 50  $\mu$ g/mL oxidized LDL, 50  $\mu$ g/mL VLDL, or 1% sera from 3-month-old WHHL rabbits. Incubation with oxidized LDL, VLDL, or WHHL rabbit serum induced significant lipid accumulation in PCPMs, and quantification of cellular lipids was performed by staining with AdipoRed assay reagent and adjusted for cell counts. RELM $\beta^{-/-}$  macrophages accumulated significantly smaller amount of lipids than RELM $\beta^{+/+}$  macrophages (Figure 3B\*). In contrast, administration of 2  $\mu$ g/mL recombinant RELM $\beta$  increased lipid accumulation in RELM $\beta^{-/-}$  PCPMs (Figure 3B $\dagger$ ), which was blocked

by coinubation with 50  $\mu$ g/mL anti-RELM $\beta$  F(ab')<sub>2</sub> antibody (Figure 3B#).

Next, 1,1-dioctadecyl-3,3,3,3-tetramethylindocarbocyanine (DiI)-acetyl LDL uptake for 4 hours was assayed and then adjusted by cell numbers (Figure 3C). 1,1-dioctadecyl-3,3,3,3-tetramethylindocarbocyanine (DiI)-acetyl LDL uptake into RELM $\beta^{-/-}$  PCPMs was significantly lower than that into RELM $\beta^{+/+}$  PCPMs. Similarly, [<sup>3</sup>H]-cholesterol efflux by high-density lipoprotein uptake was slightly but significantly decreased by RELM $\beta$  deficiency (Figure 3D).

Alterations in the expressions of lipoprotein receptors, scavenger receptors (SRs), and lipid transporters were investigated (Figure 3E). Absence of RELM $\beta$  significantly reduced protein levels of SR-A1 and VLDL receptor. SR-A2 and Lox-1 mRNA levels in the RELM $\beta^{+/+}$  PCPMs were markedly elevated by stimulation with lipopolysaccharide (LPS), and the degree of LPS-induced increase was markedly attenuated in RELM $\beta^{-/-}$  PCPMs. LDLR and CD36 expression levels did not differ between RELM $\beta^{+/+}$  and RELM $\beta^{-/-}$  PCPMs. As for lipid efflux transporters, the ATP binding cassette G1 protein level was upregulated but that of ATP binding cassette A1 was reduced, and SR-B1 also tended to be reduced in RELM $\beta^{-/-}$  PCPMs compared with RELM $\beta^{+/+}$



**Figure 2.** Resistin-like molecule (RELM)  $\beta$  promoted atherosclerosis development in apolipoprotein E-deficient (ApoE<sup>-/-</sup>) mice. **A**, Representative figures of Oil Red O-stained aortic roots from RELM $\beta$ <sup>-/-</sup> mice and their littermates, both of which were ApoE<sup>-/-</sup>, at ages 3 and 6 months. Slides of sections at the level of the aortic valve are presented. Immunofluorescence staining using anti-macrophage antibody at age 6 months is also presented using an adjacent slide with Oil Red O staining. Scale bars, 100  $\mu$ m. **B**, Representative figures of Oil Red O-stained aortic walls from RELM $\beta$ <sup>-/-</sup> mice and their littermates at age 12 months, both of which were ApoE<sup>-/-</sup>. Scale bars, 0.5 mm. **C**, Representative figures of Oil Red O-stained proximal aortic walls from RELM $\beta$ <sup>-/-</sup> mice and their littermates at age 12 months, both of which were low-density lipoprotein receptor (LDLR) deficient. **D**, Quantification of the Oil Red O-stained area in Figure 3A obtained at age 6 months. Bars represent means $\pm$ SD. **E**, Quantification of the ratios of the Oil Red O-stained area to the aortic wall area and the number of fat deposits in the aortic wall. n=15 (RELM $\beta$ <sup>+/+</sup>), 10(+/-), and 11(-/-). Plots of all samples and box plots are shown. **F**, Quantification of the number of fat deposits in the aortic wall. Plots of all samples and box plots are shown. \*Statistical significance  $P < 0.05$  compared with control.

PCPMs. The effects of recombinant RELM $\beta$  stimulation of the J774.1 cell line were generally similar to the data obtained for RELM $\beta$ <sup>+/+</sup> PCPMs compared with RELM $\beta$ <sup>-/-</sup> PCPMs, with some exceptions for CD36, LDLR, and SRB1 (Figure V in the online-only Data Supplement).

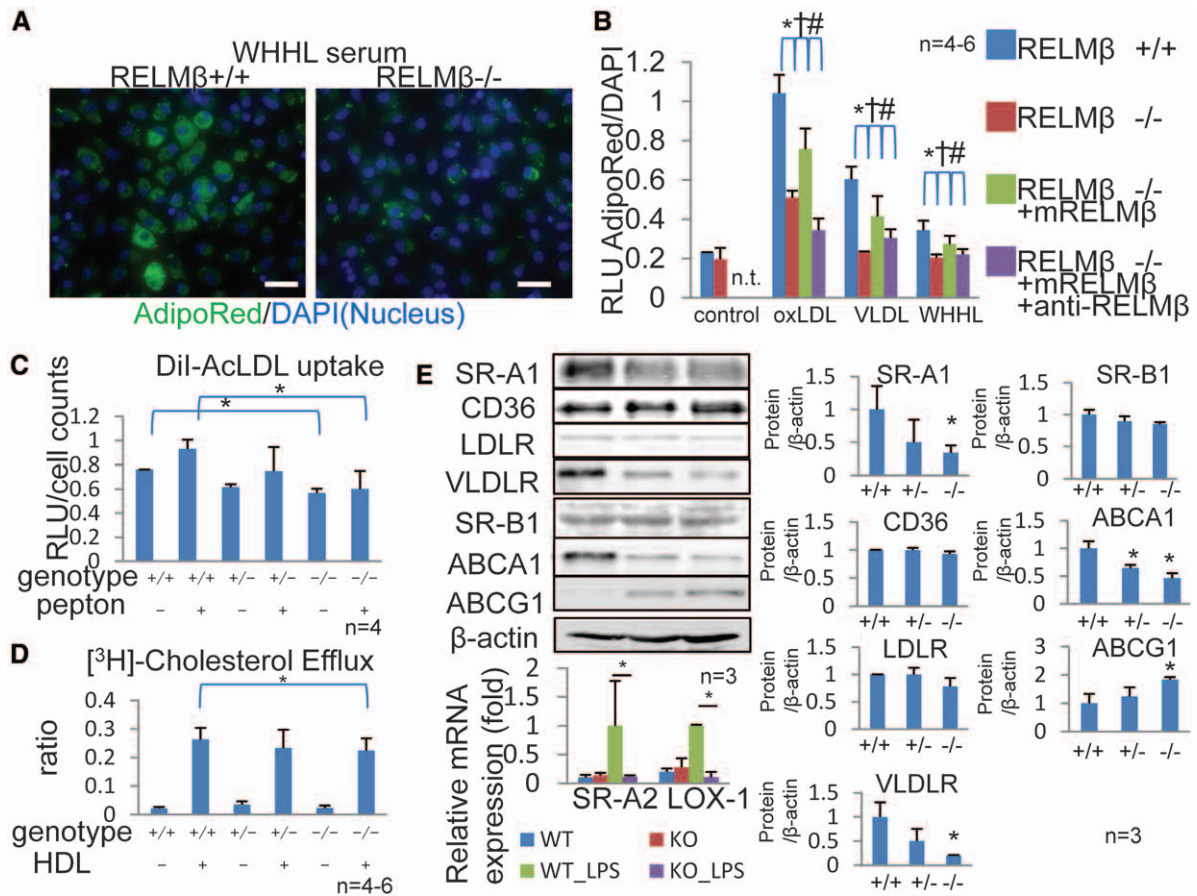
### RELM $\beta$ Enhances the Expressions of Inflammatory Cytokines and the Nuclear Factor- $\kappa$ B Pathway With LPS Stimulation in Macrophages

To reveal the proinflammatory effects of RELM $\beta$  on macrophages, LPS-induced mRNA expressions of inflammatory cytokines in PCPMs from RELM $\beta$ <sup>-/-</sup> mice and their littermates 2 days after TGC peritoneal injection were examined by quantitative polymerase chain reaction (Figure 4A). The inductions of TNF $\alpha$ , interleukin (IL)-1 $\beta$ , and IL-6 by incubation with 10 ng/mL LPS for 4 hours were very high in PCPMs from RELM $\beta$ <sup>+/+</sup> mice, and these inductions were significantly blunted in the absence of RELM $\beta$ . In addition, inductions of inflammatory cytokine secretions by recombinant human

RELM $\beta$  (hRELM $\beta$ ) administration were investigated in phorbol 12-myristate 13-acetate-differentiated human cell lines THP-1 and U-937 (Figure VIA and VIB in the online-only Data Supplement). Supernatants from THP-1 or U-937 cell culture treated with 3 g/mL hRELM $\beta$  or human resistin for 24 hours were collected, and concentrations of TNF $\alpha$ , IL-1 $\beta$ , and IL-6 were determined using a Bioplex kit. hRELM $\beta$  markedly increased the secretions of TNF $\alpha$ , IL-1 $\beta$ , and IL-6. In contrast, resistin had no effects on these cytokine secretions.

Next, LPS-induced nuclear factor (NF)- $\kappa$ B activation was investigated. After stimulation with 100 ng/mL LPS, time-dependent changes in the protein expressions and phosphorylation levels of NF- $\kappa$ B and I $\kappa$ B kinase- $\alpha/\beta$  were compared between the PCPMs from RELM $\beta$ <sup>-/-</sup> mice and their littermates (Figure 4B). Degradation of I $\kappa$ B by 100 nM LPS in the presence of cycloheximide was also investigated. Although protein expression levels of NF- $\kappa$ B and I $\kappa$ B kinase- $\alpha/\beta$  did not differ between PCPMs from RELM $\beta$ <sup>-/-</sup> mice and their littermates, phosphorylation levels of NF- $\kappa$ B and I $\kappa$ B kinase- $\alpha/\beta$  were significantly lower in the RELM $\beta$ <sup>-/-</sup> than in the RELM $\beta$ <sup>+/+</sup>





**Figure 3.** Presence of resistin-like molecule (RELM $\beta$ ) enhances macrophage foam cell formation with increased expressions of lipoprotein receptors and lipid transporters. **A**, PCPMs were obtained from RELM $\beta^{-/-}$  mice and their littermates and incubated with 1% normal and watanabe heritable hyperlipidemic (WHHL) rabbit sera for 24 hours. Representative AdipoRed staining (green) and counterstained nuclei (blue) are shown. Scale bars, 100  $\mu$ m. **B**, PCPMs were incubated with oxidized low-density lipoprotein (oxLDL), very LDL (VLDL), and WHHL serum for 48 hours. Quantification of cellular lipids stained by AdipoRed, adjusted for cellular count by DAPI staining quantified with an ARVO MX fluorimeter. PCPMs from RELM $\beta^{-/-}$  mice show decreased lipid accumulation compared with their littermates. Administration of 2  $\mu$ g/mL RELM $\beta$  to PCPMs from RELM $\beta^{-/-}$  mice restores lipid accumulation, and anti-RELM $\beta$  F(ab) $_2$  antibody blocked the effect of recombinant RELM $\beta$ . Normal rabbit IgG F(ab) $_2$  was used as the control for anti-RELM $\beta$  F(ab) $_2$ . Bars represent means $\pm$ SD (n=4-6). **C**, 1,1-dioctadecyl-3,3,3,3-tetramethylindocarbocyanine (DiI)-acetyl LDL (DiI-AcLDL) uptake for 4 hours was assayed and then adjusted by cell numbers. DiI-AcLDL uptake into RELM $\beta^{-/-}$  PCPMs was significantly lower than that into RELM $\beta^{+/+}$  PCPMs. Bars represent means $\pm$ SD (n=4). **D**, [ $^3$ H]-cholesterol efflux assay by high-density lipoprotein (HDL) uptake was slightly but significantly decreased by RELM $\beta$  deficiency. Bars represent means $\pm$ SD (n=4-6). **E**, Alterations in the expressions of lipoprotein receptors, scavenger receptors (SRs), and lipid transporters. ABC indicates ATP binding cassette; KO, knockout; LOX-1, lectin-like oxidized LDL receptor-1; LPS, lipopolysaccharide; and RLU, relative light unit.

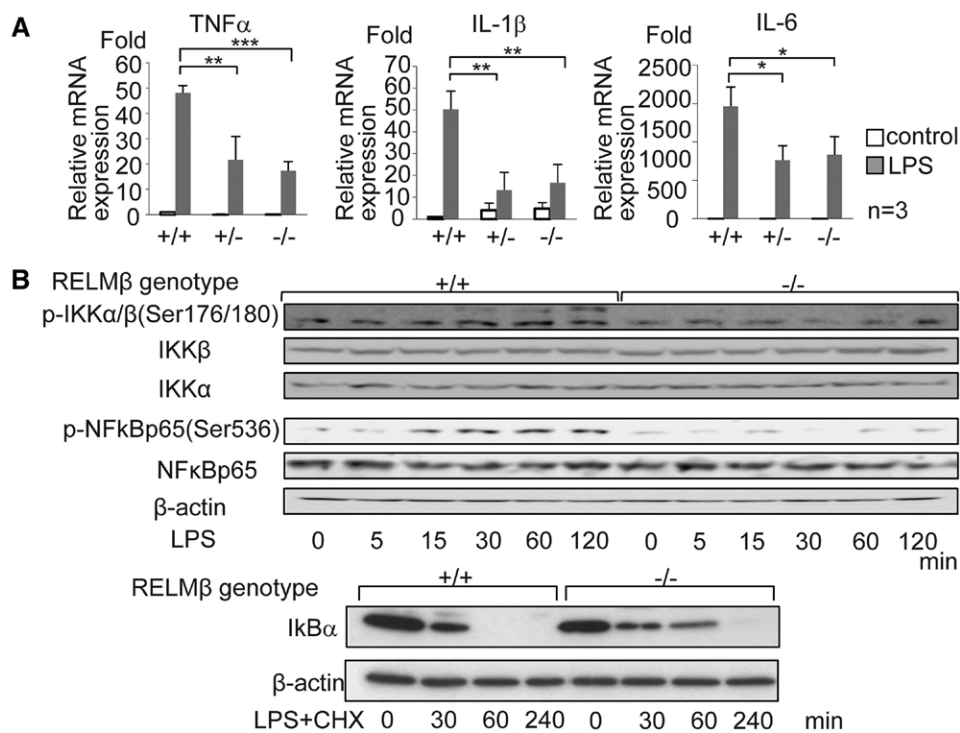
PCPMs. I $\kappa$ B degradation by LPS stimulation was also restored in the absence of RELM $\beta$  at 60 minutes in the presence of cycloheximide, thus I $\kappa$ B degradation by LPS was delayed in RELM $\beta^{-/-}$  macrophages. These data suggest that RELM $\beta$  exerts an enhancing effect on LPS-induced NF- $\kappa$ B activation.

### RELM $\beta$ Is Expressed in CD11c-Positive Cells, and the Absence of RELM $\beta$ Blunted the Inflammatory Response

Peritoneal macrophages from RELM $\beta^{+/+}$  and RELM $\beta^{-/-}$  mice were collected from RPMI1640 medium 2 days after addition of PBS, 10% proteose peptone, or 3% TGC injection. Cells were stained with F4/80, CD11c, CD206, and RELM $\beta$  and then analyzed by flow cytometry (Figure VIIA and VIIB in the online-only Data Supplement). RELM $\beta$  (+) cells were mostly F4/80(+) CD11c (+). In both F4/80 (+) CD11c (+) cells and a small population of F4/80 (+) CD206 (+) cells,

RELM $\beta$ -positive cells were inducible when stimulated with pepton or TGC to induce aseptic inflammation in the peritoneum. Absence of RELM $\beta$  significantly reduced the CD11c (+) cells, as well as CD206 (+) cells, induced by pepton/TGC (Figure VIIB in the online-only Data Supplement).

Next, we investigated whether extracellular administration of RELM $\beta$  affects the M1/M2 markers of PCPMs and whether extracellular administration of neutralizing antibody can block the effects of RELM $\beta$  on M1/M2 markers. The peritoneal macrophages from RELM $\beta^{+/+}$ ,  $+/-$ , and  $-/-$  mice were collected after stimulation with or without TGC injection, and the PCPMs obtained were incubated with 100 ng/mL of LPS for 4 hours. TGC treatment markedly increased expressions of CD11c, TNF $\alpha$ , and CD206 in RELM $\beta^{-/-}$  macrophages, whereas IL-10 decreased the expressions of these macrophages (Figure VIII\* in the online-only Data Supplement). The effects of TGC on CD11c, TNF $\alpha$ , CD206, and IL-10 were significantly suppressed in



**Figure 4. A**, The relative expressions of tumor necrosis factor (TNF)- $\alpha$ , interleukin (IL)-1 $\beta$ , and IL-6 mRNA by resistin-like molecule (RELM) $\beta$  +/+, +/-, and -/- PCPMs treated with or without 10 ng/mL lipopolysaccharide (LPS) for 4 hours. The results are presented as means ( $\pm$ SE, n=3), from 3 independent experiments (\* $P$ <0.05, \*\* $P$ <0.01). **B**, The nuclear factor (NF)- $\kappa$ B and I $\kappa$ B kinase (IKK)- $\alpha$ / $\beta$  protein expressions and phosphorylations with 100 ng/mL LPS by PCPMs from RELM $\beta$ <sup>-/-</sup> mice and their littermates and degradation of I $\kappa$ B by 100 nM LPS in the presence of cycloheximide. Although levels of NF- $\kappa$ B and IKK $\alpha$ / $\beta$  protein expression do not differ between PCPMs from RELM $\beta$ <sup>-/-</sup> mice and their littermates, phosphorylations of NF- $\kappa$ B and IKK $\alpha$ / $\beta$  are diminished in the absence of RELM $\beta$ . I $\kappa$ B degradation by LPS stimulation is restored by the absence of RELM $\beta$  at 60 minutes. CHX indicates cycloheximide.

the RELM $\beta$ <sup>-/-</sup> macrophages (Figure VIII† in the online-only Data Supplement), whereas RELM $\beta$  administration to these macrophages partially restored the effects of TGC (Figure VIII‡ in the online-only Data Supplement). Furthermore, the effects of endogenous RELM $\beta$  expression or extracellular administration of RELM $\beta$  were blocked by coincubation with anti-RELM $\beta$  F(ab')<sub>2</sub> antibody (Figure VIII# in the online-only Data Supplement).

### RELM $\beta$ Is Upregulated by Stimulation With Saturated Fatty Acids in Human Macrophage Cell Lines

The regulation of RELM $\beta$  expression in the human macrophage cell lines THP-1 and U-937 was investigated using real-time polymerase chain reaction. RELM $\beta$  expression levels in these cell lines were very low under basal conditions but showed marked induction when incubated with 0.2 mmol/L SFA, such as stearic and palmitic acids, for 16 hours. Unsaturated fatty acids were less effective for RELM $\beta$  induction. In contrast, resistin expression levels were not altered by either SFA (Figure IXA and IXB in the online-only Data Supplement). In addition, the degrees of SFA-induced TNF $\alpha$  and IL-6 expressions were significantly lower in RELM $\beta$ <sup>-/-</sup> than in RELM $\beta$ <sup>+/+</sup> PCPMs (Figure IXC in the online-only Data Supplement).

### Discussion

RELM $\beta$  expression was formerly thought to be limited to epidermal, particularly goblet, cells of the small and large intestines and bronchial tracts.<sup>9,12</sup> In these tissues, RELM $\beta$  reportedly contributes to protection against infections with parasitic worms or bacteria and also functions to maintain barrier integrity.<sup>14,20</sup> In agreement with such functions, RELM $\beta$

expression in the gut is reportedly induced on exposure to a conventional environment after being in a germ-free environment.<sup>21</sup> Furthermore, RELM $\beta$  has been suggested to be involved in a variety of human inflammatory gastrointestinal conditions, including allergic gastroenteropathies,<sup>20</sup> airway remodeling,<sup>22</sup> and pulmonary hypertension.<sup>23</sup>

In contrast to the aforementioned reports, the first surprising finding of this study was the high expression of RELM $\beta$  in the foam cells of atherosclerotic lesions, which was demonstrated in both human autopsy samples and the aortas of ApoE<sup>-/-</sup> and LDLR<sup>-/-</sup> mice. RELM $\beta$  detection in atherosclerotic lesions was limited to the large, foam cell macrophages in plaques, with none being found in the small CD68-positive macrophages scattered in the surrounding normal adipose tissue. In this study, it was shown that very LDL-rich WHHL serum in murine PCPMs and SFA in human macrophage cell lines markedly upregulated the expression of RELM $\beta$ . Thus, in contrast to the continuous expression of resistin in macrophages, RELM $\beta$  expression in macrophages is likely to be induced by certain stimulants, such as specific pathological states.

To examine the contribution of RELM $\beta$  to the pathogenesis of atherosclerosis, we generated RELM $\beta$ <sup>-/-</sup> mice with the ApoE<sup>-/-</sup> or LDLR<sup>-/-</sup> background. Indeed, these mice showed significantly less lipid accumulation in the aortic root and aortic wall than RELM $\beta$ <sup>+/+</sup> with ApoE<sup>-/-</sup> or LDLR<sup>-/-</sup> mice. Absence of RELM $\beta$  does not result in significant changes in serum lipid profiles, which indicates that macrophage-derived RELM $\beta$  directly contributes to lipid accumulation in macrophages and the development of atherosclerosis.

It is well known that atherosclerosis develops through multiple processes, among which foam cell formation is a key event, because it leads to the secretion of inflammatory cytokines<sup>24</sup> and plaque instability.<sup>25</sup> It was revealed in vitro that



RELM $\beta$  induced macrophages to induce NF- $\kappa$ B signaling and express inflammatory cytokines, lipid uptake, and also promoted their transformation into foam cells. NF- $\kappa$ B signaling is reportedly involved in not only inflammatory responses but also foam cell formation.<sup>26</sup>

In this study, it was demonstrated that RELM $\beta$  induces the transformation of macrophages into foam cells. Furthermore, cellular mechanisms lead to foam cell transformations, such as lipid uptake, metabolism, efflux, and, governed by their respective SRs, expressions of lipases, hydroxylase, and ATP binding cassette transporters.<sup>27,28</sup> It was previously documented that intraperitoneal injection of RELM $\beta$  enlarged murine peritoneal macrophages.<sup>29</sup> We speculated that lipid accumulation would be the explanation for this observation and then demonstrated that RELM $\beta^{-/-}$  PCPMs accumulated smaller amounts of lipid when incubated with various lipoproteins. In the lipid uptake assay, RELM $\beta^{-/-}$  showed diminished lipoprotein uptake, but lipid efflux was also diminished by RELM $\beta$  deficiency. Extracellular recombinant RELM $\beta$  administration to RELM $\beta^{-/-}$  PCPMs and J774.1 cells results in expression changes of SRs and lipid transporters, such as lipoprotein receptors (LDLR, VLDL receptor) and class A (SR-A1) and B (CD36) SRs. Class A is a group of receptors for oxidized LDL and acLDL. Cholesterol efflux and high-density lipoprotein-dependent lipid transporters (SR-B1, ATP binding cassette A1) were downregulated in RELM $\beta^{-/-}$  macrophages, whereas lipid uptake, especially of modified LDL and VLDL, was phenotypically predominant.

RELM $\beta$  activates the classical M1 macrophage marker CD11c and inflammatory cytokines, and absence of RELM $\beta$  reduces inducible CD11c-positive and CD206-positive cells. In contrast, major portion of inflammation-induced CD11c- or CD206-positive cells are RELM $\beta$  positive. CD11c-positive cells are reportedly important for foam cell formation<sup>30</sup> and constitute a major cell population in atherosclerotic plaque.<sup>31</sup> RELM $\beta$  is essential for the induction of CD11c-positive cells, and a direct effect was demonstrated by administration of RELM $\beta$ . The number of CD206-positive macrophages is paradoxically increased by peritoneal TGC injection, although IL-10, an anti-inflammatory cytokine, is decreased. The latter effect is partially reversed by neutralization of extracellular RELM $\beta$ . CD206-positive, alternatively activated M2 macrophages are generally anti-inflammatory, but also have a role in foam cell formation.<sup>32</sup> Induction of CD206-positive cells is speculated to provide a degree of compensation but is not sufficient to overcome the inflammatory phenotype, and RELM $\beta$  in CD206-positive cells suggests inhibition of IL-10 expression.

Taking into consideration that macrophages themselves produce RELM $\beta$  and that serum RELM $\beta$  (on the order of several nanograms per milliliter in mice) concentrations would not reach those necessary to function in macrophages, it is possible that RELM $\beta$  produced by foam cells functions in macrophages in either a paracrine or an autocrine manner. We demonstrated that extracellular RELM $\beta$  can induce inflammation, lipid accumulation, and gene regulations by recombinant RELM $\beta$  administration and neutralization of inflammation by its blocking antibody.

Our results strongly suggest that RELM $\beta$ , which is abundantly expressed in the foam cells of atherosclerotic lesions, functions in both autocrine and paracrine manners in M1/M2 macrophages, endothelial cells, and fibroblasts (data not shown), thereby contributing to atherosclerosis development and plaque instability (Figure X in the online-only Data Supplement). Thus, an agent suppressing RELM $\beta$  expression or an antagonist of the as yet unidentified receptor might be used to prevent or treat atherosclerosis. In addition, serum RELM $\beta$  concentrations might serve as a marker for judging the degree of atherosclerosis. Further study, particularly for identification of the RELM $\beta$  receptor and to assess the value of measuring the serum RELM $\beta$  concentration, is needed.

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A. Kushiya designed the research, performed experiments, and wrote the article; N. Oue, Y. Nakatsu, H. Ono, T. Fukushima, H. Kamata, T. Kikuchi, M. Fujishiro, K. Nishiyama, H. Aburatani, S. Kushiya, M. Iizuka, N. Taki, J. Encinas, K. Sentani, N. Ogonuki, A. Ogura, and W. Yasui performed experiments; H. Sakoda, F. Nishimura, S. Kawazu, H. Katagiri, Y. Higashi, and H. Kurihara designed the research; and T. Asano designed the research and wrote the article. We thank Masako Fujita, Yuriko Muta, and Hirokazu Sato for technical assistance.

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### Disclosures

None.

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### Significance

This study demonstrated, for the first time, the expression and involvement of resistin-like molecule (RELM)  $\beta$  derived from macrophages in atherosclerotic lesions in both human tissues and mice. Deficiency of RELM $\beta$  expression reduces the size of lesions composed of macrophages and lipid accumulation in atherosclerotic model mice, without altering serum lipid profiles. RELM $\beta$  directly induces lipid cycling and results in foam cell formation. Furthermore, RELM $\beta$  is expressed only in activated inflammatory macrophages and activates both classical and alternative pathways. The lipopolysaccharide effect on inflammatory cytokine expression is enhanced by RELM $\beta$ , and it is suggested that the presence of RELM $\beta$  promotes the nuclear factor- $\kappa$ B pathway. Interventions aimed at preventing the effects of RELM $\beta$  might be the key to inhibiting atherosclerosis development.

## SUPPLEMENTAL MATERIALS

### **Resistin like molecule $\beta$ is abundantly expressed in foam cells and is involved in atherosclerosis development**

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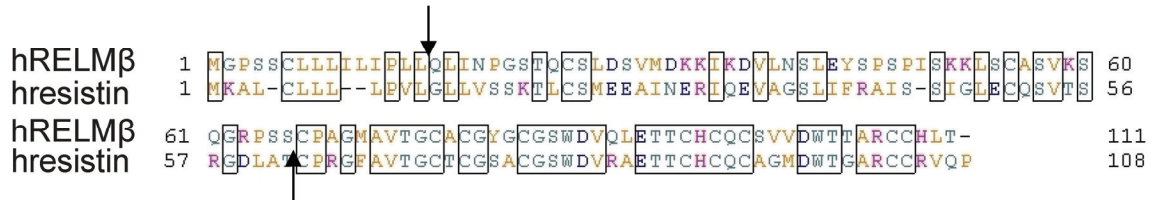
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Supplementary Fig. I

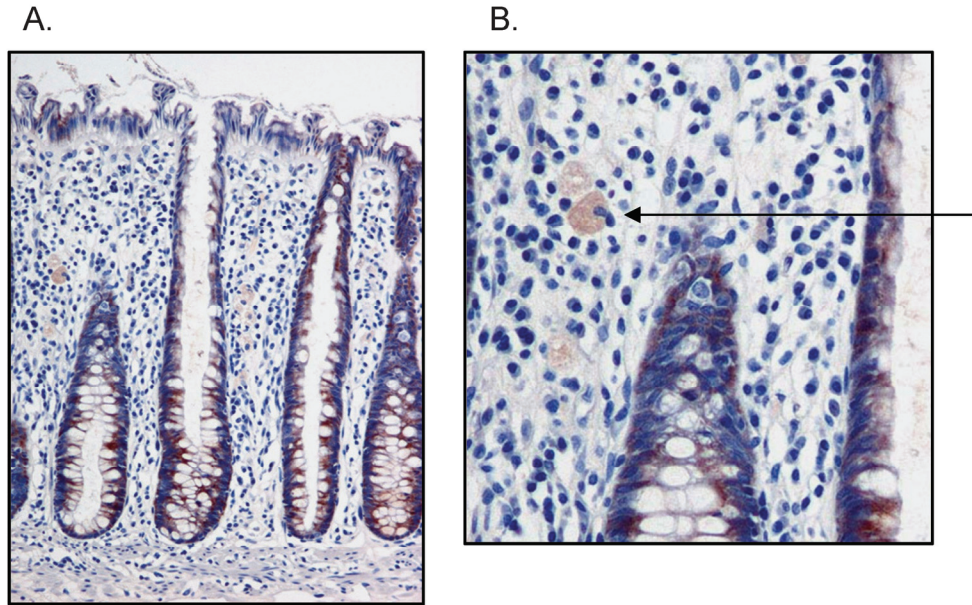


Antigen: amino acids 14-66 of human RELM $\beta$

Supplementary Fig. I

Alignment of amino acid sequences for human RELM $\beta$  and resistin, and peptides for generating monoclonal antibodies against human RELM $\beta$ . Balb/c mice were immunized with amino acids 14-66 of RELM $\beta$  fused with GST. Among the isolated monoclonal antibodies, we selected two, 1C1-A and 3A-6, which can be used for immunoblotting and immunostaining. These antibodies did not cross-react with resistin (data not shown), as predicted by amino acids 14-66 of RELM $\beta$  not being conserved in the sequence of resistin.

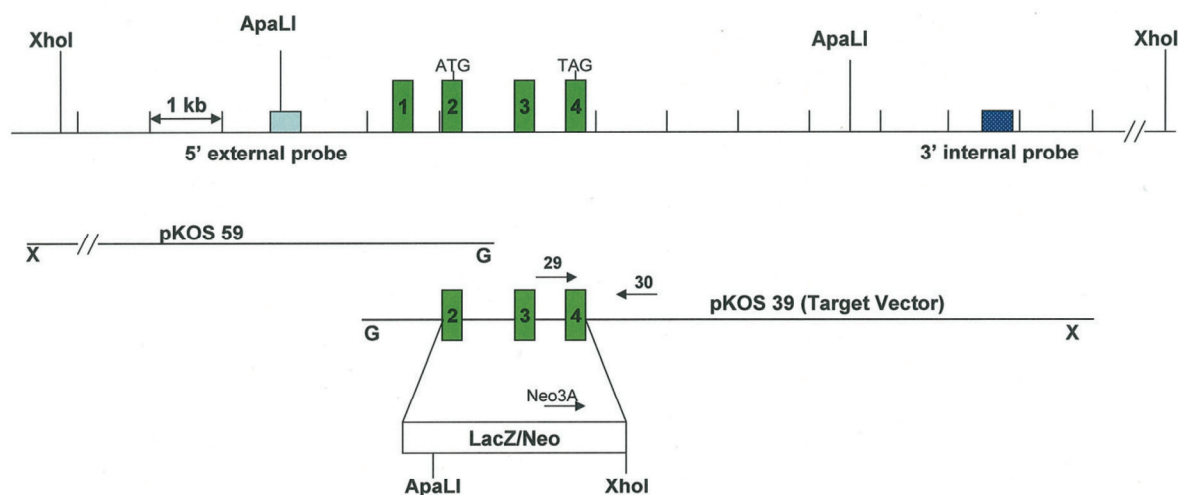
Supplementary Fig. II



Supplementary Fig. II

Immunohistochemistry of the human colon with 3A-6 antibody. A. Normal colon tissue was routinely embedded in paraffin. Four- $\mu$ m-thick slices obtained from these samples were immunostained with anti-hRELM $\beta$  antibody. Bars in panel A indicate 100 $\mu$ m. B. Arrow indicates a RELM $\beta$  positive macrophage. These staining reactions were blunted by co-incubation with recombinant antigen (data not shown), suggesting this staining to be specific.

Supplementary Fig. III

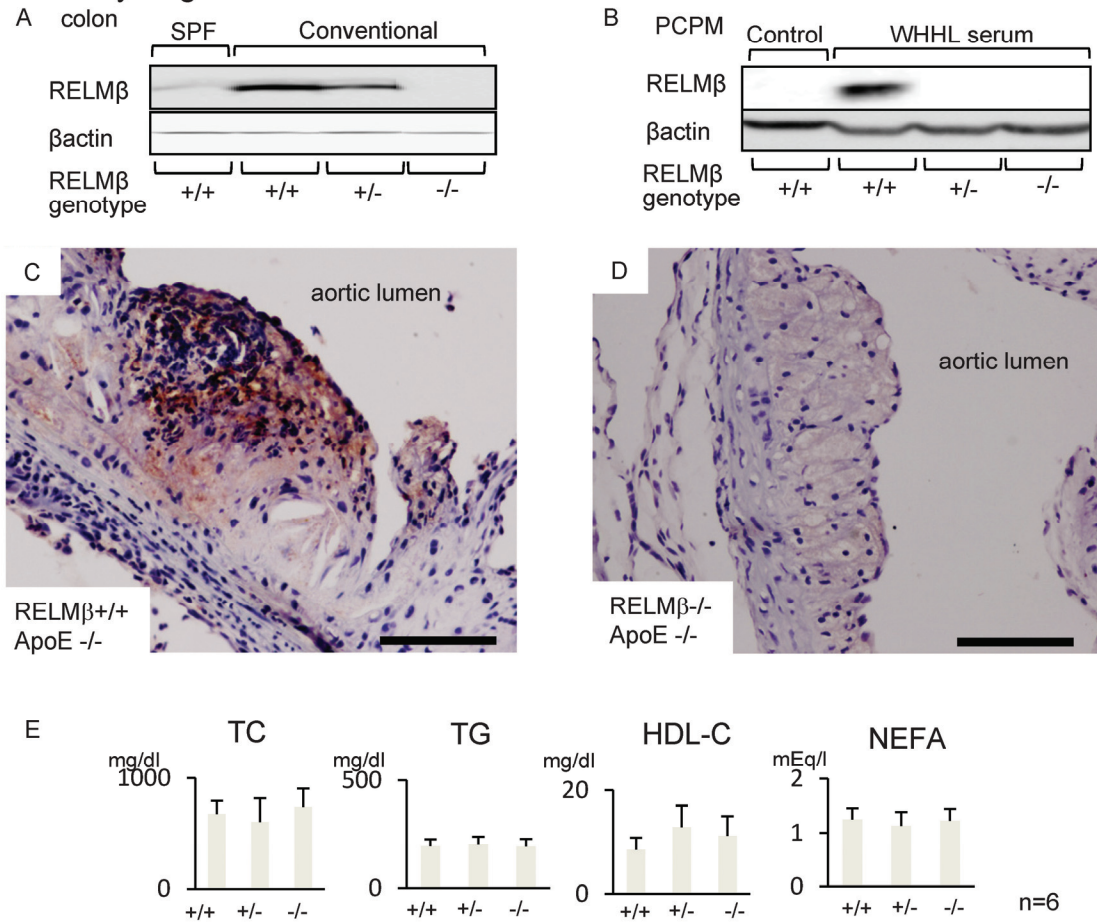


Supplementary Fig. III

Construction of RELM $\beta$  gene targeting vector. The Lambda KOS phage library was screened by PCR using exon 2-specific primers. pKOS-39 was isolated, then transfected into yeast, and clones that had undergone homologous recombination to replace exons 2-4 were isolated. The linearized targeting vector was electroporated into 129/SvEv<sup>Brd</sup> (Lex-1) ES cells. G418/FIAU-resistant ES cell clones were isolated, and correctly targeted clones were identified and confirmed by Southern analysis.



Supplementary Fig. IV

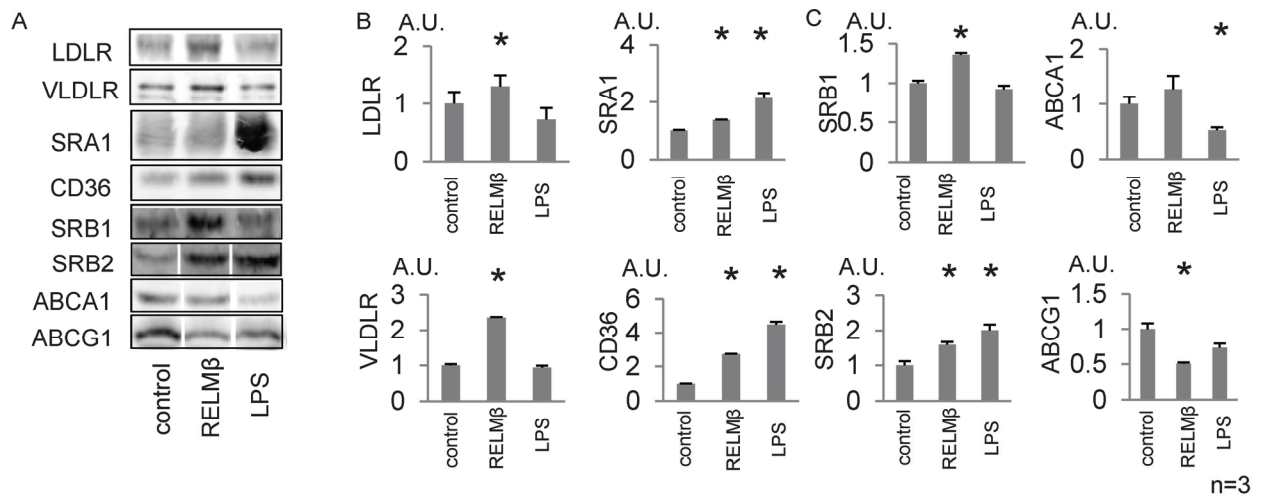


Supplementary Fig. IV.

RELMβ promoted atherosclerosis development in ApoE<sup>-/-</sup> mice.

A. RELMβ expression in the colons of RELMβ<sup>+/+</sup> mice under specific pathogen free conditions and RELMβ<sup>+/+</sup>, RELMβ<sup>+/-</sup> and RELMβ<sup>-/-</sup> mice under conventional circumstances. B. RELMβ expression in PCPMs of RELMβ<sup>+/+</sup> treated with control rabbit serum, and PCPMs of RELMβ<sup>+/+</sup>, RELMβ<sup>+/-</sup> and RELMβ<sup>-/-</sup> mice treated with WHHL serum. Bands in the upper panel from western blotting represent RELMβ expression, the lower panel β-actin expression. C. RELMβ expression by macrophages in the aortic root. Aortic roots from RELMβ<sup>+/+</sup> ApoE<sup>-/-</sup> mice were routinely embedded in OTC compound. Five-μm-thick slices of the aortic root were processed for immunohistochemical staining using anti-mRELMβ. Hematoxylin staining was used to detect the nuclei. D. RELMβ absence in RELMβ<sup>-/-</sup> ApoE<sup>-/-</sup> mouse aortic root. Scales in panels C and D indicate 50μm. E. Serum investigation of lipid profiles. Bars indicate means±SD.

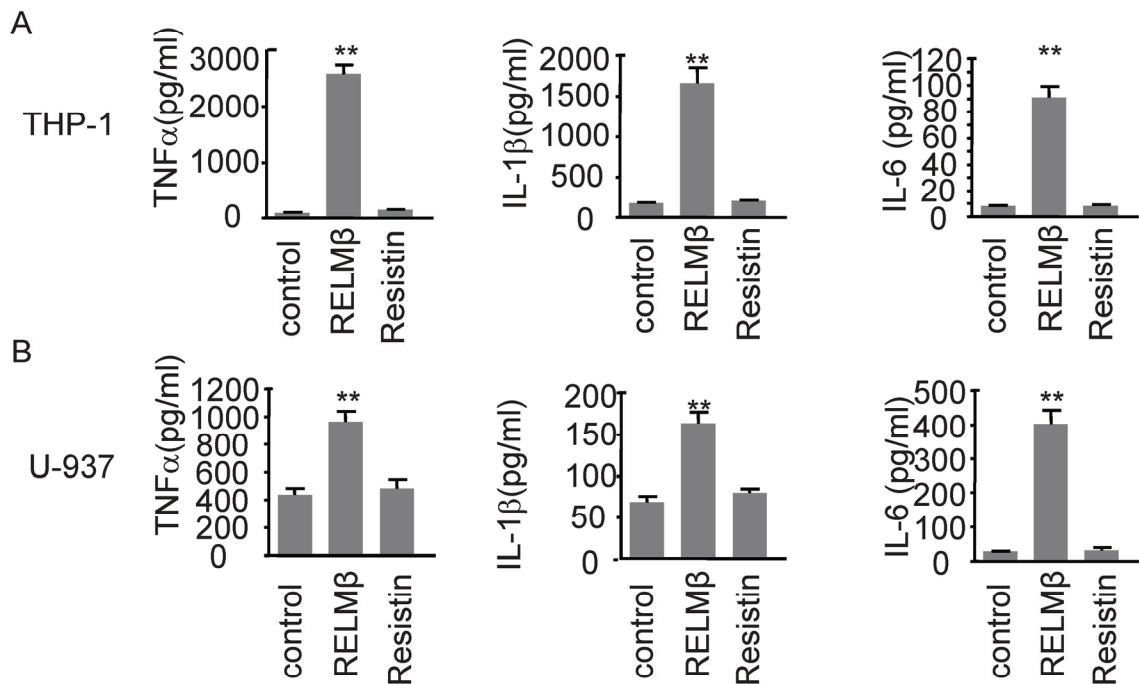
Supplementary Fig. V



Supplementary Fig. V.

Stimulation with mRELM $\beta$  enhances macrophage foam cell formation with effects on expressions of lipoprotein receptors and lipid transporters. J774.1 cells were incubated with or without 100ng/ml mRELM $\beta$  or 10ng/ml LPS for 16 hours. Thirty micrograms of cellular protein were immunoblotted. A. Representative bands are presented. B. Quantification of triplicate test results is presented. Note that effects of mRELM $\beta$  administration are almost the opposite of those obtained in the absence of RELM $\beta$  as shown in Fig. 5E. Bars indicate means $\pm$ SEM. \*Statistical significance at p<0.05

Supplementary Fig. VI



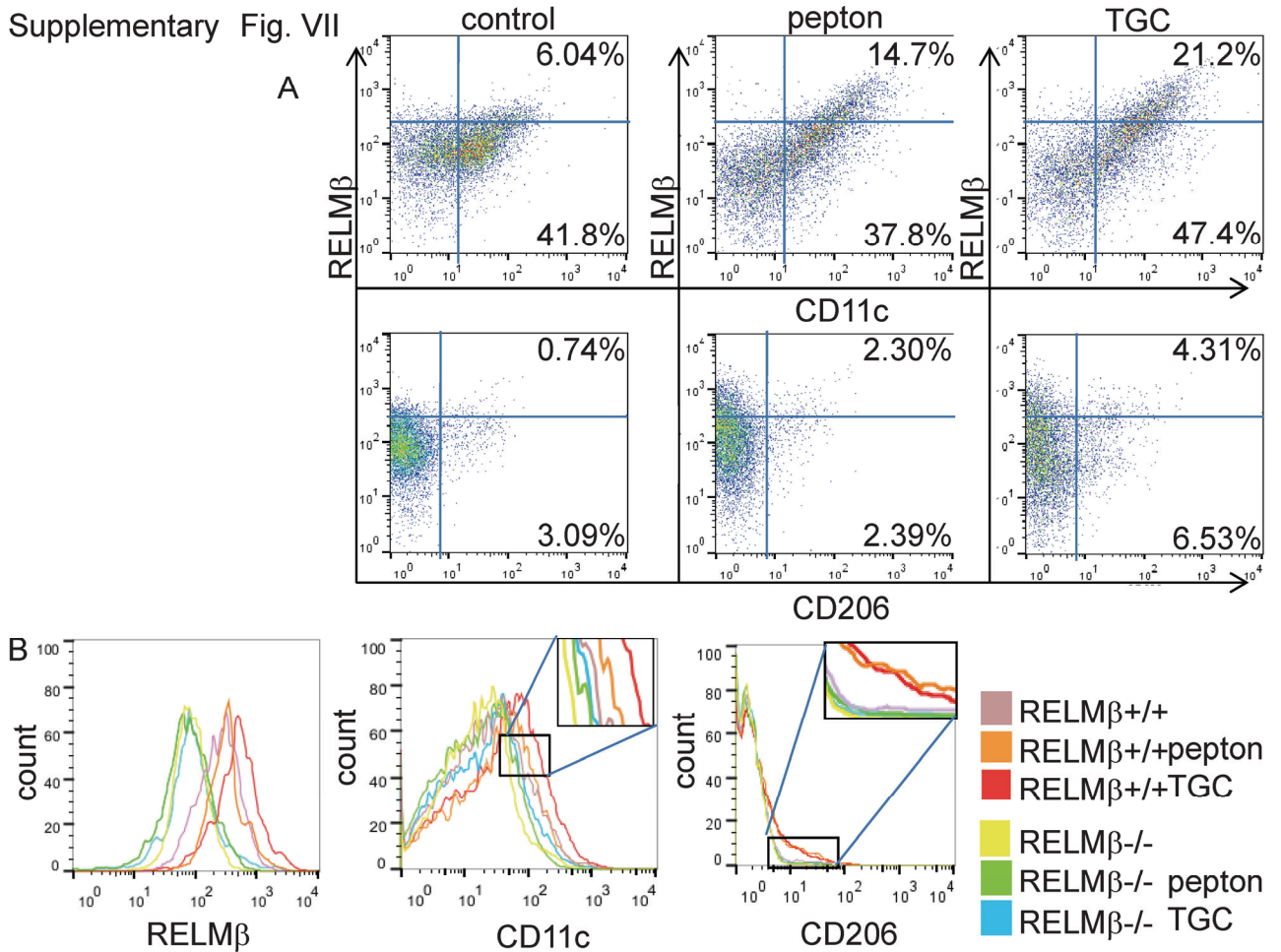
n=3

Supplementary Fig. VI. RELM $\beta$  and LPS but not resistin upregulated inflammatory cytokine secretions from macrophages

A. The concentrations of TNF $\alpha$ , IL-1 $\beta$  and IL-6 in the culture media from THP-1 cells treated with or without 3 $\mu$ g/ml hRELM $\beta$  or 3 $\mu$ g/ml human resistin for 24 hours were measured using Bioplex. B. The media from the U-937 cell cultures treated with or without RELM $\beta$  or resistin for 4 hours were collected and the concentrations of TNF $\alpha$ , IL-1 $\beta$  and IL-6 were determined using Bioplex. (\*\*P<0.01). Bars indicate means $\pm$ SD. \*\*Statistical significance at p<0.01.

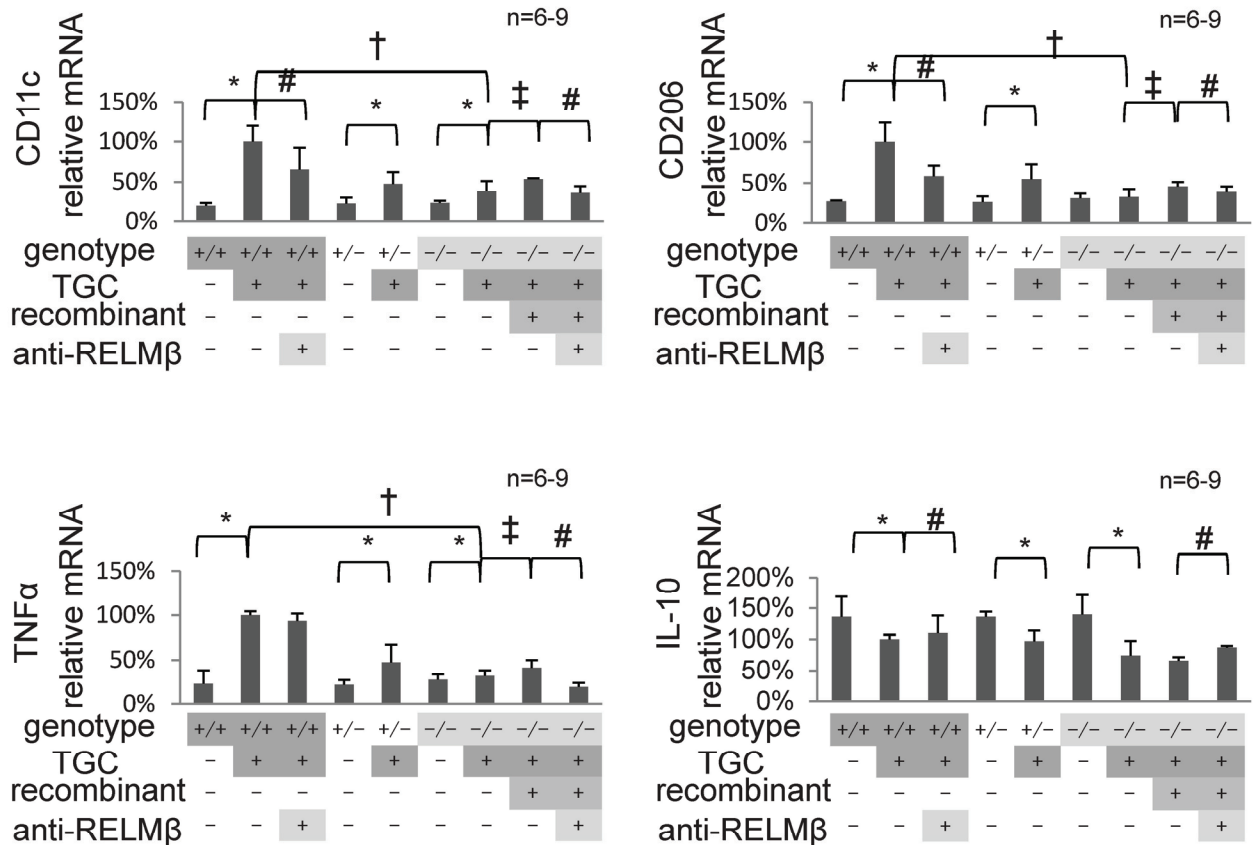


Supplementary Fig. VII



Supplementary Fig. VII. Peritoneal macrophages from RELM $\beta$  <sup>+/+</sup> and <sup>-/-</sup> mice were collected by RPMI1640 medium supplementation or not with 10% proteose pepton (pepton) or 3% thioglycolate (TGC). Cells were stained for F4/80, CD11c, CD206 and RELM $\beta$ , and then analyzed by flowcytometry. F4/80 (+) CD11c (+) cells were mostly RELM $\beta$  (+), and a small but significant population of F4/80 (+) CD206 (+) cells was also RELM $\beta$  (+). Both populations of RELM $\beta$  positive cells were inducible when stimulated by pepton or TGC to induce aseptic inflammation in the peritoneum. Absence of RELM $\beta$  significantly reduced pepton/TGC induction of CD11c (+) cells and also blunted that of CD206 (+) cells.

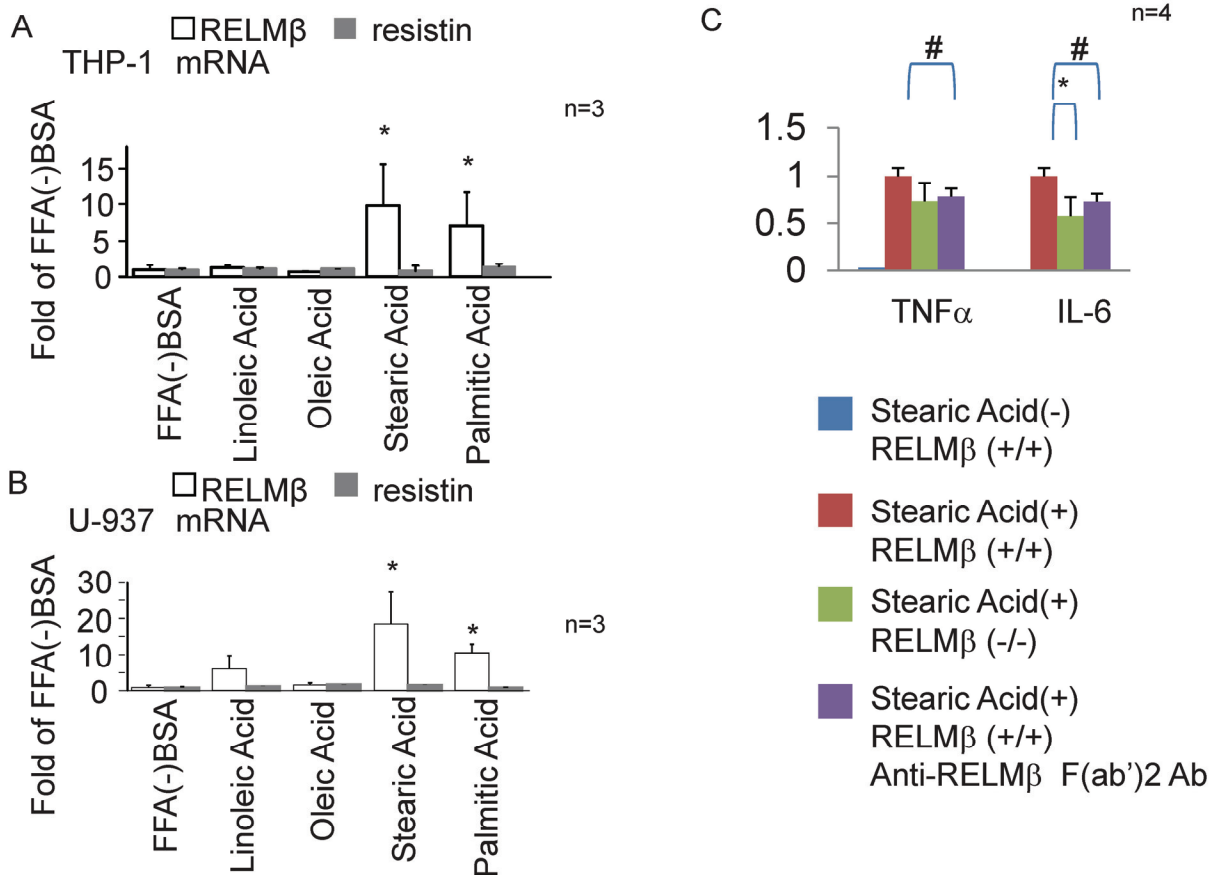
Supplementary Fig. VIII



Supplementary Fig. VIII. Differences in the expressions of M1 and M2 macrophage markers among RELMβ genotypes (+/+, +/-, or -/-), extracellular administration of mRELMβ and extracellular administration of mRELMβ neutralizing antibody

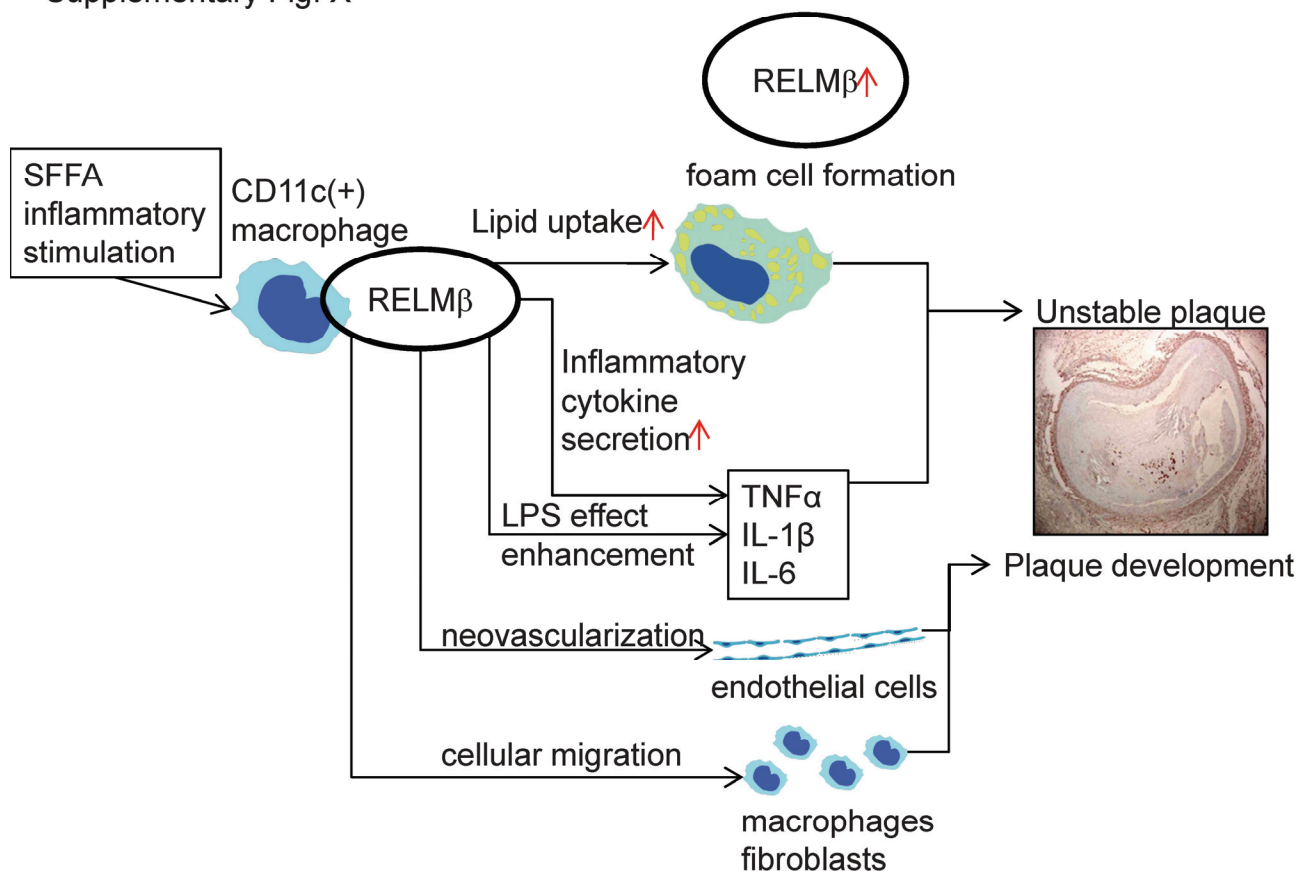
PCPMs were collected 2 days after phosphate-buffered saline injection, supplemented or not with 3% TGC. Then, the cells were incubated with 100ng/ml LPS for 4 hours. Relative mRNA expression to that of β-actin was expressed as 100% for RELMβ +/+ and TGC(+). Bars represent means±SD. n=6-9. \*statistically significant (p<0.05) for TGC induction of mRNA expression. †statistically significant (p<0.05) for RELMβ absence. ‡statistically significant for mRELMβ administration. #statistically significant (p<0.05) for mRELMβ neutralization by anti-RELMβ F(ab')<sub>2</sub> antibody.

Supplementary Fig. IX



Supplementary Fig. IX. RELMβ expression is increased by stimulation with fatty acids, and free fatty acid (FFA)-induced inflammatory cytokine expressions were enhanced by extracellular RELMβ. A. THP-1 cells were incubated with 0.2 mM of the indicated fatty acid for 16 hours. The mRNA amounts of RELMβ, resistin and β-actin cDNA were quantified by qPCR. B. U-937 cells were incubated with 0.2mM of the indicated fatty acid for 16 hours. The same processes as those in Figure 3A were performed. \*statistically significant (p<0.05) compared with control. C. PCPMs were incubated with 0.2mM stearic acid for 16 hours and TNFα, IL-6, and β-actin mRNA expressions were quantified by qPCR. Fold changes in the expressions of TNFβ and IL-6 mRNA by PCPMs, to which FFA had been added, from RELMβ +/+ mice were determined in triplicate. PCPMs, with RELMβ +/+, -/- and -/- phenotypes, were co-incubated with 2 μg/ml mRELMβ, or -/- cells were co-incubated with both 2 μg/ml of recombinant mRELMβ and 50μg/ml of anti-mRELMβ F(ab')2 antibody. The results are presented as means±SE, from three independent experiments. \*statistically significant (p<0.05) for stearic acid (+) RELMβ -/- compared to stearic acid (+) RELMβ +/+. #statistically significant (p<0.05) for stearic acid (+) RELMβ -/- compared to stearic acid (+) anti-RELMβ F(ab')2 antibody.

Supplementary Fig. X



Supplementary Fig. X

Scheme illustrating the involvement of RELM $\beta$  in the development of atherosclerosis

- 1) SFA or inflammatory signals stimulate RELM $\beta$  expression in macrophages.
- 2) RELM $\beta$  expressing macrophages increase lipid uptake, develop into foam cells, inflammatory cytokine secretion increases, enhancing LPS effects. RELM $\beta$  induces neovascularization of endothelial cells and cellular migration of invading cells.
- 3) These RELM $\beta$  mediated effects lead to atherosclerotic plaque development and induce plaque instability.



## METHODS and MATERIALS

### *Reagents*

Recombinant m- and h-RELM $\beta$  proteins were purchased from Peprtech (NJ, USA). Lipopolysaccharide (LPS) from *Escherichia coli* 0111:B4 was purchased from Sigma-Aldrich Japan. Serum of 3-month-old Watanabe heritable hyperlipidemic (WHHL) rabbits was purchased from Oriental Yeast (Tokyo, Japan). Human oxLDL (Biomedical Technologies, MA, USA, #BT-910), human HDL (Calbiochem, CA, USA, #437641), VLDL (Sigma-Aldrich, MO, USA, #L7527), human acetylated (ac) LDL (Kalen Biomedical, MD, USA, #770201), and Dil conjugated AcLDL (Biomedical Technologies, #BT-902) were also purchased. (1,2,<sup>3</sup>H)-cholesterol was purchased from PerkinElmer (NJ, USA, NET139250UC(9.25 MBq)). All reagents were of analytical grade.

### *Cell culture*

Primary cultured peritoneal macrophages (PCPMs) from mice were collected 4 days after peritoneal 10% proteose pepton (pepton) or 3% thioglycolate (TGC) injection, as described previously <sup>1</sup>, purified using MSP-P (JIMRO, Gunma, Japan), and cultured in RPMI1640 supplemented with 10% fetal calf serum. J774.1, murine monocyte/macrophage cell lines, and THP-1 and U-937, human monocyte/macrophage cell lines, J774.1, THP-1 and U-937, were cultured in RPMI 1640 (Sigma-Aldrich) medium supplemented with 10% fetal calf serum (Invitrogen, CA, USA). All media contained Penicillin 100U/ml and Streptomycin 100 $\mu$ g/ml (Invitrogen) and all cells were cultured at 37°C in 5% CO<sub>2</sub>.

### *Antibody List*

The affinity-purified antibodies against murine RELM $\beta$  (mRELM $\beta$ ) were prepared as previously described <sup>2</sup>. Anti-mRELM $\beta$  F(ab')<sub>2</sub> antibody was prepared using a F(ab')<sub>2</sub> preparation kit (Thermo Scientific, IL, USA) to block the effects of extracellular RELM $\beta$ . The aim of using the F(ab')<sub>2</sub> antibody is to prevent macrophage activation via the Fc receptor portion. The monoclonal antibodies against human RELM $\beta$  (3A6 and 1C1) were prepared using amino acids 14-66 of RELM $\beta$ .

Two of the isolated monoclonal antibodies, 1C1-A and 3A-6, were revealed to be applicable to immunoblotting and immunostaining, and in this study, mainly 3A-6 was used. Primary antibodies for western blotting and immuno-fluorescent staining were purchased, as follows; low density lipoprotein receptor (LDLR) (Cayman Chemical, MI, USA, #1007665), very low density lipoprotein (VLDL) R, scavenger receptor (SR)-A1 (R&D Systems, MN, USA, #AF2258, 1797), CD36 (Lifespan Bioscience, WA, USA, #LS-B662/10019), SR-B1, -B2, ATP-binding cassette (ABC)G1 (Novus Biologicals, CO, USA, #NB400-101, 102, 132), ABCA1 (Thermo Scientific, MA, USA, #PA1-16789), anti-macrophage (Abcam #56297, Cambridge, UK). The NF $\kappa$ B Pathway Sampler Kit was purchased from Cell Signaling

Technology (MA, USA, #9936). Normal rabbit IgG for generating the control F(ab')<sub>2</sub> antibody was purchased from MBL (Nagoya, Japan #PM035).

For flowcytometry, rat anti-mouse CD16/32 antibody (Ab) (BD Pharmingen, NJ, USA, #553142), anti-CD11c antibody-PE conjugated Ab (Abcam #ab86901), Alexa Fluor 488 anti-mouse CD206 Ab (Biolegend, CA, USA, #BL141710), anti-F4/80-PE/Cy5 conjugated Ab (eBioscience, CA, USA, #154801), and anti-rabbit Ab-PE/Cy7 conjugated Ab (Santa Cruz, #SC-3845) were used.

### *Human tissue samples*

Tissue samples of the coronary artery and colon were obtained at autopsy from 6 men (age range, 74-88 years) and 4 women (age range, 72-92 years) diagnosed with acute myocardial infarction. These patients had been treated at the Hiroshima University Hospital or an affiliated hospital. Written informed consent was obtained from an authorized representative. For strict privacy protection, identifying information for all samples was removed before analysis. This procedure was in accordance with the Ethical Guidelines for Human Genome/Gene Research of the Japanese Government.

Routinely embedded paraffin sections were incubated either with a mouse monoclonal anti-hRELM $\beta$  antibody (1C1-A or 3A-6) and/or anti-CD68 antibody (a macrophage marker, Dako Cytomation) and then visualized employing the Dako Envision+ Mouse Peroxidase Detection System (Dako Cytomation, Carpinteria, CA, USA), or with 3A-6 and CD86 antibody pre-labeled with Alexafluor 546 and 488, respectively.

### *Immunohistochemical analysis for human RELM $\beta$*

For immunohistochemical analysis, we used archival formalin-fixed, paraffin-embedded tissues. Immunohistochemical analysis was performed with a Dako Envision+ Mouse Peroxidase Detection System (Dako Cytomation, Carpinteria, CA, USA). Antigen retrieval was performed by microwave heating in citrate buffer (pH 6.0) for 30 minutes. Peroxidase activity was blocked with 3% H<sub>2</sub>O<sub>2</sub>-methanol for 10 minutes, and sections were incubated with normal goat serum (Dako Cytomation) for 20 minutes to block nonspecific antibody binding sites. Sections were incubated with a mouse monoclonal anti-hRELM $\beta$  antibody (1C1-A or 3A-6) or anti-CD68 antibody (a macrophage marker, Dako Cytomation) for 1 hour at room temperature, followed by incubation with Envision+ anti-mouse peroxidase for 1 hour. For color reaction, sections were incubated with the DAB Substrate-Chromogen Solution (Dako Cytomation) for 10 minutes. Sections were counterstained with 0.1% hematoxylin. Negative controls were created by omission of the primary antibody.

For double-immunofluorescence staining, the anti-hRELM $\beta$  antibody (3A-6) and anti-CD68 antibody were pre-labeled with Alexafluor 546 and 488, respectively, using the

Zenon Labeling Kit (Invitrogen), and confocal imaging was performed using a Zeiss confocal microscope (Carl Zeiss, Thornwood, NY, USA).

### *Animals*

This study was approved by the Ethics Committee of the Institute for Adult Diseases, Asahi Life Foundation. All animal experiments were conducted in accordance with the Guidelines for the Care and Use of Laboratory Animals of the same institution. ApoE  $-/-$  mice (B6.129P2-ApoE<sup>tm1Unc/J</sup>) and LDLR $-/-$  mice were purchased from Charles River Co (MA, USA). Mice were maintained under conventional conditions, unless otherwise indicated, and a 12-hour light/dark cycle with free access to food and water. High cholesterol diet (20.1% fat, 1.25% cholesterol) was purchased from Research Diet Inc. (NJ, USA, #D12801C) and fed to LDLR  $-/-$  mice for 8 weeks. The water was replaced every 2 days. Food was withdrawn 12 hours before the experiment.

### *Generation of RELM $\beta$ $-/-$ mice*

The RELM $\beta$  mutant mice were generated (**Supplementary Fig. III**) in collaboration with Lexicon Pharmaceuticals, Inc. (The Woodlands, TX, USA).

The RELM $\beta$  targeting vector was derived using the Lambda KOS system<sup>3</sup>. The Lambda KOS phage library, arrayed into 96 super-pools, was screened by PCR using exon 2-specific primers for RELM $\beta$ -2 (Supplementary Table).

The PCR-positive phage super-pools were plated and screened by filter hybridization using the 347 bp amplicon derived from primers RELM $\beta$ -2 as a probe. Two pKOS genomic clones, pKOS-39 and pKOS-59, were isolated from the library screen and confirmed by sequence and restriction analyses. Gene-specific arms using RELM $\beta$ -3 primers (The primer list is in the Supplementary Table) were appended by PCR to a yeast selection cassette containing the URA3 marker.

The yeast selection cassette and pKOS-39 were co-transformed into yeast, and clones that had undergone homologous recombination to replace a 1713 bp region containing exons 2-4 with the yeast selection cassette were isolated. The yeast cassette was subsequently replaced with a LacZ/Neo selection cassette to complete the RELM $\beta$  targeting vector.

The Not I linearized targeting vector was electroporated into 129/SvEv<sup>Brd</sup> (Lex-1) ES cells. G418/FIAU resistant ES cell clones were isolated, and correctly targeted clones were identified and confirmed by Southern analysis using a 493 bp 5' external probe (26/21), generated by PCR using primers RELM $\beta$ -4, and a 479 bp 3' internal probe (22/24), amplified by PCR using primers RELM $\beta$  -22 and -24.

Southern analysis using probe 26/21 detected a 7.8 Kb wild type band and a 3.4 Kb mutant band in *Apa*L1 digested genomic DNA while probe 22/24 detected a 17.2 Kb wild type band and a 9.0 Kb mutant band in *Xho*I digested genomic DNA. One targeted ES cell clone was identified and microinjected into C57BL/6 (albino) blastocysts to generate chimeric animals which were bred to C57BL/6 (albino) females, and the resulting heterozygous offspring were interbred to produce homozygous RELM $\beta$  deficient mice. Mice heterozygous for the deleted exon2 were bred to generate homozygous knockout mice.

The genetic background was then changed to C57BL/6 by a marker-assisted speed congenic strategy <sup>4</sup>. B6 background RELM $\beta$  *-/-* mice were crossed with ApoE *-/-* or LDLR *-/-* mice to generate RELM $\beta$  *-/-* mice with ApoE *-/-* or LDLR *-/-* background. *Generation of RELM $\beta$  *-/-* mice*

#### *Preparation of murine tissue samples*

At the indicated ages, the mice were euthanized. The heart and entire aorta were removed en bloc, then formalin-fixed. Samples were rinsed with phosphate-buffered saline (PBS) and aortic roots were routinely embedded in OCT compound (Sakura Finetek Japan, Tokyo, Japan). Five- $\mu$ m-thick slices of the aortic root were obtained sequentially. The aortic tree was incised longitudinally, and then rinsed with PBS.

#### *Histological analyses of mice*

The aortic root slices and opened aortic trees were subjected to oil red O staining, as previously described <sup>5</sup>. Hematoxylin staining was used to detect the nuclei. In addition, these samples were processed for immunofluorescence staining using anti-macrophage antibody (Abcam). Alexa-Fluor 488 anti-rat IgG was used as the secondary antibody. Digital images of lesions were obtained with a Nikon Eclipse Ti microscope. Areas of lipid accumulation were histomorphometrically analyzed using MultiGauge ver. 3.1 (FujiFilm, Tokyo, Japan), according to the manufacturer's instructions. The ratios of lesion area or number stained by oil red O, to the whole aortic wall area, were calculated as previously described <sup>6</sup>.

#### *Serum investigation*

Serum triglyceride, cholesterol and free fatty acids were assayed with Triglyceride E test Wako, Cholesterol E test Wako, and NEFA C test Wako (Wako Chemicals, Osaka, Japan), respectively, according to the manufacturer's instructions.

#### *Quantification of lipid accumulation in macrophages*

PCPMs were cultured on 96-well plates for lipid accumulation assays as previously described <sup>7</sup>. After incubation with 50mg/ml oxLDL, 50mg/ml VLDL, or 1% Watanabe heritable



hyperlipidemic (WHHL) rabbit serum for 48h, the cells were stained with AdipoRed™ assay reagent (Nile Red) with DAPI counter-staining for nuclei, and then subjected to lipid accumulation quantification using an ARVO MX fluorimeter (Perkin Elmer, MA, USA), according to the manufacturer's instructions. Lipid accumulation was adjusted for cell counts.

#### *1,1-dioctadecyl-3,3,3,3-tetramethylindocarbocyanine (Dil)-acLDL uptake assay*

The Dil-acLDL assay was performed as previously described with slight modification<sup>8</sup>. PCPMs were incubated with 10 mg/mL Dil-acLDL and 1 µg/ml DAPI for 4 hours at 37°C. After the cells were counted, they were washed, Dil was then extracted with isopropyl alcohol, and relative fluorescence intensity was determined at 524 nm (excitation) and at 567 nm (emission). Experiments were performed 3 times, and representative values are shown as means±SEM.

#### *Cholesterol efflux measurements*

Cholesterol efflux was measured as previously described<sup>8</sup>. PCPMs were incubated with 100 µg/mL acLDL and 1.2 µCi/mL (1,2-<sup>3</sup>H)-cholesterol in RPMI1640 medium containing 0.2% bovine serum albumin for 24 hours at 37°C. Cells were then incubated with RPMI1640 medium containing 0.2% bovine serum albumin supplemented or not with 50 µg/mL of high-density lipoprotein (HDL). After a further 48-hour incubation, medium and cell lysates were collected and radioactivity was determined by liquid scintillation counting. Cholesterol efflux was expressed as the percentage of radioactivity released into the medium relative to total radioactivity.

#### *Quantification of mRNA expression*

The mRNA was prepared from cells using the RNeasy-mini kit (QIAGEN, Hilden, Germany) and quantitative real-time PCR was run with a LightCycler480 (Roche) using FastStart SYBR Green Master (Roche) and the primers shown in the **Supplementary Table**.

#### *Western blotting*

Western blot analysis was carried out as described previously<sup>9</sup>. Band images were obtained by LAS-4000 (FujiFilm) and quantified by MultiGauge ver. 3.1. Fold changes in protein expressions as compared with β-actin were determined in triplicate. Cells were lysed with lysis buffer: 10mM Tris-HCl pH 7.4, 150mM NaCl, 2mM EDTA, 1% Triton-X, 10mM NaF, 10mM Na<sub>4</sub>P<sub>2</sub>O<sub>7</sub>, 1mM o-Vanadate, 1x Complete Protease Inhibitor Cocktail (Roche). Supernatants from cell lysates were boiled in Laemmli sample buffer containing 100 mmol/l dithiothreitol. Then, 10µg of protein were separated by SDS-PAGE and electrophoretically transferred to membranes, which were then incubated with a specific antibody. The

antigen-antibody interactions were visualized using horseradish peroxidase-conjugated secondary antibody and SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific). Protein expressions as compared with  $\beta$ -actin were determined in triplicate.

#### *Flowcytometry analysis of peritoneal macrophages*

Flowcytometric analysis was performed as previously described<sup>10</sup>. The Fc receptor on peritoneal macrophages was blocked by incubation with anti-CD16/32 Ab for 10 min, and macrophages were incubated with fluorochrome conjugated primary antibodies (F4/80/CD11c/CD206) for 30 min at 4°C. For RELM $\beta$  detection, cells were permeabilized with methanol, and anti-RELM $\beta$  Ab incubation was followed by secondary anti-rabbit IgG Ab-PE/Cy7. Cells were subjected to FACSVantage SE (BD Biosciences). The data analysis was performed using FlowJo (Tree Star, OR, USA).

#### Quantification of cytokine secretions

Inflammatory cytokines secreted into the media were quantified using Bioplex (Bio-Rad, CA, USA) according to the manufacturer's instructions.

#### *Statistical analysis*

Statistical analyses were performed using Jmp 8.0.1 (SAS institute, NC, USA) and R version 2.13.1 (The R Foundation for Statistical Computing). Results are expressed as means  $\pm$  SE, SD, or median and quartiles, and statistical significance was assessed using unpaired Student's *t* tests and ANOVA followed by the Tukey HSD test, unless otherwise indicated.

## Supplementary Table

## Primer lists

	Forward	Reverse
CD11c	CTGGATAGCCTTTCTTCTGCTC	GCACACTGTGTCCGAACTC
TNF $\alpha$	GCCACCACGCTCTTCTGTCT	GTCTGGGCCATAGAACTGAT
Il-6	GATGCTACCAAACCTGGATATAATC	CTGGCACCACCTAGTTGGTTGTC
Il-1 $\beta$	TCGCTCAGGGTCACAAGAAA	CATCAGAGGGCAAGGAGGAAAAC
IL-10	GCTCTTACTGACTGGCATGAG	CGCAGCTCTAGGAGCATGTG
CD206	CAGGTGTGGGCTCAGGTAGT	TGTGGTGAGCTGAAAGGTGA
cyclophilinA	GTGGTCTTTGGGAAGGTGAA	TTACAGGACATTGCGAGCAG
RELM $\beta$ -2	GAGTGAATCTGCTCTTAGGG	CCTTGCCCCTTCTCTTACC
RELM $\beta$ -3	CGCGTTGTTTCCCTTTGTCTTCTAGCT CCAGGAGG	TTCTGACAACAGACACTCAAACC TAGGAGAAATAA
RELM $\beta$ -4	CCCTGCACAATATTTGTTTCC	AACAGACCAAAGATGAAGCC
RELM $\beta$ -22/24	CTGTTGGTCATCTCTGAAGC	GAAGTATGAAGTGGCTACCC
hRELM $\beta$	CACCCAGGAGCTCAGAGATCTAA	ACGGCCCCATCCTGTACA
h36B4	CCACGCTGCTGAACATGCT	TCGAACACCTGCTGGATGAC

## Reference

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