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Purinergic Receptor Y₆ Deficiency Limits Vascular Inflammation and Atherosclerosis in Mice

Peter Stachon,* Alexander Peikert,* Nathaly Anto Michel, Sonja Hergeth, Timoteo Marchini, Dennis Wolf, Bianca Dufner, Natalie Hoppe, Cemil Korcan Ayata, Melanie Grimm Sanja Cicko, Lisa Schulte, Jochen Reinöhl, Constantin von zur Muhlen, Christoph Bode, Marco Idzko,† Andreas Zirlik†

Objective—Nucleotides such as ATP, ADP, UTP, and UDP serve as proinflammatory danger signals via purinergic receptors on their release to the extracellular space by activated or dying cells. UDP binds to the purinergic receptor Y₆ (P2Y₆) and propagates vascular inflammation by inducing the expression of chemokines such as monocyte chemoattractant protein 1, interleukin-8, or its mouse homologs CCL1/keratinocyte chemokine, CXCL2/macrophage inflammatory protein 2, and CXCL5/LIX, and adhesion molecules such as vascular cell adhesion molecule 1 and intercellular cell adhesion molecule 1. Thus, P2Y₆ contributes to leukocyte recruitment and inflammation in conditions such as allergic asthma or sepsis. Because atherosclerosis is a chronic inflammatory disease driven by leukocyte recruitment to the vessel wall, we hypothesized a role of P2Y₆ in atherogenesis.

Approach and Results—Intraperitoneal stimulation of wild-type mice with UDP induced rolling and adhesion of leukocytes to the vessel wall as assessed by intravital microscopy. This effect was not present in P2Y₆-deficient mice. Atherosclerotic aortas of low-density lipoprotein receptor-deficient mice consuming high-cholesterol diet for 16 weeks expressed significantly more transcripts and protein of P2Y₆ than respective controls. Finally, P2Y₆^{-/-}/low-density lipoprotein receptor-deficient mice consuming high-cholesterol diet for 16 weeks developed significantly smaller atherosclerotic lesions compared with P2Y₆^{+/-}/low-density lipoprotein receptor-deficient mice. Bone marrow transplantation identified a crucial role of P2Y₆ on vascular resident cells, most likely endothelial cells, on leukocyte recruitment and atherogenesis. Atherosclerotic lesions of P2Y₆-deficient mice contained fewer macrophages and fewer lipids as determined by immunohistochemistry. Mechanistically, RNA expression of vascular cell adhesion molecule 1 and interleukin-6 was decreased in these lesions and P2Y₆-deficient macrophages took up less modified low-density lipoprotein cholesterol.

Conclusions—We show for the first time that P2Y₆ deficiency limits atherosclerosis and plaque inflammation in mice. (*Arterioscler Thromb Vasc Biol.* 2014;34:00-00.)

Key Words: atherosclerosis ■ inflammation ■ receptors, purinergic P2 ■ uridine diphosphate ■ vascular cell adhesion molecule-1

Atherosclerosis and its sequelae such as myocardial infarction, coronary heart disease, and stroke represent the leading cause of noncommunicable death worldwide.¹ Vast experimental research and clinical science led to the appreciation of atherosclerosis as a chronic inflammatory disease.²⁻⁶ However, despite the knowledge of its inflammatory nature, we still lack anti-inflammatory or immune-modulatory treatment options beyond statins.^{7,8}

Increasing evidences point toward an important role of extracellular nucleotides such as ATP, ADP, UTP, or UDP in cardiovascular physiology and pathophysiology, via activating

purinergic P2 receptors, which can be subdivided into 2 families: (1) the G-protein-coupled P2YR (P2Y1–14) and (2) the ligand-gated ion channels P2XR (P2X1–7) receptors.⁹⁻¹¹ Among the P2YR subtypes, the P2Y₆R is particularly expressed on stromal cells such as vascular endothelial cells (ECs) and epithelial cells and is almost selectively activated by UDP. However, functional expression has also been reported on various leukocytes such as human monocytes, dendritic cells, eosinophils, and neutrophils. Activation of P2Y₆R triggers chemokine/cytokine release (such as interleukin-8 [IL-8] or its mouse homologs CCL1/keratinocyte chemokine [KC], CXCL2/macrophage inflammatory

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Nonstandard Abbreviations and Acronyms

ECs	endothelial cells
IL-8	interleukin 8
LDL	low-density lipoprotein
LDLR	low-density lipoprotein receptor
MCP-1	monocyte chemoattractant protein 1
P2Y	purinergic receptors Y (G-protein coupled)
VCAM-1	vascular cell adhesion molecule 1

protein 2, and CXCL5/LIX, CCL20, vascular cell adhesion molecule 1 [VCAM-1]) from epithelial/ECs, monocytes, dendritic cells, eosinophils, and neutrophils, implicating a role of the P2Y₆R in promoting the recruitment of inflammatory cells to sites of inflammation or infection.^{12–15} Indeed, using a murine peritonitis model, Zhang et al¹⁶ demonstrated that P2Y₆ promotes host defense by augmenting the expression of monocyte chemoattractant protein 1 (MCP-1) and guiding monocytes to the location of infection. Besides the chemokine-driven recruitment, also a direct chemoattractant activity of the UDP–P2Y₆R axis has been described¹⁷ for leukocytes.

Thus, stimulation of the P2Y₆R has been implicated with chronic inflammatory diseases. Indeed, selective inhibition or knockdown of P2Y₆R reduced all cardinal features of experimental acute and chronic allergic asthma in mice.¹⁸ Furthermore, P2Y₆ contributes to inflammation of intestinal mucosa promoting IL-8–dependent neutrophil recruitment.¹⁹ Pyridoxal phosphate-6-azophenyl-2',4'-disulfonic acid, a synthetic substance blocking several purinergic receptors including P2Y₆, P2Y₄, P2Y₁₁, and purinergic receptors X (ion channels), reduced atherosclerosis in apolipoprotein E–deficient mice suggesting that purinergic signaling is also relevant during the inflammatory processes propagating atherogenesis.²⁰

In the light of these data, we hypothesized that P2Y₆ plays an important role in leukocyte recruitment to the vessel wall and that its deletion may limit experimental atherosclerosis.

Materials and Methods

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

Results

UDP Induces Leukocyte Rolling, Adhesion, and Transmigration via P2Y₆

P2Y₆ activation induces the expression of several chemokines and adhesion molecules.^{12–14,16,18,19} However, the role of UDP and P2Y₆ in leukocyte recruitment to the inflamed vessel wall in vivo has not been addressed to date. To examine the latter, P2Y₆-competent mice received peritoneal injections with 200 nmol UDP. Two hours later, the visceral peritoneum was prepared and leukocyte rolling and adhesion were analyzed by intravital microscopy. Stimulation with UDP significantly induced leukocyte rolling and adhesion compared with PBS-challenged control mice. Rolling further increased after 4 hours and decreased after 6 hours, indicating a fast regulation of adhesion molecules by UDP ($n \geq 11$; $P = 0.002$; $P = 0.001$). Conversely, UDP-induced leukocyte rolling and adhesion to the vessel wall were comparable between P2Y₆-deficient mice

after 2, 4, and 6 hours of UDP stimulation and wild-type controls without UDP stimulation (Figure 1A and 1B).

Leukocyte recruitment is mediated by the interaction between ECs and leukocytes. P2Y₆-competent and P2Y₆-deficient mice were either reconstituted with P2Y₆-competent or -deficient bone marrow. To test whether P2Y₆ on leukocytes or ECs is crucial for leukocyte recruitment, mice were stimulated with 200 nmol UDP and intravital microscopy was performed ($n = 6$ per group). As shown in Figure 1C, UDP-induced leukocyte adhesion was reduced in mice with P2Y₆-deficient ECs compared with those with P2Y₆-competent ECs. However, rolling was only slightly affected. Thus, P2Y₆ influences expression of adhesion molecules on ECs and endothelial P2Y₆ promotes leukocyte recruitment to the inflamed vessel wall. However, P2Y₆ deficiency on hematopoietic cells does not affect leukocyte recruitment.

To test for a role of UDP–P2Y₆ axis beyond adhesion in leukocyte migration, extracellular matrix (Matrigel) was mixed with UDP or vehicle and injected subcutaneously. After 3 days, Matrigel was removed and macrophage content was determined by F4/80 staining. UDP increased macrophage migration into Matrigel of P2Y₆-competent mice, whereas macrophage migration into Matrigel of P2Y₆-deficient mice was not altered by UDP (Figure 1D). These data demonstrate a crucial role of P2Y₆ in UDP-dependent leukocyte recruitment to the endothelium.

P2Y₆ Is Expressed in Atherosclerosis

Because leukocyte recruitment to the vessel is a crucial step in atherogenesis, we hypothesized that P2Y₆ is also involved in the development of atherosclerotic lesions. To examine the expression of P2Y₆ in murine atherosclerotic lesions, low-density lipoprotein receptor–deficient (LDLR^{-/-}) mice consumed high-cholesterol diet to induce atherosclerosis. LDLR^{-/-} mice consuming chow diet served as control. After 16 weeks of feeding, atherosclerotic aortic tissue expressed significantly more P2Y₆ than respective nondiseased aortic tissue ($n = 4$; $P = 0.05$; Figure 2A) as assessed by quantitative real-time polymerase chain reaction. Similarly, atherosclerotic lesions in the aortic root stained more intensely for P2Y₆ than respective sections of control animals ($n = 4$; $P = 0.009$; Figure 2B and 2C) suggesting a role for P2Y₆ in atherosclerosis. Distribution of P2Y₆ within the atherosclerotic lesion was assessed by 3-color immunofluorescence for cell nuclei (DAPI), ECs (CD31), and P2Y₆. As shown in Figure 2D and 2E, P2Y₆-positive staining mainly colocalized with CD31-positive ECs.

P2Y₆ Deficiency Reduces Atherosclerosis

To test for a functional role of P2Y₆ in atherogenesis, P2Y₆^{-/-} mice were crossed with LDLR^{-/-} mice and consumed high-cholesterol diet for 16 weeks. P2Y₆^{+/-}/LDLR^{-/-} littermate mice on a C57/BL6J background served as control. Subsequently, intimal lesion size was determined in sections of the aortic root and aortic arch. P2Y₆^{-/-}/LDLR^{-/-} (aortic root: 0.33 ± 0.03 mm², $n = 17$; aortic arch: 0.13 ± 0.03 mm², $n = 9$) mice developed significantly smaller atherosclerotic lesions than P2Y₆^{+/-}/LDLR^{-/-} animals (aortic root: 0.51 ± 0.04 mm², $n = 14$, $P = 0.003$; aortic arch: 0.26 ± 0.03 mm², $n = 9$, $P = 0.01$) in both locations. Additionally, P2Y₆-deficient mice accumulated less lipids in the abdominal aorta as assessed by en face analysis after oil-red-O staining (Figure 3A–3C). To

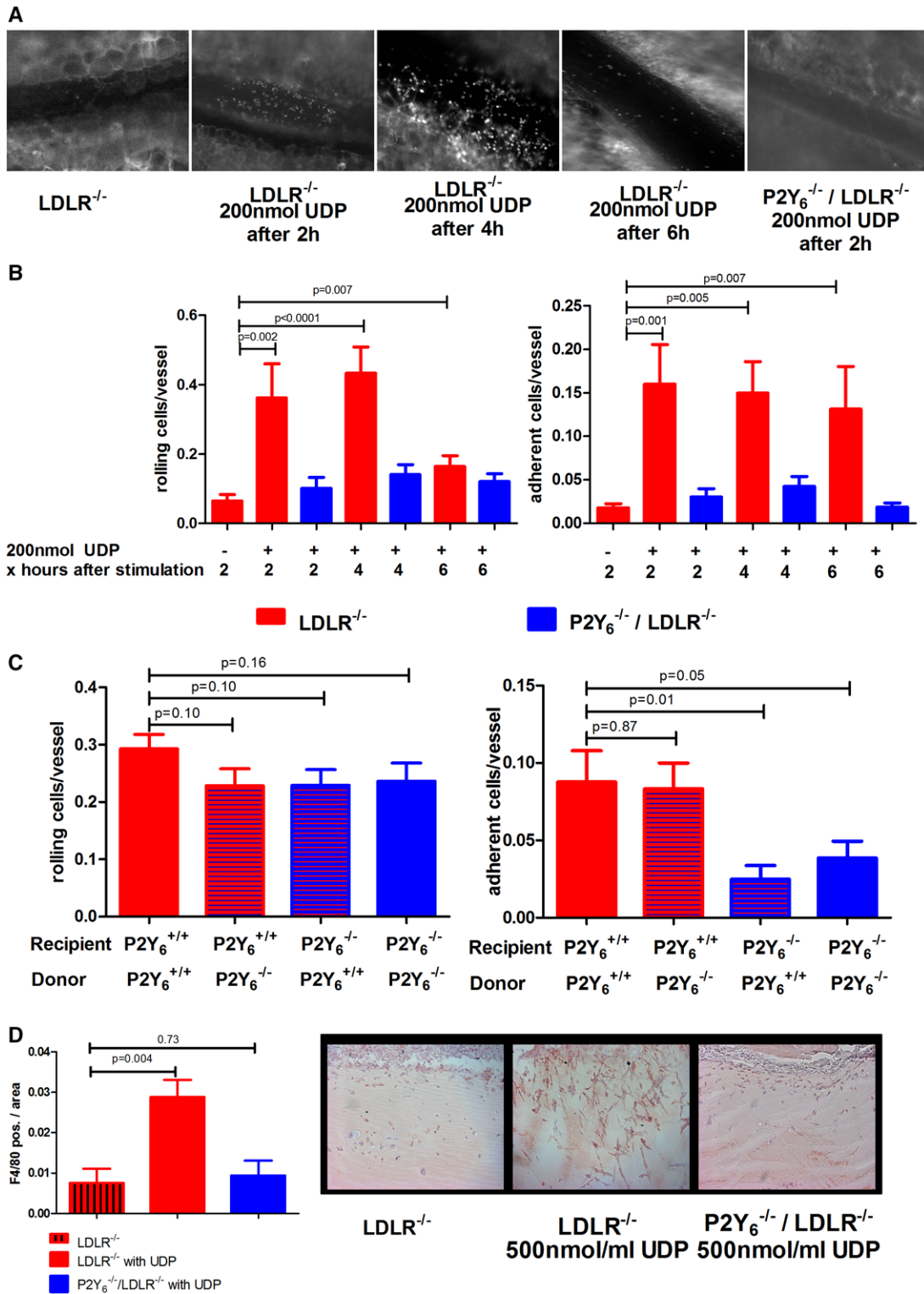


Figure 1. UDP increases leukocyte recruitment. P2Y₆^{+/+}/low-density lipoprotein receptor-deficient (LDLR^{-/-}) mice were stimulated with vehicle or 200 nmol UDP and P2Y₆^{-/-}/LDLR^{-/-} mice with 200 nmol UDP intraperitoneally. After 2, 4, and 6 hours, leukocyte rolling and adhesion were quantified in peritoneal vessels by intravital microscopy (n=15 for vehicle in P2Y₆^{+/+}/LDLR^{-/-}, n=11 for UDP in P2Y₆^{+/+}/LDLR^{-/-}, n=15 for UDP in for P2Y₆^{-/-}/LDLR^{-/-}). Representative images are shown (A); rolling and adhesion (B) were quantified. P2Y₆^{+/+}/LDLR^{+/+} mice were irradiated and reconstituted with either P2Y₆^{+/+}/LDLR^{+/+} or P2Y₆^{-/-}/LDLR^{-/-} bone marrow. Furthermore, P2Y₆^{-/-}/LDLR^{+/+} were reconstituted with mice P2Y₆^{+/+}/LDLR^{+/+} or P2Y₆^{-/-}/LDLR^{-/-} bone marrow. Two hours after UDP stimulation, intravital microscopy was performed and rolling and adhesion were quantified. C, Matrigel either mixed with UDP or vehicle was injected subcutaneously in LDLR^{-/-} or P2Y₆^{-/-} mice. After 3 days, amount of migrated F4/80-positive cells was determined by immunohistochemistry. D, Quantification of migration (left) and representative images (right). Data are presented as pooled mean±SEM.

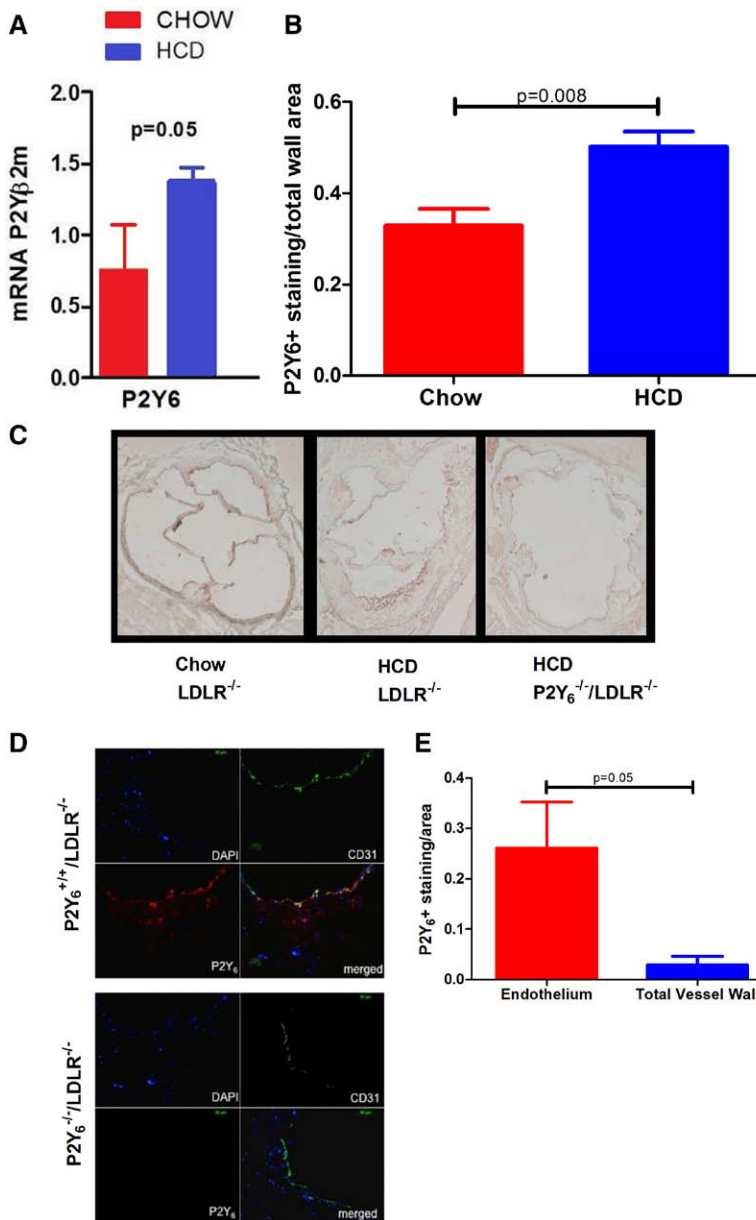


Figure 2. Purinergic receptor Y₆ (P2Y₆) expression is increased in murine atherosclerotic lesions. Low-density lipoprotein receptor-deficient (LDLR^{-/-}) mice were fed a chow diet (n=4) or high-cholesterol diet (HCD; n=6) for 16 weeks. RNA from aortic arches was isolated, and P2Y₆ expression was determined by quantitative polymerase chain reaction (A). Aortic roots were stained with anti-P2Y₆ for immunohistochemistry (C; magnification, ×4) and quantified for positive staining (B). P2Y₆ distribution in atherosclerotic lesions from LDLR^{-/-} was quantified by 3-color immunohistochemistry. Nuclei were stained with DAPI (blue), endothelial for CD31 (green), and P2Y₆ (red). Atherosclerotic lesions from P2Y₆^{-/-}/LDLR^{-/-} mice served as controls. Magnification, ×40 (D). E, Quantification of P2Y₆ distribution within the atherosclerotic lesion is shown. Data are presented as pooled mean±SEM.

investigate the contribution of P2Y₆ on vascular resident such as ECs or hematopoietic cells to atherogenesis P2Y₆-competent and -deficient LDLR^{-/-} mice, mice were reconstituted with P2Y₆^{+/+}/LDLR^{-/-} or P2Y₆^{-/-}/LDLR^{-/-} bone marrow (n=6 per group). Mice with deficiency of P2Y₆ on vascular resident cells, most likely ECs, developed smaller atherosclerotic lesions compared with mice with P2Y₆-competent vascular resident cells (Figure 3D). Leukocyte count, differential blood count, total cholesterol, and triglycerides did not differ between both study groups after diet (Table). No phenotypic differences regarding life span, reproduction, behavior, or health occurred between the study groups.

P2Y₆ Deficiency Limits Plaque Inflammation

Because certain phenotypic features of an atherosclerotic plaque such as content of inflammatory cells and lipids coincide with plaque rupture in humans, we analyzed not only mere lesion size but also plaque composition. Parallel sections of atherosclerotic lesions were stained for lipids, macrophages,

smooth muscle cells, and collagen. Interestingly, plaques from P2Y₆^{-/-}/LDLR^{-/-} mice contained a significantly reduced amount of lipids ($P=0.005$) and macrophages ($P=0.03$) compared with plaques from P2Y₆^{+/+}/LDLR^{-/-} control animals. Smooth muscle cells and collagen stabilize atherosclerotic lesions by protecting the necrotic core from exposure to the blood stream and thus may help to prevent plaque rupture. Both smooth muscle cells and collagen content were increased in atherosclerotic lesions from P2Y₆-deficient mice ($P=0.04$ for smooth muscle cells; $P=0.02$ for collagen) compared with respective controls. Taken together, P2Y₆ animals develop plaques of smaller size that display features commonly associated with more favorable and stable atherosclerotic lesions in humans (Figure 4).

P2Y₆-Deficient Atherosclerotic Arteries Express Less VCAM-1 and IL-6

P2Y₆ promotes IL-6, MCP-1, KC, VCAM-1, and intercellular cell adhesion molecule 1 [ICAM-1] expression in vitro.^{13,18,19,21}

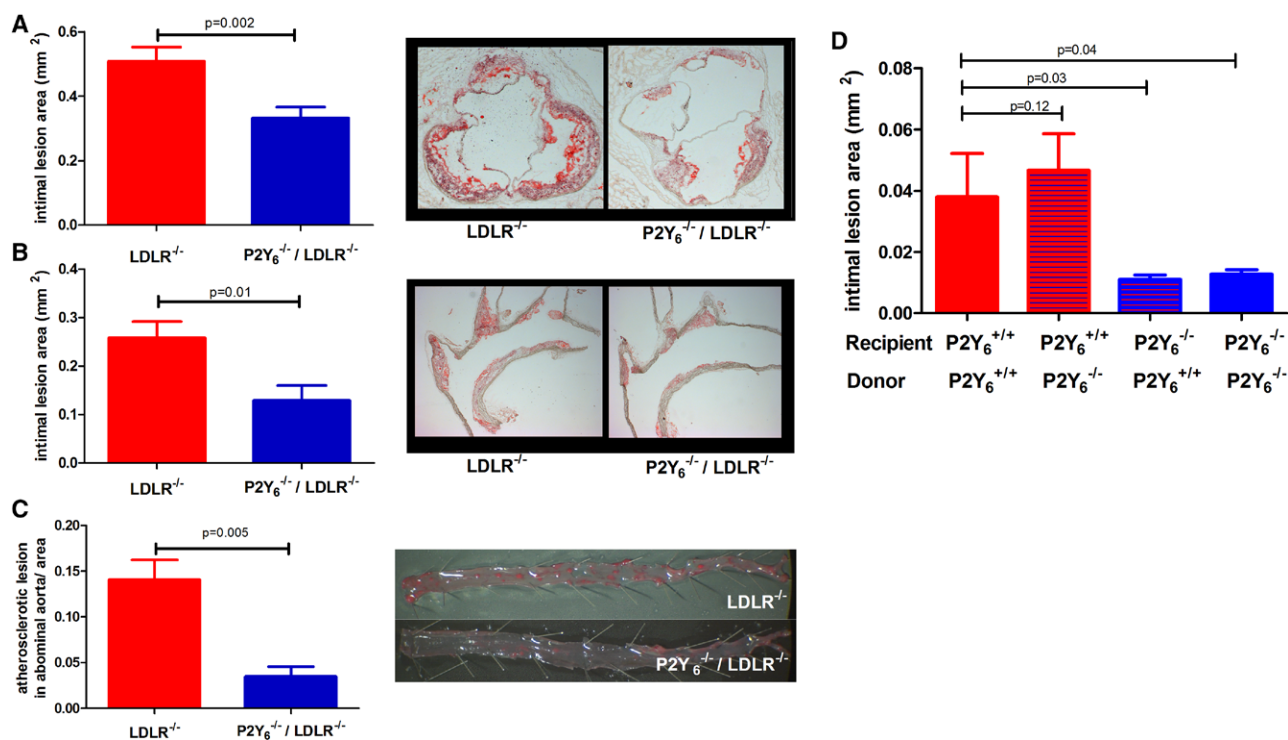


Figure 3. Purinergic receptor Y₆ (P2Y₆)-deficient mice develop decreased atherosclerosis. P2Y₆^{-/-}/low-density lipoprotein receptor-deficient (LDLR^{-/-}) (n=15) and P2Y₆^{+/+}/LDLR^{-/-} (n=15) mice consumed a high-cholesterol diet (HCD) for 16 weeks, and atherosclerotic lesion size was determined in aortic root (A) and aortic arch (B). Lipid distribution was analyzed with en face analysis of abdominal aortas after Oil-Red-O staining - (C). P2Y₆^{+/+}/LDLR^{-/-} and P2Y₆^{-/-}/LDLR^{-/-} mice were irradiated and reconstituted with P2Y₆^{+/+}/LDLR^{-/-} or P2Y₆^{-/-}/LDLR^{-/-} bone marrow. Atherosclerosis in aortic arch was analyzed after 8 weeks of HCD (D). Data are presented as pooled mean±SEM. Representative images of aortic root, aortic arch, and abdominal aortas stained for lipids are displayed aside.

To investigate the potential mechanisms of our findings that P2Y₆ deficiency limits atherogenesis, plaque inflammation, and inflammatory cell recruitment to the vessel wall, we evaluated the expression of these molecules in arterial tissue from P2Y₆^{+/+}/LDLR^{-/-} (n=5) and P2Y₆^{-/-}/LDLR^{-/-} (n=4) mice after 16 weeks of high-cholesterol diet by quantitative real-time polymerase chain reaction. Expression of IL-6 and VCAM-1 was significantly reduced in P2Y₆-deficient atherosclerotic lesions, whereas MCP-1 and ICAM-1 did not differ when compared with respective controls. KC expression tended to be decreased (Figure 5). Increased VCAM-1 expression was confirmed by immunohistochemistry. These data suggest VCAM-1 expression as potential mechanism limiting leukocyte recruitment to the vessel wall and attenuating atherogenesis in P2Y₆-deficient mice.

P2Y₆-Deficient Macrophages Take up Less Modified LDL Cholesterol

Modified LDL cholesterol uptake and foam cell formation are key processes in atherogenesis. Atherosclerotic lesions from P2Y₆-deficient mice contained significantly reduced amounts of lipids indicating a role of P2Y₆ in cellular cholesterol accumulation. Therefore, we isolated P2Y₆-competent and -deficient macrophages and incubated them with ALEXA488-marked acetylated LDL cholesterol. Acetylated LDL cholesterol uptake was quantified by fluorescence-activated cell sorting analysis. As shown in Figure 5, macrophages from P2Y₆-deficient showed reduced lipid accumulation and decreased foam cell formation compared with respective controls.

Discussion

The present study reports an atheroprotective effect of P2Y₆ deficiency in mice. We show that the UDP-P2Y₆ axis induces leukocyte recruitment to the vessel wall. Accordingly, fewer macrophages accumulated in atherosclerotic lesions of P2Y₆-deficient mice.

Leukocyte migration into nascent atherosclerotic lesions is a crucial and constant event throughout atherogenesis.^{2,22}

Table.

	LDLR ^{-/-}	P2Y ₆ ^{-/-} /LDLR ^{-/-}	P Value
Total leukocytes before diet, tsd/μL	7.6	9.7	0.01
Total leukocytes after diet, tsd/μL	2.8	3.3	0.15
Total T cells, tsd/μL	0.78	0.65	0.06
CD4 ⁺ cells/total T cells	0.47	0.43	0.59
CD8 ⁺ cells/total T cells	0.10	0.12	0.63
Total B cells, tsd/μL	1.1	1.0	0.38
Monocytes/leukocytes	0.04	0.05	0.12
Inflammatory monocytes/monocytes	0.56	0.64	0.14
Total platelets before diet, tsd/μL	337	327	0.64
Total platelets after diet, tsd/μL	158	161	0.85
Total cholesterol after diet	373	405	0.81

Before and after 16 weeks of high-cholesterol diet, blood was taken and total leukocytes and platelets were measured in thousand per microliter (tsd/μL). Furthermore, leukocyte subsets were determined by fluorescence-activated cell sorting analysis after diet. Plasma total cholesterol was measured after diet. LDLR^{-/-} indicates low-density lipoprotein receptor deficient.

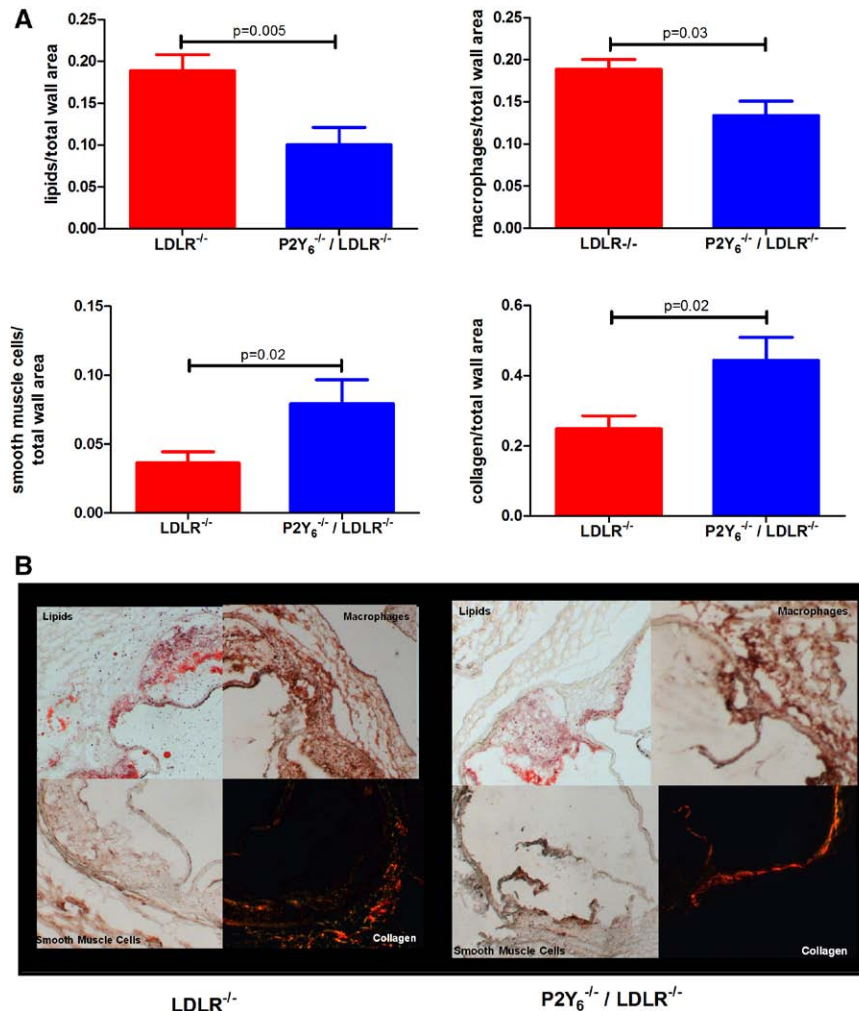


Figure 4. Purinergic receptor Y_6 ($P2Y_6$)-deficient murine atherosclerotic lesions show a stable plaque phenotype. After 16 weeks of high-cholesterol diet, aortic roots were stained with Oil-Red-O for lipids, with anti-MAC3 for macrophages, anti- α -actin for smooth muscle cells, and sirius red for collagen. Positive staining was quantified and referred to total wall area (A). Data are presented as pooled mean \pm SEM. Representative sections are shown in the merged image (B).

Previous studies identified extracellular nucleotides, such as ATP, ADP, UTP, and UDP, ligands of purinergic receptors, as inducers of chemokine and adhesion molecule expression.^{10,23,24} Activation of $P2Y_6$ attracts leukocytes to sites of inflammation by promoting MCP-1, IL-8 expression in humans, and their analogs MCP-1 and KC/macrophage inflammatory protein 2/LIX in mice.^{12,16,18,19} Furthermore, pharmacological inhibition of $P2Y_6$ reduced the expression of the adhesion molecules VCAM-1 and ICAM-1 on an EC line after tumor necrosis factor- α stimulation, implicating that $P2Y_6$ contributes to leukocyte adhesion.^{25,26} Administration of UDP aggravates colitis-like disease by inducing neutrophil recruitment to the bowel indicating an important role of UDP and $P2Y_6$ in inflammation.¹⁹ Accordingly, in bacterial peritonitis, UDP injection results in a more efficacious clearance of *Escherichia coli*, suggesting again a proinflammatory role of the UDP- $P2Y_6$ axis.¹⁶ In the present study, we provide evidence that UDP induces leukocyte rolling on and adhesion to inflamed vessels via $P2Y_6$ in vivo. Leukocyte adhesion in bone marrow chimera with $P2Y_6$ deficiency on resident cells was markedly reduced compared with mice with $P2Y_6$ -competent resident cells assessed by intravital microscopy. These data suggest that endothelial $P2Y_6$ accounts for leukocyte recruitment in vascular inflammation.

Because UDP and $P2Y_6$ signaling contribute to chronic inflammatory diseases such as allergic asthma, colitis, and gout

by leukocyte activation, we postulated a role of $P2Y_6$ in atherosclerosis.^{14,18,19} $P2Y_6$ is overexpressed in inflamed tissue such as lipopolysaccharide- and monosodium urate-mediated inflammation.²⁶ Atherosclerotic lesions of apolipoprotein E-deficient mice contain more $P2Y_6$ mRNA compared with wild-type mice.²⁰ Our data confirm overexpression of $P2Y_6$ in atherosclerotic lesions of LDLR^{-/-} mice consuming 16 weeks of high cholesterol both by quantitative polymerase chain reaction and immunohistochemistry. Furthermore, $P2Y_6$ mainly colocalized with the endothelial marker CD31. Guns et al²⁰ reported in a short-time atherosclerosis study that a 4-week treatment with pyridoxal phosphate-6-azophenyl-2',4'-disulfonic acid, a broad purinergic receptor antagonist blocking $P2Y_6$, $P2Y_4$, $P2Y_{11}$, and purinergic receptors X (ion channels), reduced atherosclerosis in apolipoprotein E-deficient mice. In the present study, we demonstrate that LDLR^{-/-} mice specifically deficient in $P2Y_6$ developed significantly smaller atherosclerotic lesions than respective controls. Atherosclerosis was markedly reduced in mice with $P2Y_6$ deficiency on vascular resident cells, most likely ECs. This is in line with the recent finding in murine lung inflammation that epithelial but not hematopoietic $P2Y_6$ accounts for leukocyte recruitment.¹⁸ Analysis of plaque composition revealed a decreased amount of macrophages and lipids in $P2Y_6$ -deficient mice and a relatively increased amount of smooth muscle cells and collagen. These are features commonly associated with a more stable plaque

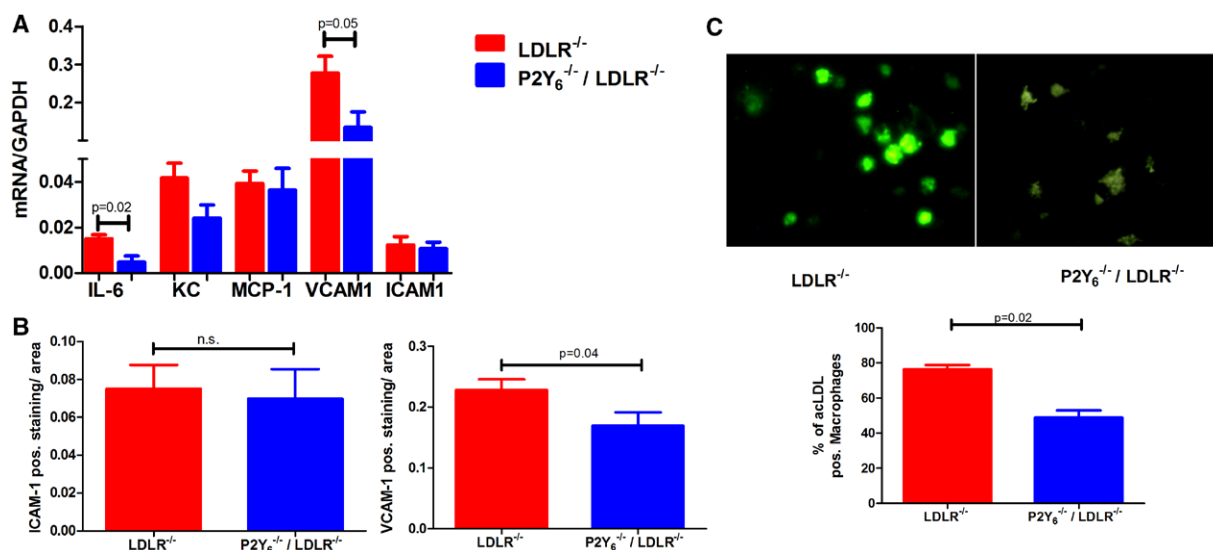


Figure 5. Purinergic receptor Y₆ (P2Y₆)-deficient atherosclerotic lesions express less vascular cell adhesion molecule 1 (VCAM-1) and interleukin-6 (IL-6). RNA from atherosclerotic lesion of P2Y₆^{-/-}/low-density lipoprotein receptor-deficient (LDLR^{-/-}; n=5) and P2Y₆^{+/-}/LDLR^{-/-} (n=4) mice was isolated, mRNA concentrations of IL-6, VCAM-1, keratinocyte chemokine (KC), monocyte chemoattractant protein 1 (MCP-1), and intercellular cell adhesion molecule 1 (ICAM-1) were determined with real-time polymerase chain reaction, and results were referred to GAPDH as housekeeping gene (A). Sections from aortic roots were stained for ICAM-1 and VCAM-1 with immunohistochemistry and quantified for positive staining (B). Macrophages from P2Y₆-deficient or competent mice were isolated 3 days after sterile peritonitis and incubated with ALEXA488-marked acetylated low-density lipoprotein cholesterol (acLDL). Fluorescence was determined by fluorescence-activated cell sorting analysis. Representative fluorescence pictures are shown (C, upper). Data are presented as pooled mean±SEM.

phenotype in humans.²⁷ The reduced amount of lipids within the atherosclerotic plaques of P2Y₆-deficient animals can be driven by less lesional macrophage content or disturbed foam cell formation. Beyond the role of P2Y₆ in leukocyte recruitment, we observed a reduced lipid uptake of P2Y₆-deficient macrophages in vitro indicating a role of P2Y₆ in foam cell formation. But this might not affect plaque growth in vivo because atherosclerosis was dependent on nonhematopoietic P2Y₆.

Based on our findings that (1) P2Y₆-deficient mice are resistant to UDP-induced vascular inflammation and (2) atherosclerotic lesions of P2Y₆-deficient mice contain less inflammatory cells than respective controls, we tested whether some key adhesion molecules, chemokines, and cytokines in atherosclerotic lesions are regulated by P2Y₆ using quantitative polymerase chain reaction. Interestingly, the adhesion molecule VCAM-1 was decreased in P2Y₆-deficient atherosclerotic lesions indicating a crucial role of P2Y₆ in VCAM-1-induced leukocyte recruitment to the atherosclerotic lesion. VCAM-1 is known to be critical atherosclerotic lesion formation, whereas the role of ICAM-1 in atherosclerosis is controversially discussed.^{28,29} Although the UDP-P2Y₆ axis seems to regulate both VCAM-1 and ICAM-1 on ECs in vitro, in the present study, only the downregulation of VCAM-1 in P2Y₆-deficient lesions likely reduces leukocyte adhesion and recruitment to the plaque.²¹ Chemokines activate leukocytes and facilitate leukocyte rolling and trigger leukocyte arrest.³⁰ Because in allergic asthma epithelial cells drive the anti-inflammatory effect of P2Y₆ deficiency, one could speculate that endothelial P2Y₆ deficiency accounts for the antiatherogenic effect by reduced endothelial VCAM-1 expression.¹⁸ Beside its role in adhesion molecule regulation, P2Y₆ is also involved in chemokine expression by inducing MCP-1 and KC/macrophage inflammatory protein 2 in mice.^{12,21} We demonstrate a slight reduction of KC expression in P2Y₆-deficient atherosclerotic lesions, whereas MCP-1 expression does

not differ between the study groups. Furthermore, plaques of P2Y₆-deficient mice are less inflamed because of decreased IL-6 expression, a proatherogenic cytokine.³¹

Purinergic receptors are ubiquitously expressed throughout the vasculature, and thus, it is supposed that extracellular nucleotides as danger signals and their receptors participate in atherosclerosis.³² Antagonists of ADP such as ticlopidine, clopidogrel, and ticagrelor, which block the P2Y₁₂ receptor on platelets, are already established in treatment of coronary heart disease.³³ Li et al³⁴ reported that P2Y₁₂ deficiency on platelets reduces atherosclerosis in mice by augmenting inflammatory cell recruitment via decreased platelet factor 4 and P-selectin expression. Deficiency of the ADP receptor P2Y₁ on ECs diminished atherosclerotic lesions and tumor necrosis factor- α -induced vascular inflammation in mice.^{35,36}

In conclusion, the present study demonstrates that UDP induces leukocyte recruitment to the vessel wall via endothelial P2Y₆ receptor. Because leukocyte recruitment is a key process in atherosclerosis, P2Y₆ deficiency reduces atherosclerotic lesions in mice by decreased VCAM-1 expression. Our data suggest the UDP-P2Y₆ axis as promising potential target for prevention or treatment of vascular inflammation as seen with atherosclerosis.

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Disclosures

None.

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Significance

Atherosclerosis is a chronic inflammatory disease, but an anti-inflammatory treatment beyond statins is still lacking. Extracellular nucleotides such as ATP, ADP, UTP, and UDP serve as proinflammatory danger signals once released to the extracellular space by activated or dying cells. They bind to purinergic receptors and induce vascular inflammation. In the present study, we provide evidence that the UDP–purinergic receptor Y_6 axis contributes to vascular inflammation and atherosclerosis: UDP induces leukocyte rolling and adhesion via purinergic receptor Y_6 . Accordingly, atherosclerotic lesions of purinergic receptor Y_6 -deficient mice are smaller and contain less macrophages because of decreased vascular cell adhesion molecule 1 expression. Overall, our data suggest the UDP–purinergic receptor Y_6 axis as promising potential target for prevention or treatment of vascular inflammation as seen with atherosclerosis.

Supplemental methods

In vivo study

P2Y₆-deficient mice crossed with LDLR^{-/-} mice. LDLR^{-/-} and P2Y₆^{-/-} / LDLR^{-/-} mice are both on C57bl/6 background. Every mouse used for the *in vivo* study or mechanistic experiments was genotyped with the polymerase chain reaction and employed the following primers: LDLR, 5'-CCA TAT GCA TCC CCA GTC TT-3' (common primer), 5'-GCG ATG GAT ACA CTC ACT GC-3' (wild-type primer), 5'-AAT CCA TCT TGT TCA ATG GCC GAT C-3' (mutant primer); P₂Y₆ wild type, 5'-CAC GTT CAC ATC CAT TTG CC-3' (3s), 5'-AGG TGT TGT GAC AGA AGT GTG-3' (4s), P2Y₆ mutant, 5'-ATT CTC ACC GGA TTC AGT CG-3' (sense), 5'-GGC TGC TGA TCT CGT TCT TC-3' (antisense). After four weeks male P2Y₆^{-/-} / LDLR^{-/-} mice (n=18) and LDLR^{-/-} mice (n=16) were fed with high-cholesterol diet for 16 weeks (sniff EF R/M acc. D12108 mod., sniff Spezialdiäten GmbH, Soest, Germany, #E15749-34). Subsequently after 16 weeks of HCD, mice were anesthetized with an intraperitoneal injection of 80% 100mg/ml Ketamine hydrochloride (Freiburg Inresa Arzneimittel, Freiburg, Germany, #07714091) and 20% 20mg/ml Xylazin (Rompun 2%, Bayer Vital GmbH, Leverkusen, Germany, #1320422) (Dose at 0,1ml/10g of body weight). In order to bleed the animal, the portal vein was opened and a perfusion with sterile 0.9% NaCl was started. Epididymal fat pads were removed and measured. Organs and the aorta were prepared under the microscope (Zeiss, STEMI 2000-C, Carl Zeiss Inc., Oberkochen, Germany). Connective and adipose tissue were removed. Aortic arch and roots were embedded in OCT (Tissue-Tec O.C.T. Compound, Sakura Finetek Europe GmbH, Alphen aan den Rijn, Netherlands, #4583) and stored at -80°C. The abdominal aorta was given in 10% buffered formalin and stored at 4°C. For immunohistological analysis of plaque size and plaque composition, aortic roots and arches were cut in 6µm sections at -20°C on a cryostat (CM 1510S Leica Microsystems Nussloch GmbH, Nussloch, Germany). Sections of the aortic root and arch underwent analysis for the total wall area (= intima + media), intimal lesion area (intima), and medial area (media) as described previously¹. To analyze plaque composition, the percentage of positively stained area for macrophages (anti-mouse Mac-3), lipids (Oil-Red-O), collagen (Picrosirius red), and smooth muscle cells (anti-α-actin), was calculated by blinded investigators employing computer-assisted image analysis software (Image Pro, Media Cybernetics, Rockville, MD, USA). All mice were housed under specific pathogen-free conditions and procedures were approved by the Animal Care Committee of the University of Freiburg.

Histology

Oil Red O staining

For immunohistochemical staining of lipids 0.5% Oil-red-O solution was prepared one day before the staining procedure. Therefore 2.5 g pure Oil red O (Sigma-Aldrich, St. Luis, MO, USA, #O0625) were solved under stirring in 500ml propylene glycol (1,2-Propandiol, Fisher Scientific, Waltham, MA, USA, #S25769) at 95°C. The obtained

suspension was filtered through 185mm filter paper (Whatman GmbH, Dassel, Germany, #10314714) and cooled down to room temperature. Before staining, filtration was repeated with a 0.2µm filter (Nalgene vacuum filtration system, Sigma-Aldrich, St. Luis, MO, USA, # 568-0020). Sections of the aortic root and arch were adapted to room temperature. Afterwards they were fixed for 10min in 10% formalin. After washing under floating water dehydration was performed in 100% propylene glycol. Sections were stained in 0.5% Oil Red O for 25min at 60°C. After 10min washing under floating water cell nuclei were counterstained for 5sec in 25% hematoxylin (Sigma-Aldrich, St. Luis, MO, USA, #H3136-100G) and dipped 0.25% ammonium (Ammonia water 0.25%, Electron Microscopy Sciences, Hatfield, PA, USA, #26123-10). Finally they were embedded in glycerol gelatin (Sigma-Aldrich, St. Luis, MO, USA, #GG1) and covered with a cover slip.

Picrosirius Red staining

For immunohistological staining of collagen, a 0.1% solution of sirius red powder (Polyscience inc., Warrington, PA, USA, #09400) was prepared in saturated aqueous picric acid (Ricca Chemical Company, Arlington, TX, USA, #5860-32) and filtered. Slides were air dried and fixed in 10% buffered formalin for 10 min at RT. After rinsing with tap water, the slides were incubated for 3-4 hours in picrosirius red solution. Later, rinsed twice for 1 min in 0.01 N HCl, the slides were dehydrated in different concentrations of Ethanol (70% ethanol for 30-45 sec, 95% ethanol about 5min, and 100% ethanol about 5 min) and xylenes (5 min).

Anti-Mac-3 and α-actin staining

For immunohistochemical staining of Mac-3 (Anti- Mac-3 (rat anti-mouse), BD Pharmingen, Franklin Lakes, NJ, USA, #553322) and α-actin (Anti-actin, α-Smooth Muscle-FITC conjugate , Sigma-Aldrich, St. Luis, MO, USA, #F-3777), sections of the aortic arch and root were adapted to RT and fixed in acetone for 9min. To avoid leak of staining roots and arches were surrounded with polysiloxane (15% Dimethylpolysiloxane, Sigma-Aldrich, St. Luis, MO, USA, #DMPS-12M), 84% propanol(Iso-propyl-alcohol, VWR International GmbH, Darmstadt, Germany, # VW3250-4) 1% H₂SO₄(Fisher Scientific, Schwerte, Germany, # A300-500) at 65°C. Sections were incubated in 0.3% H₂O₂ (EMD Chemicals, Merck KGaA, Darmstadt, Germany, # HX0635-1) for 15min at RT. After 3 times washing with PBS for 5min at RT, anti-Mac-3 sections were incubated with 50µl 5% rabbit serum (Vector Laboratories, Burlingame, CA, USA, #S-5000) for 20 min, while α-actin sections were incubated with 50 µl horse serum(Vector Laboratories, Burlingame, CA, USA, #S-2000) for 20min, to avoid unspecific binding. According to the manufacturer's protocol, serum was removed and staining was performed with the first antibody. After three times washing with PBS for 5min at RT, anti-Mac-3 sections were overlaid with biotinylated Rabbit anti-Rat (Vector Laboratories, Burlingame, CA, USA, #BA-4001) for 45 min at RT, while α-actin sections were overlaid with anti-FITC-biotin conjugated (Sigma-Aldrich, St. Luis, MO, USA, #B0287) for 45 min at RT. This step was followed by 3 times washing with PBS for 5min at RT. Sections were subsequently incubated with 50µl of Elite PK-6100 Vectastain ABC kits (Vector Laboratories, Burlingame, CA, USA, #PK-6100) for 30min at RT. After washing with PBS 3 times for 5min at RT, staining was developed with a drop of Chromagen AEC Substrat (DAKO, Hamburg, Germany, # K3464) for 1 to 3min. Stained sections were washed for 20min under floating water and cell nuclei were counterstained with hematoxylin.

Intravital microscopy

2 h before surgery the three groups of mice were pretreated with an intraperitoneal injection of 300 μ l PBS (LDLR^{-/-} control group) or respectively 200 nmol UDP (5-OMe-EDP-Trisodium salt, Tocris, Ellisville, MO, USA, #4376) in 300 μ l PBS (LDLR^{-/-} group and P2Y₆^{-/-} group). All mice were anesthetized by intraperitoneal injection of 80% 100mg/ml Ketamine hydrochloride (Freiburg Inresa Arzneimittel, Freiburg, Germany, #07714091) and 20% 20mg/ml Xylazin (Rompun 2%, Bayer Vital GmbH, Leverkusen, Germany, #1320422) (Dose at 0,1ml/10g of body weight). Before surgery 60 μ l Rhodamin 6G (1mg/ml) were injected retro orbitally. After disinfection of the abdominal area, epigastric vessels were localized, and the peritoneum was opened with a median cut. Now, the mice were moved to a tempered Petri dish, where a part of the intestines was gently taken out of the peritoneum and the mesenteric vessels were exposed. An intravital microscope (AxioScope Vario, Carl Zeiss Inc., Oberkochen, Germany) was used to make microscopic observations of the exposed mesenteric vessels. 6 venules of diameters between 30 and 50 μ m from each mouse were observed and recorded through a high sensitivity camera system (AxioCam MRm, Carl Zeiss Inc., Oberkochen, Germany) for 60 seconds. Rolling and adhering leucocytes were quantified by a blinded investigator applying Axiovision Rel. 4.6 Software (Carl Zeiss Inc., Oberkochen, Germany).

Quantitative reverse transcript polymerase chain reaction of murine samples

From the performed feeding study, harvested organs meant for expression analysis were stored in RNAlater (Qiagen, Venlo, Netherlands, #76140) at -80°C. RNA was extracted from murine aortic arches using TRIzol Reagent (Invitrogen, life technologies, Carlsbad, CA, #15596-026) utilizing a modified protocol. Homogenization was performed using a rotor stator dispergator (IKA, Wilmington, NC, USA). Purified RNA was transcribed to cDNA using Transcriptor First Strand cDNA Synthesis Kit (Roche, Basel, Switzerland). The cDNA for real-time PCR was obtained with Roche Lightcycler 480 (Roche, Basel, Switzerland) as previously described².

Primer Sequences:

mKC fow.: 5'-GCT GGG ATT CAC CTC AAG AA-3'
mKC rev.: 5'-TGG GGA CAC CTT TTA GCA TC-3'
mMCP-1 fow.: 5'-CCC ACT CAC CTG CTG CTA CT-3'
mMCP-1 rev.: 5'-TTC CTT CTT GGG GTC AGC AC-3'
mICAM-1 fow.: 5'-CAC GCT ACC TCT GCT CCT G-3'
mICAM rev.: 5'-TCT GGG ATG GAT GGA TAC CT-3'
mVCAM-1 fow.: 5'-GAA CCC AAA CAG AGG CAG AG-3'
mVCAM rev.: 5'-GGT ATC CCA TCA CTT GAG CAG-3'

FACS Analysis of peripheral blood leukocytes

Leukocytes were isolated from total blood and were incubated with anti-mouse FcR11/III for 10 min at 4°C in FACS buffer. Then they were stained for monocyte and granulocyte characterization with the following anti mouse antibodies: CD45-eflour 780, CD11b-FITC, CD115-APC, Gr-1-PeCy7 and for B and T-cells we used the following antibodies: CD45-eflour 780, CD3-AmCyan, CD4-FITC, CD8-PE, CD19-PeCy7 and CD11c-APC. Cells were analyzed on a FACSCanto II cytometer (BD) using Diva software and the next gating strategy: Resident monocytes CD45+/CD11b+/CD115+/Gr-1-; Inflammatory monocytes

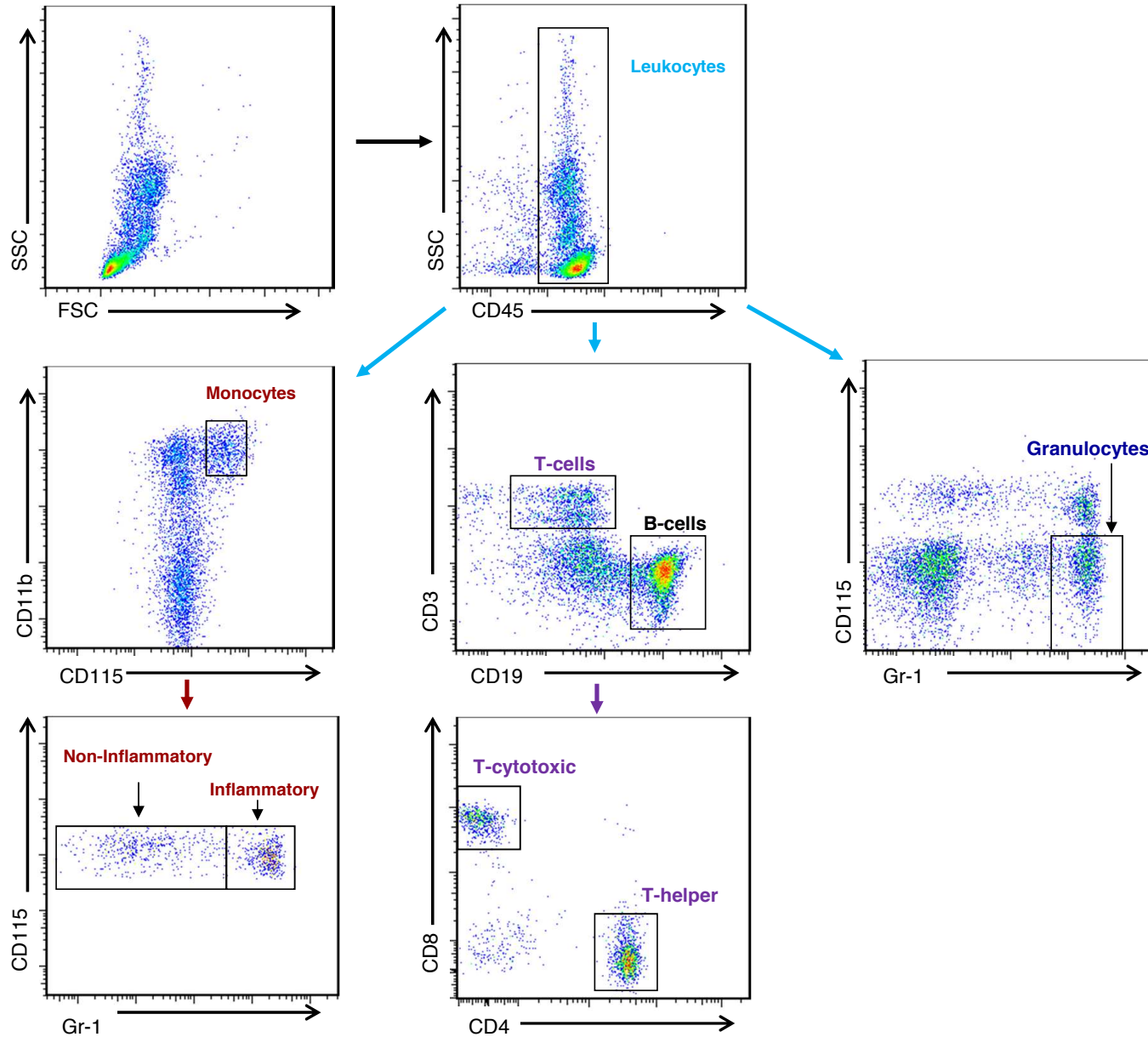
CD45+/CD11b+/CD115+/Gr-1+; Granulocytes CD45+/CD11b+/CD115-/Gr-1+; T-cells CD45+/CD3+; T-helper CD45+/CD3+/CD4+; T-cytotoxic CD45+/CD3+/CD8+; B-cells CD45+/CD19+/CD11c- (Supplemental Figure I).

Data Analysis

Data of at least 3 experiments were pooled and presented as means±SEM.

Statistical analysis used the Student's 2-tailed *t* test for paired or unpaired values. A probability value ≤0.05 was considered as statistically significant.

1. Stachon P, Missiou A, Walter C, Varo N, Colberg C, Wolf D, Buchner M, von Zur Muhlen C, Zirlik K, Bode C, Zirlik A. Tumor necrosis factor receptor associated factor 6 is not required for atherogenesis in mice and does not associate with atherosclerosis in humans. *PLoS one*. 2010;5:e11589
2. Missiou A, Kostlin N, Varo N, Rudolf P, Aichele P, Ernst S, Munkel C, Walter C, Stachon P, Sommer B, Pfeifer D, Zirlik K, MacFarlane L, Wolf D, Tsitsikov E, Bode C, Libby P, Zirlik A. Tumor necrosis factor receptor-associated factor 1 (traf1) deficiency attenuates atherosclerosis in mice by impairing monocyte recruitment to the vessel wall. *Circulation*. 2010;121:2033-2044

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Supplemental figure I: Gating strategy for peripheral blood leukocytes: Resident monocytes CD45⁺/CD11b⁺/CD115⁺/Gr-1⁻; Inflammatory monocytes CD45⁺/CD11b⁺/CD115⁺/Gr-1⁺; Granulocytes CD45⁺/CD11b⁺/CD115⁻/Gr-1⁺; T-cells CD45⁺/CD3⁺; T-helper CD45⁺/CD3⁺/CD4⁺; T-cytotoxic CD45⁺/CD3⁺/CD8⁺; B-cells CD45⁺/CD19⁺/CD11c⁻.