Extracellular ATP Induces Vascular Inflammation and Atherosclerosis via Purinergic Receptor Y_2 in Mice

Peter Stachon, Serjosha Geis, Alexander Peikert, Adrian Heidenreich, Nathaly Anto Michel, Fatih Ünal, Natalie Hoppe, Bianca Dufner, Lisa Schulte, Timoteo Marchini, Sanja Cicko, Korcan Ayata, Andreas Zech, Dennis Wolf, Ingo Hilgendorf, Florian Willecke, Jochen Reinöhl, Constantin von zur Mühlen, Christoph Bode, Marco Idzko,* Andreas Zirlik*

Objective—A solid body of evidence supports a role of extracellular ATP and its P2 receptors in innate and adaptive immunity. It promotes inflammation as a danger signal in various chronic inflammatory diseases. Thus, we hypothesize contribution of extracellular ATP and its receptor $P2Y_2$ in vascular inflammation and atherosclerosis.

Approach and Results—Extracellular ATP induced leukocyte rolling, adhesion, and migration in vivo as assessed by intravital microscopy and in sterile peritonitis. To test the role of extracellular ATP in atherosclerosis, ATP or saline as control was injected intraperitoneally 3× a week in low-density lipoprotein receptor$^{-/-}$ mice consuming high cholesterol diet. Atherosclerosis significantly increased after 16 weeks in ATP-treated mice (n=13; control group, 0.26 mm2; ATP group, 0.33 mm2; P=0.01). To gain into the role of ATP-receptor $P2Y_2$ in ATP-induced leukocyte recruitment, ATP was administered systemically in $P2Y_2$-deficient or $P2Y_2$-competent mice. In $P2Y_2$-deficient mice, the ATP-induced leukocyte adhesion was significantly reduced as assessed by intravital microscopy. $P2Y_2$ expression in atherosclerosis was measured by real-time polymerase chain reaction and immunohistochemistry and demonstrates an increased expression mainly caused by influx of $P2Y_2$-expressing macrophages. To investigate the functional role of $P2Y_2$ in atherogenesis, $P2Y_2$-deficient low-density lipoprotein receptor$^{-/-}$ mice consumed high cholesterol diet. After 16 weeks, $P2Y_2$-deficient mice showed significantly reduced atherosclerotic lesions with decreased macrophages compared with $P2Y_2$-competent mice (n=11; aortic arch: control group, 0.25 mm^2; $P2Y_2$-deficient, 0.14 mm2; P=0.04). Mechanistically, atherosclerotic lesions from $P2Y_2$-deficient mice expressed less vascular cell adhesion molecule-1 and intercellular adhesion molecule-1 RNA.

Conclusions—We show that extracellular ATP induces vascular inflammation and atherosclerosis via activation of $P2Y_2$. (***Arterioscler Thromb Vasc Biol*. 2016;36:1577-1586. DOI: 10.1161/ATVBAHA.115.307397.)**

Key Words: adenosine triphosphate ■ atherosclerosis ■ immunity ■ leukocytes ■ mice ■ receptors, purinergic P2Y2

ATP is the crucial universal energy carrier in cellular respiration,[1] thus intracellular concentrations can reach up to 8 mmol/L. Of note, many forms of cellular irritation such as cell death, hypoxia, or inflammation promotes the release of ATP from intracellular storage pool into the extracellular compartment. Thereby active and passive modes can be involved; for instance, platelet aggregation triggers ATP release via vesicular release, whereas activated neutrophils can spill ATP via connexin hemichannels. Pannexin channels seem to be one of the major modes of ATP release from apoptotic cells. Once in the extracellular compartment, ATP can bind to purinergic receptors acting either as a find-me signal or danger signal modulating/inducing inflammatory responses.[2–5] Purinergic receptors are almost ubiquitously expressed in all kinds of tissues and contribute to innate and adaptive immunity.[6,7] Extracellular ATP activates 2 kinds of purinergic receptors: purinergic receptors Y (P2Y-receptors) are G-protein–coupled receptors ($P2Y_{1,2,4,6,11,12,13,14}$) and purinergic receptors X (P2X receptors; $P2X_{1-7}$) are plasma membrane ion-channels.[8,9] Purinergic receptors contribute to various chronic inflammatory diseases, including chronic obstructive pulmonary disease, allergic dermatitis, or graft versus host disease.[10–13]

Atherosclerosis is a chronic inflammatory disease and accounts for tremendous morbidity and mortality worldwide.[14] Innate and adaptive immunity drives atherogenesis in every step from atheroma formation, to progression, and complication.[15,16] Several previous studies suggest a role of extracellular nucleotides and purinergic receptors in atherosclerosis and vascular inflammation: $P2Y_1$ deficiency reduces atherosclerosis and limits leukocyte adhesion in mice.[17,18] The UDP

Received on: May 14, 2016; final version accepted on: June 2, 2016.

From the Atherogenesis Research Group, University Heart Center Freiburg, Department of Cardiology and Angiology I (P.S., S.G., A.P., A.H., N.A.M., F.Ü., N.H., B.D., L.S., T.M., D.W., I.H., F.W., J.R., C.v.z.M., C.B., A.Z.) and Department of Pneumology (S.C., K.A., A.Z., M.I.), University of Freiburg, Freiburg, Germany.

*These authors share senior authorship.

The online-only Data Supplement is available with this article at http://atvb.ahajournals.org/lookup/suppl/doi:10.1161/ATVBAHA.115.307397/-/DC1.

Correspondence to Andreas Zirlik, MD, Atherogenesis Research Group, Heart Center, University of Freiburg, Hugstetterstrasse 55, 79106 Freiburg, Germany. E-mail andreas.zirlik@universitaets-herzzentrum.de

Nonstandard Abbreviations and Acronyms	
HCD	high cholesterol diet
ICAM-1	intercellular cell adhesion molecule 1
LDLR	low-density lipoprotein receptor
P2X	purinergic receptors X
P2Y	purinergic receptors Y
SMC	smooth muscle cells
VCAM-1	vascular cell adhesion molecule 1

receptor $P2Y_6$ contributes to experimental atherosclerosis.[19] $P2Y_{12}$ antagonists such as clopidogrel, prasugrel, or ticagrelor are clinically used in patients with coronary heart disease to inhibit platelet aggregation.[20,21] But beyond its role in platelet aggregation, $P2Y_{12}$-deficient mice are protected from vascular inflammation and atherosclerosis.[22,23]

Extracellular ATP and UTP activate equipotentially mitogen-activated protein kinases via the purinergic receptor $P2Y_2$.[24] The ATP–$P2Y_2$ interaction increases levels of chemokines such as keratinocyte-derived chemokine and macrophage inflammatory protein 2 levels, and $P2Y_2$-deficient mice express reduced vascular cell adhesion molecule 1 (VCAM-1).[25–28] Thus, activation of the ATP- $P2Y_2$ axis guides leukocyte to sites of inflammation.

Because leukocyte recruitment to the vessel wall is a crucial step in atherogenesis,[29,30] we hypothesized that extracellular ATP plays an important role in leukocyte recruitment to the inflamed vessel wall and atherosclerosis via $P2Y_2$, and that $P2Y_2$ deletion limits experimental atherosclerosis.

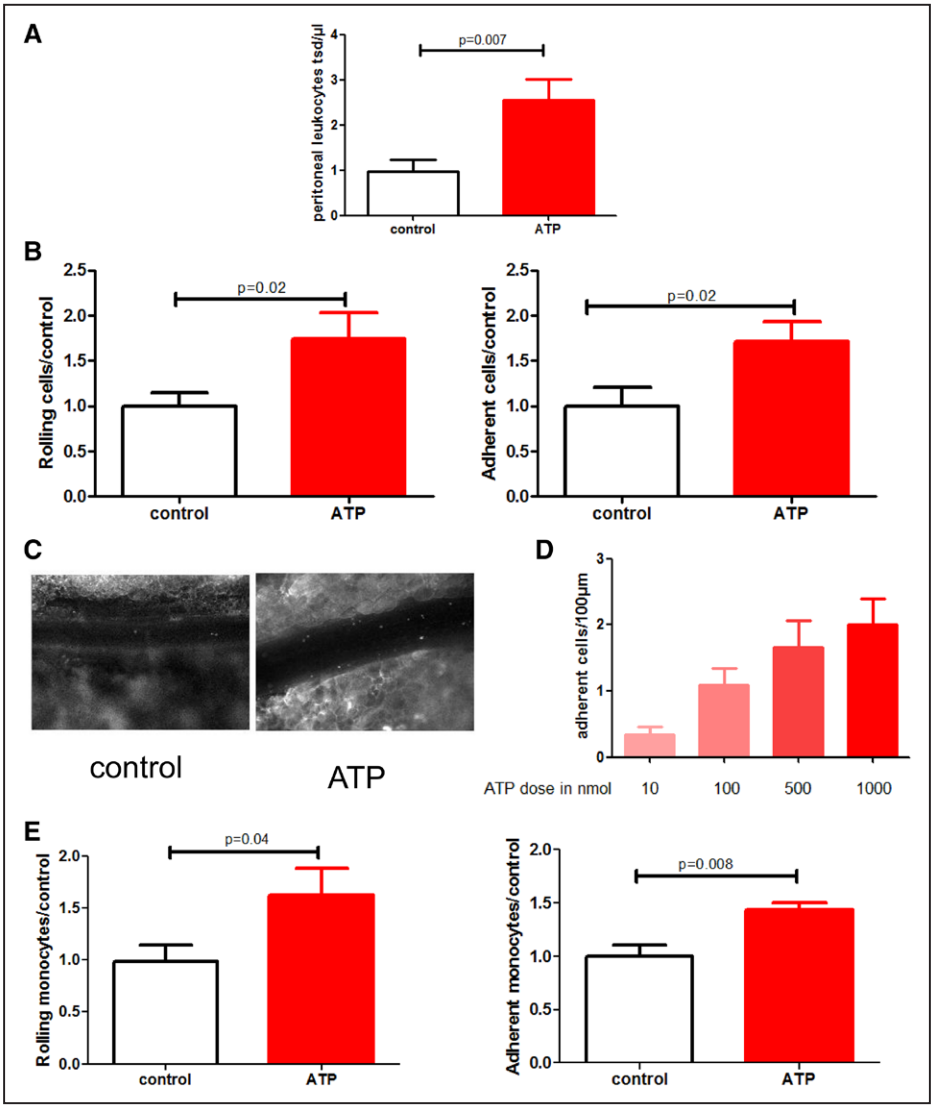

Figure 1. Extracellular ATP in leukocyte recruitment. C57/BL6 mice were stimulated with thioglycolate intraperitoneally for induction of sterile peritonitis. ATP (100 nmol) was injected additionally in the peritoneal cavity (ATP group), PBS served as control (PBS). Peritoneal leukocytes were counted after 4 h (**A**). C57/BL6 mice were intraperitoneally stimulated with either PBS or 100 nmol ATP. Intravital microscopy of mesenteric vessels was performed after 2 hours, and leukocyte rolling and adhesion were measured (**B**). Representative images are shown in **C**. Adherent cells to mesenterial vessels were determined with intravital microscopy after stimulation with increasing doses of ATP (**D**). CX3CR1-GFP (green fluorescence protein) mice were intraperitoneally stimulated with either PBS or 100 nmol ATP. Intravital microscopy was performed after 2 hours, and leukocyte rolling and adhesion were measured (**E**). Data are presented as pooled mean±SEM. Statistical analysis used the Student 2-tailed *t* test for paired or unpaired values.

Materials and Methods

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

Results

Extracellular ATP Induces Leukocyte Rolling, Adhesion, and Migration

Leukocyte recruitment to the vessel wall is a crucial step in atherogenesis. To elucidate the role of extracellular ATP in leukocyte migration to the site of inflammation, sterile peritonitis was induced in C57/BL mice. Whenever extracellular ATP was injected into mice, significantly more leukocytes entered the peritoneal cavity compared with saline-injected control mice (Figure 1A). Intravital microscopy of mesenteric vessels was performed to functionally dissect leukocyte recruitment after ATP stimulation. Extracellular ATP induced both leukocyte rolling and adhesion (Figure 1B and 1C). ATP-mediated leukocyte adhesion proved to be dose-dependent because more leukocytes adhered with increasing doses of ATP (Figure 1D).

Because monocytes are the predominant leukocyte cell fraction recruited into the atherosclerotic vessel wall, we evaluated the role of extracellular ATP in monocyte recruitment by intravital microscopy of CX3CR-1-GFP (green fluorescence protein) mice. In accord with the previous findings, extracellular ATP promoted monocyte rolling and adhesion to the vessel wall (Figure 1E).

Atherogenesis Increases Plasma Level of Extracellular ATP

To elucidate the contribution of extracellular ATP in atherosclerosis, ATP levels were measured in nonatherosclerotic

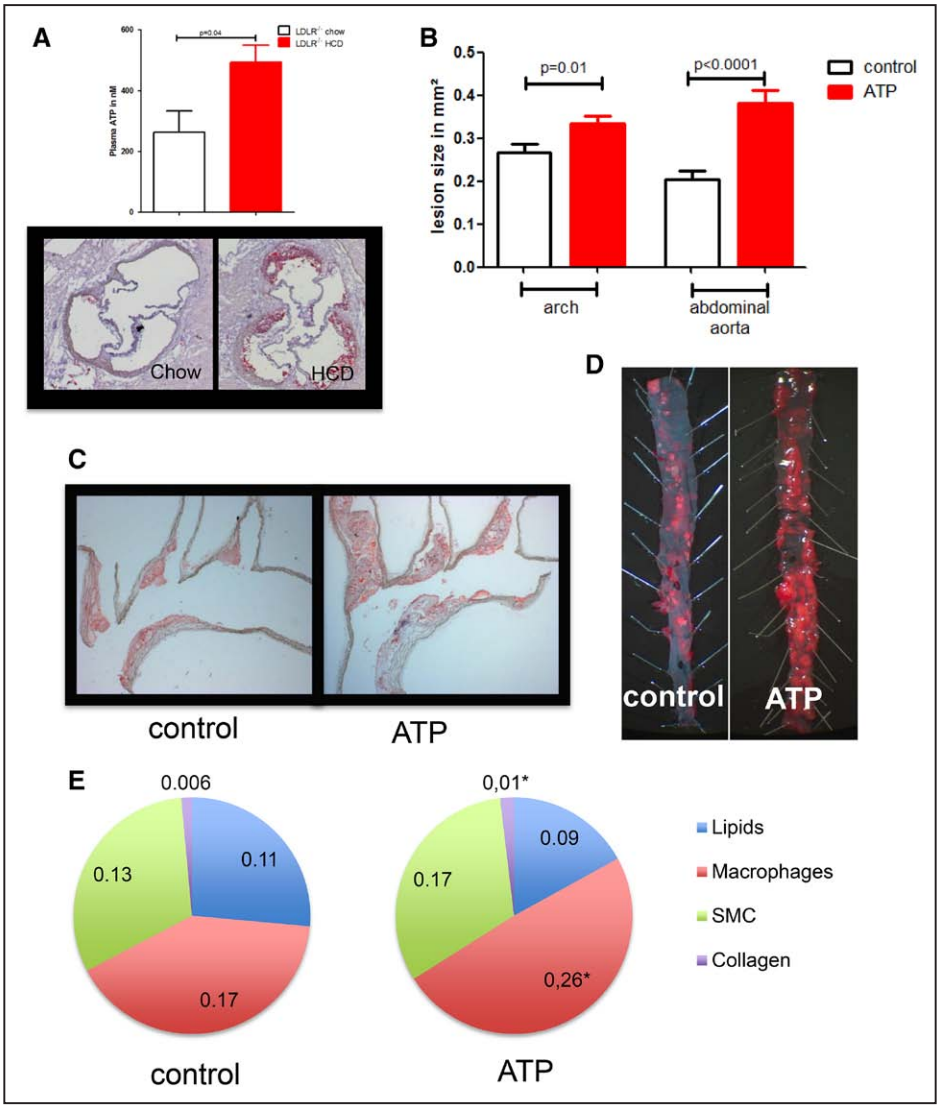

Figure 2. Extracellular ATP in atherosclerosis. Low-density lipoprotein receptor (LDLR)$^{-/-}$ mice consumed a chow or high cholesterol diet (HCD) for 8 wk. ATP levels were measured after 4 wk; representative sections of aortic root are shown at the **bottom** of **A**. LDLR$^{-/-}$ mice were consumed a HCD for 16 wk. The control group was injected with 300-μL PBS (n=13), the ATP group with 10-nmol ATP in 300-μL PBS (n=13) 3× per wk. Size of atherosclerotic lesions was analyzed in aortic arch and abdominal aorta. Analysis is shown in **B**, representative images of aortic arch in **C** and abdominal aorta in **D**. Plaque composition was determined by immunohistochemistry for lipids (Oil-red-O), macrophages (anti–Mac-3), smooth muscle cells (SMCs; anti–α-actin), and collagen (Picrosirius Red). Quantification is shown in **E**. Data are presented as pooled mean±SEM. Statistical analysis used the Student 2-tailed *t* test for paired or unpaired values.

Table 1. Baseline Characteristics of ATP Injection Study

	Control LDLR$^{-/-}$	ATP Stimulation LDLR$^{-/-}$	*P* Value
Total leukocytes after diet, tsd/μL	7.7	9.4	0.07
Lymphocytes/leukocytes	0.62	0.61	0.17
T cells/lymphocytes	0.23	0.29	0.12
T-helper cells/T cells	0.57	0.55	0.25
T-killer cells/T cells	0.38	0.39	0.07
B cells/lymphocytes	0.59	0.64	0.22
Monocytes/leukocytes	0.09	0.20	0.08
Inflammatory monocytes/monocytes	0.58	0.54	0.50
Weight, g, after diet	35.3	31.2	<0.01
Triglycerides after diet, mg/dL	189	170	0.67
Cholesterol after diet, mg/dL	530	468	0.26

After 16 weeks of high cholesterol diet blood was taken, and total leukocytes were measured in thousand per microliter (tsd/μL). Furthermore, leukocyte subsets were determined by fluorescence-activated cell sorter analysis after diet. Weight and plasma total cholesterol were measured after diet.

low-density lipoprotein receptor (LDLR)$^{-/-}$ mice consuming a chow diet and atherosclerotic LDLR$^{-/-}$ mice consuming high cholesterol diet (HCD). After 8 weeks of HCD, animals showed atherosclerotic lesions in the aortic arch. The plasma levels of ATP were significantly higher in atherosclerotic mice (492±57 nmol/L) than in chow fed nonatherosclerotic mice (263±70 nmol/L; *P*=0.04; Figure 2A).

Extracellular ATP Promotes Atherosclerosis

Because extracellular ATP increased during atherogenesis and induced monocyte rolling and adhesion, we hypothesized a role for extracellular ATP in atherogenesis. LDLR$^{-/-}$ mice consumed HCD. ATP or saline was injected 3× per week. After 16 weeks of treatment, ATP-stimulated mice developed significantly increased atherosclerotic lesions in the aortic arch (n=13; control group, 0.26 mm^2; ATP group, 0.33 mm^2; *P*=0.01) and the abdominal aorta (n=13; control group, 0.20 mm^2; ATP group, 0.38 mm^2; *P*<0.0001) compared with saline-stimulated animals (Figure 2B through 2D).

Analyzing the plaque composition by immunohistochemistry revealed ATP as a mediator of plaque inflammation. Atherosclerotic lesions of ATP-simulated LDLR$^{-/-}$ animals contained more macrophages (control group, 0.11 mm^2 pos. staining/total area versus 0.26 mm^2 positive staining/total area; *P*=0.03) without affecting content of lipids or smooth muscle cells (SMCs). Interestingly, collagen content was enhanced in atherosclerotic lesions of ATP-stimulated mice (control group, 0.006 mm^2 pos. staining/total area versus 0.012 mm^2 pos. staining/total area; *P*=0.04; Figure 2E).

Leukocytes in peripheral blood, differential blood count, total cholesterol, and triglycerides did not differ in the study groups, but ATP-treated animals gained less weight during the HCD (Table 1).

ATP-P2Y$_2$ Interaction Mediates Leukocyte Adhesion

Extracellular ATP binds to several purinergic receptors. It is known that ATP–P2Y$_2$ interaction promotes expression of cell adhesion molecules such as VCAM-1 and chemokines indicating an important role of P2Y$_2$ in inflammation. To test the ATP–P2Y$_2$ axis in vascular inflammation, P2Y$_2$-deficient and competent LDLR$^{-/-}$ mice were stimulated IP with ATP. As shown in Figure 3, administration of ATP in P2Y$_2$-competent LDLR$^{-/-}$ mice again induced leukocyte rolling and adhesion. However, extracellular ATP mediated leukocyte rolling, but not leukocyte adhesion in P2Y$_2$-deficient LDLR$^{-/-}$ mice. ATP is a subject to breakdown, and its metabolites such as ADP or AMP activate purinergic receptors. To rule out that these metabolites mediated the induction of leukocyte adhesion, P2Y$_2$-competent and P2Y$_2$-deficient were treated with ADP and AMP. Both metabolites slightly increased leukocyte adhesion irrespective of P2Y$_2$, suggesting that the main effect was because of the administration of ATP. This suggests that the ATP–P2Y$_2$ interaction affects particularly leukocyte adhesion by regulating cell adhesion molecule expression in vascular inflammation (Figure 3A and 3B).

P2Y$_2$ Is Expressed in Macrophages and Endothelial Cells in Atherosclerotic Plaques

Based on our finding that the ATP–P2Y$_2$ interaction induced leukocyte recruitment, we determined the mRNA and protein expression levels of P2Y$_2$ in atherosclerotic lesions. Markedly more relative P2Y$_2$-mRNA was detectable in atherosclerotic vessels of LDLR$^{-/-}$ mice consuming HCD than in respective control mice consuming chow (Figure 3C). Immunohistochemistry confirmed these results (Figure 3D and 3E).

Three color-immunofluorescence imaging revealed coexpression of the endothelial marker CD31 and P2Y$_2$. However, endothelial P2Y$_2$ expression and amount of P2Y$_2$ expressing SMCs were similar in nonatherosclerotic and atherosclerotic vessels. In contrast, we observed increased numbers of P2Y$_2$-expressing macrophages, suggesting that these macrophages promote enhanced atherosclerotic P2Y$_2$ expression (Figure 3F and 3G).

P2Y$_2$ Deficiency Limits Atherosclerosis and Plaque Inflammation

To investigate the functional role of P2Y$_2$ in atherosclerosis, P2Y$_2$-deficient animals were crossed with LDLR$^{-/-}$ animals. LDLR$^{-/-}$ littermates served as control. After 16 weeks of HCD, P2Y$_2^{-/-}$ /LDLR$^{-/-}$ mice showed significantly smaller atherosclerotic lesions than P2Y$_2^{+/+}$ /LDLR$^{-/-}$ mice in the aortic arch (control group, 0.25 mm^2; P2Y$_2^{-/-}$ group, 0.14 mm^2; *P*=0.04) and the abdominal aorta (control group, 0.14 mm^2; P2Y$_2^{-/-}$ group, 0.04 mm^2; *P*=0.02; Figure 4A through 4C).

Consistent with the results from intravital microscopy and the ATP-atherosclerosis study, atherosclerotic lesions of P2Y$_2$-deficient mice contained less macrophages and relatively more SMCs, but similar amount of lipids. Also collagen was similar in both groups (Figure 4D). Leukocytes in peripheral blood, differential blood count, weight, total cholesterol, and triglycerides did not differ in the study groups (Table 2). Because

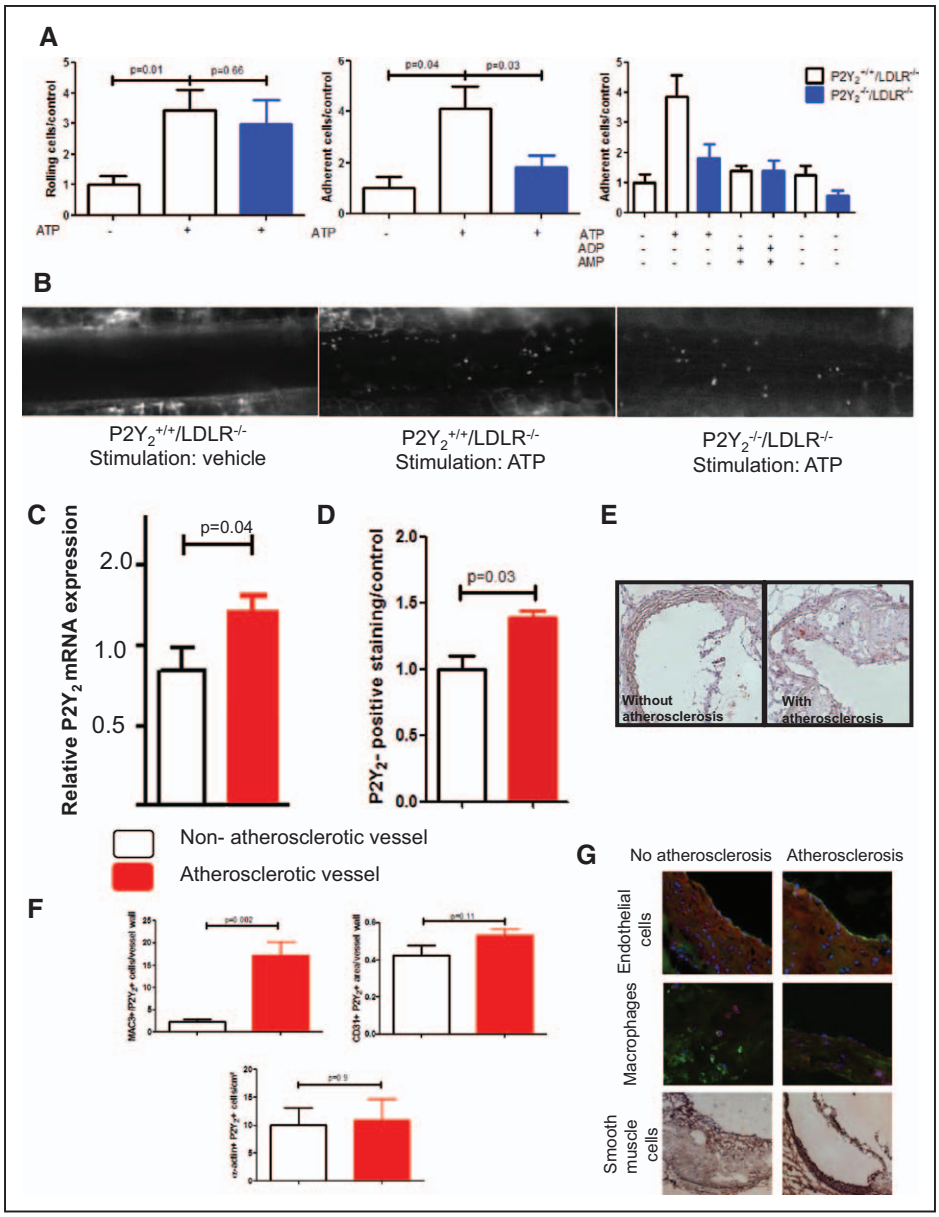

Figure 3. $P2Y_2$ in extracellular ATP induced leukocyte recruitment and atherosclerosis. $P2Y_2$-competent low-density lipoprotein receptor $(LDLR)^{-/-}$ mice were stimulated with saline as control or 100-nmol ATP. $P2Y_2$-deficient $LDLR^{-/-}$ mice were stimulated with ATP. Wild-type mice were stimulated with ATP, ADP, and AMP. Leukocyte rolling and adhesion were assessed by intravital microscopy. Quantification is shown in **A**, representative images in **B**. $LDLR^{-/-}$ mice consumed a chow diet or high cholesterol diet for 16 wk. RNA from aortic arches was isolated, and $P2Y_2$ expression was determined by quantitative polymerase chain reaction (**C**). Aortic roots were stained with anti-$P2Y_2$ for immunohistochemistry and quantified for positive staining (**D**). Representative images are shown in **E**. $P2Y_2$ distribution in atherosclerotic lesions from $LDLR^{-/-}$ was quantified by 3-color immunohistochemistry. Nuclei were stained with DAPI (blue), endothelial with anti-CD31 or macrophages with anti–Mac-3 (green), and $P2Y_2$ (red). Smooth muscle cells were stained with anti–α-actin (blue) and $P2Y_2$ in brown. Quantification of $P2Y_2$ distribution within the atherosclerotic lesion is shown in **F**. Representative images with ×20 magnification in **G**. Data are presented as pooled mean±SEM. Statistical analysis used the Student 2-tailed *t* test for paired or unpaired values.

ATP binds to different purinergic receptors, $P2Y_2$-deficient and $P2Y_2$-competent $LDLR^{-/-}$ mice were stimulated with ATP or PBS for 8 weeks and fed HCD. To exclude breakdown of subcutaneously applied ATP, plasma levels were determined in both groups. Indeed, the administration of ATP increased plasma levels of extracellular ATP (plasma level in PBS-treated mice: 496 nmol/L, in ATP-treated mice 693 nmol/L) and induced early atherosclerotic lesions in $P2Y_2$-competent $LDLR^{-/-}$ mice (lesion in aortic arch: PBS in $P2Y_2^{+/+}/LDLR^{-/-}$: 0.02 mm^2; ATP in $P2Y_2^{+/+}/LDLR^{-/-}$: 0.05 mm^2; P=0.0009). ATP-stimulated $P2Y_2$-deficient $LDLR^{-/-}$ mice developed only slightly larger atherosclerotic lesions compared with saline-treated $P2Y_2$-deficient $LDLR^{-/-}$ mice (lesion in aortic arch: PBS in $P2Y_2^{-/-}/LDLR^{-/-}$: 0.01 mm^2; ATP in $P2Y_2^{-/-}/LDLR^{-/-}$: 0.02 mm^2; P=0.07). In contrast, $P2Y_2$-competent animals developed significantly greater atherosclerotic lesions demonstrating a crucial role of $P2Y_2$ in ATP-mediated atherosclerosis (Figure 4E).

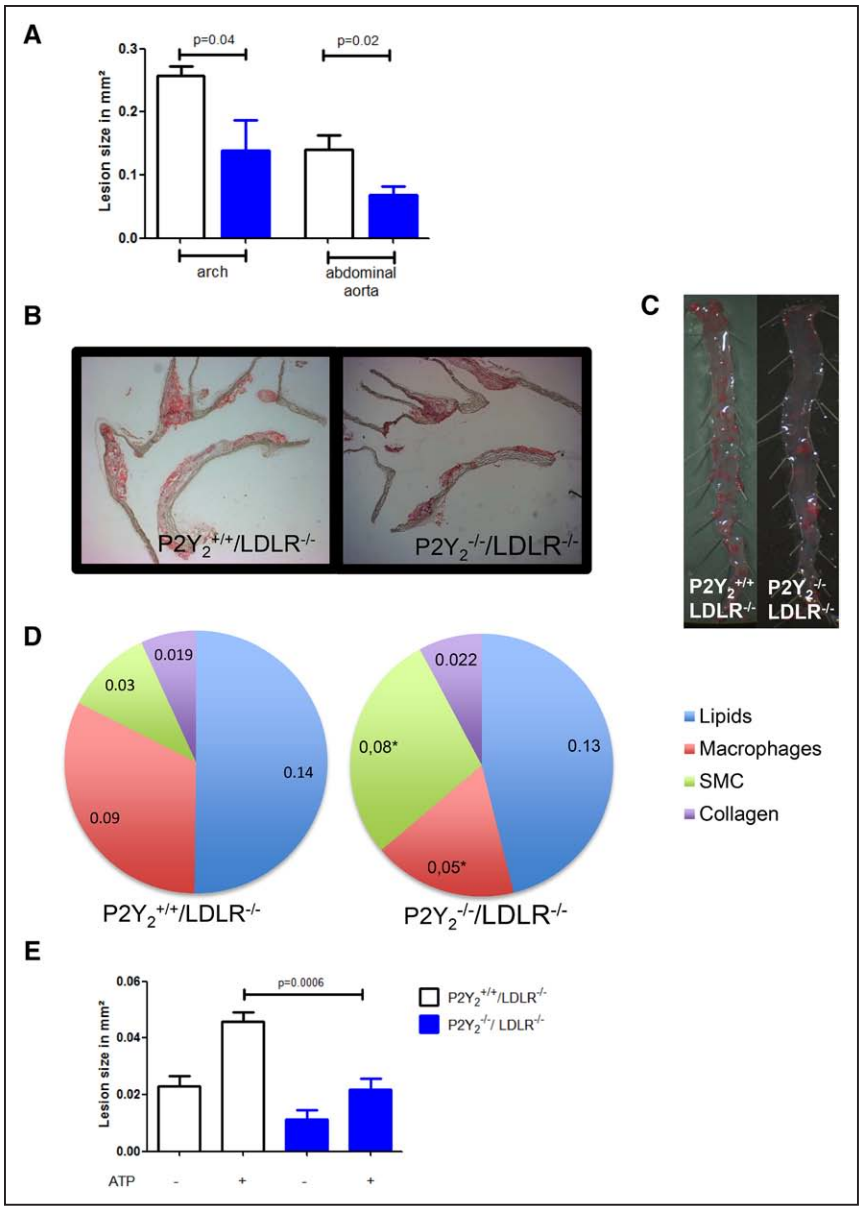

Figure 4. $P2Y_2$ in atherosclerosis. $P2Y_2^{+/+}$/$LDLR^{-/-}$ (n=15) and $P2Y_2^{-/-}$/$LDLR^{-/-}$ (n=15) mice consumed a high cholesterol diet (HCD) for 16 wk, and atherosclerotic lesion size was determined in aortic arch and abdominal aorta. Analysis is shown in **A**, representative images of aortic arch and abdominal aorta in **B** and **C**. Plaque composition was determined by immunohistochemistry for lipids (Oil-red-O), macrophages (anti–Mac-3), smooth muscle cells (SMCs; anti-α-actin), and collagen (Picrosirius Red). Quantification is shown in **D**. $P2Y_2^{+/+}$/ $LDLR^{-/-}$ and $P2Y_2^{-/-}$/$LDLR^{-/-}$ received osmotic pumps subcutaneously containing PBS or ATPγS (5 nmol/d) and consumed a HCD for 8 wk. Atherosclerotic lesion size was determined in aortic arch (**E**). Data are presented as pooled mean±SEM. Statistical analysis used the Student 2-tailed *t* test for paired or unpaired values.

$P2Y_2$ Regulates Adhesion Molecule Expression

Because $P2Y_2$ deficiency reduced leukocyte recruitment to sites of inflammation and also the content of macrophages in atherosclerotic lesions, we determined RNA expression of chemokines and cell adhesion molecules in atherosclerotic lesions of $P2Y_2$-competent and $P2Y_2$-deficient mice. The expression of both adhesion molecules intercellular cell adhesion molecule (ICAM)-1 and VCAM-1 was significantly reduced in atherosclerotic lesions of $P2Y_2$-deficient mice. This was confirmed by immunohistochemistry. The lesional expression of integrins lymphocyte function–associated antigen 1, very late antigen 4, L-selectin, and E-selectin were not altered, whereas P-selectin was decreased in $P2Y_2$-deficient mice (Figure 5B, representative pictures are shown in Figure I in the online-only Data Supplement). RNA of chemokines such as keratinocyte-derived chemokine, RANTES (regulated on activation normal T-cell–expressed and secreted), or MCP-1 (monocyte chemoattractant protein-1) was slightly decreased (Figure 5A). Because atherosclerotic lesions from $P2Y_2$-deficient mice contained more SMC, we investigated markers for apoptosis and proliferation in atherosclerotic lesions. No differences in KI67 or caspase-3 expression occurred between $P2Y_2$-deficient and $P2Y_2$-competent mice (Figure 5C).

Furthermore, intraperitoneal stimulation with ATP induced VCAM-1 expression in aortic endothelial cells of wild-type mice, but did not in endothelial cells of $P2Y_2$-deficient mice. E-selectin expression was slightly increased, whereas ICAM-1 and P-selectin expressions were not altered. Integrin expression on monocytes was not influenced by ATP stimulation, indicating a crucial role of the ATP–$P2Y_2$ axis in endothelial VCAM-1 expression (Figure 5D).

Discussion

The present study reports the novel finding that extracellular ATP promotes vascular inflammation and atherosclerosis, whereas the deficiency of the ATP-receptor $P2Y_2$ reduces both.

Table 2. Baseline Characteristics $P2Y_2$-Knockout Study

	$P2Y_2^{+/+}$/ $LDLR^{-/-}$	$P2Y_2^{-/-}$/ $LDLR^{-/-}$	*P* Value
Total leukocytes before diet, tsd/μL	7.6	7.8	0.74
Total leukocytes after diet, tsd/μL	2.7	3.3	0.21
Lymphocytes/leukocytes	0.6	0.6	0.54
T cells/lymphocytes	0.18	0.16	0.33
T helper cells/T cells	0.47	0.44	0.73
T-killer cells/T cells	0.10	0.12	0.54
B cells/lymphocytes	0.54	0.46	0.24
Monocytes/leukocytes	0.04	0.06	0.07
Inflammatory monocytes/monocytes	0.56	0.64	0.39
Total platelets after diet, tsd/μL	157	196	0.08
Total platelets before diet, tsd/μL	337	345	0.72
Weight, g, before diet	20.1	22.4	0.06
Weight, g, after diet	29.2	30.8	0.26
Weight visceral fat pad, g, after diet	0.29	0.43	0.12
Triglycerides after diet, mg/dL	85	81	0.89
Cholesterol after diet, mg/dL	798	769	0.86

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 sorter analysis after diet. Weight and plasma total cholesterol were measured after diet

Only few studies investigated the role of extracellular nucleotides in vascular inflammation and atherosclerosis although their proinflammatory properties are well-established.[2] Leukocyte recruitment after tumor necrosis factor-α stimulation was reduced in $P2Y_1$-deficient mice.[18] Recently, we could show that extracellular UDP induced leukocyte rolling and adhesion via endothelial $P2Y_6$.[19] Intraperitoneal injection of ATP enhanced total neutrophil and macrophage count in peritoneal lavage by inducing macrophage inflammatory protein 2 via $P2X_7$ and $P2Y_2$.[25] Accordingly, $P2Y_2$ induced chemotaxis of human neutrophils.[28,31,32] In the present study, we observed increased leukocytes in thioglycolate-induced sterile peritonitis if extracellular ATP was additionally injected. To further characterize the role of extracellular ATP in leukocyte rolling and adhesion, we performed intravital microscopy. We show in vivo that extracellular ATP induced rolling and adhesion of leukocytes. Besides the role of extracellular ATP in neutrophil migration, it guides dendritic cells, macrophages, and eosinophils in allergic lung inflammation.[27,33] We provide evidence that extracellular ATP activates monocyte rolling and adhesion, which is a crucial step in atherogenesis.[2]

To gain further insight into the role of extracellular ATP in atherosclerosis, we measured plasma levels of extracellular ATP in atherosclerotic and nonatherosclerotic mice showing an increased release of ATP on development of atherosclerosis. To test the functional role of extracellular ATP in atherosclerosis, we stimulated $LDLR^{-/-}$ mice consuming HCD with ATPgammaS (ATPγS), a nonhydrolyzable, more stable form of ATP. The selected dose of 10 nmol is roughly equivalent to a doubling of the natural extracellular ATP concentration in plasma, which is between 400 and 700 nmol/L.[34] The intraperitoneal administration of ATP increased plasma levels from 500 to 700 nmol/L in our study compared with the control group. Mice stimulated with extracellular ATP developed larger atherosclerotic lesions although they gain less weight compared with control animals. This is in accord with a previous study showing that infusion of UTP and ATP increases intimal hyperplasia in collared rabbit carotid arteries.[35]

Atherosclerotic lesions of ATP-stimulated animals are more inflamed with a higher content of macrophages. This suggests that ATP guides monocytes to the atherosclerotic lesion as we observe in intravital microscopy. Interestingly, collagen content is also increased, which could account for to a feedback mechanism to the release of ATP by dying cells. This is in line with the finding, that extracellular ATP is involved in lung and pancreas fibrotic remodeling.[36,37]

Extracellular ATP binds to all P2X- and P2Y-receptors except $P2Y_6$ and $P2Y_{14}$.[2,38] Because $P2Y_2$ contributes to chemokine expression such as MCP-1 or macrophage inflammatory protein 2 and cell adhesion molecule expression such as VCAM-1,[26,39] we hypothesized that extracellular ATP induces leukocyte recruitment via $P2Y_2$ in vivo. Intraperitoneal stimulation of $P2Y_2$-competent mice again induced leukocyte rolling and adhesion. However, in $P2Y_2$-deficient mice, leukocyte adhesion was significantly reduced without affecting rolling. This can be explained by the fact that extracellular ATP regulates selectin function and thereby rolling by $P2X_7$,[40,41] but increases adhesion molecule expression and thereby adhesion by $P2Y_2$. Metabolites of ATP such as ADP and AMP, which activate purinergic receptors, increased leukocyte adhesion only slightly compared with ATP administration. This could not be reversed in $P2Y_2$-deficient animals indicating a specific role of $P2Y_2$ in ATP-mediated leukocyte adhesion. Thus, we investigated the role of $P2Y_2$ in atherosclerosis.

To elucidate the contribution of $P2Y_2$ in atherosclerosis, we measured $P2Y_2$ expression in nonatherosclerotic and atherosclerotic vessels. $P2Y_2$ RNA was relatively increased in atherosclerotic lesions determined by real-time polymerase chain reaction and immunohistochemistry. Accordingly, $P2Y_2$ was upregulated in neointima formation in collared rabbit carotid arteries.[35] Immunofluorescence of atherosclerotic lesions imaging revealed that $P2Y_2$ was coexpressed with the endothelial markers CD31 and SMC α-actin in nonatherosclerotic and atherosclerotic vessels indicating a functional role of $P2Y_2$ in these cells, but intensity of coexpression did not significantly change during atherogenesis. However, we observe a marked increase of $P2Y_2$-expressing macrophages indicating that macrophages contribute to the enhanced $P2Y_2$ expression in atherosclerotic lesions.

The role of danger signals such as extracellular nucleotides in atherosclerosis is not sufficiently clarified. Treatment with the broad purinergic inhibitor purinergic receptor antagonist reduced atherosclerosis in $ApoE^{-/-}$ mice.[42] Deficiency of $P2Y_1$, $P2Y_6$, or $P2Y_{12}$ receptors limited experimental atherosclerosis.[17,19,22] We demonstrate that $P2Y_2$-deficient $LDLR^{-/-}$ mice develop significantly smaller plaques than $P2Y_2$-competent control $LDLR^{-/-}$ mice. In line with the results of the intravital

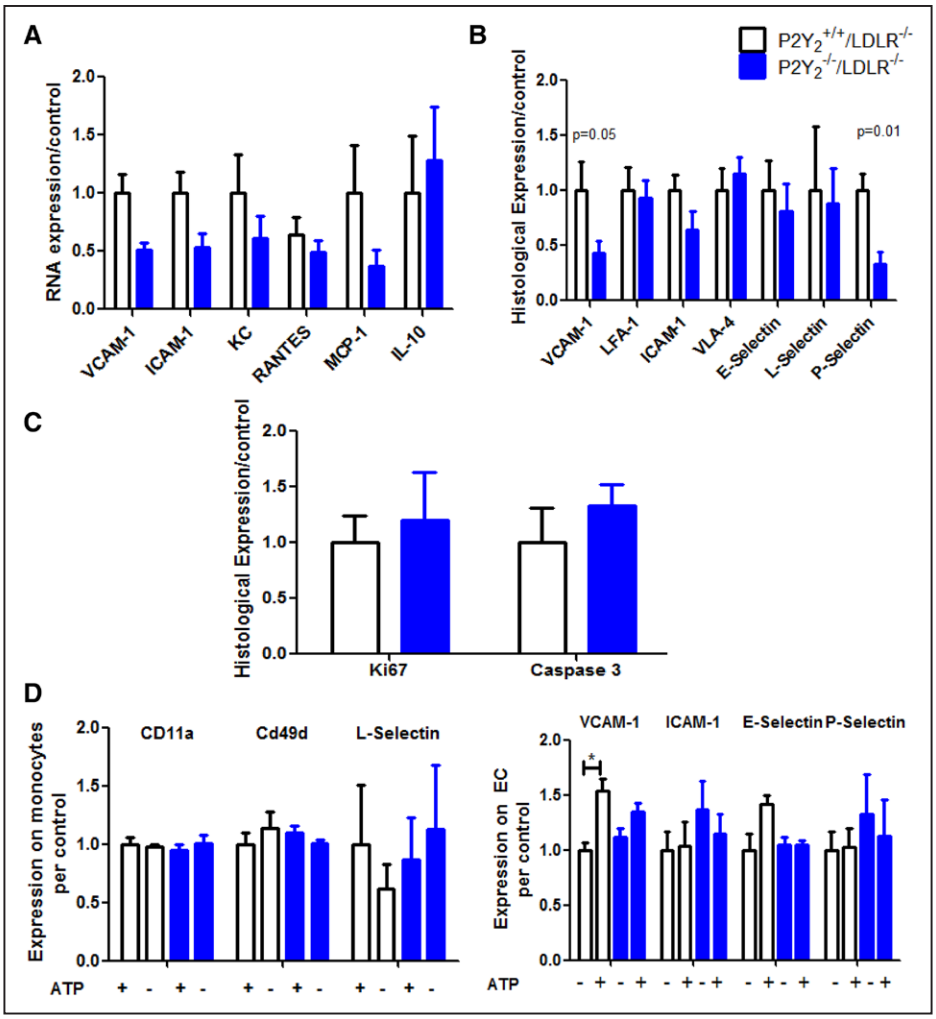

Figure 5. Adhesion molecule and chemokine expression in atherosclerotic lesions from $P2Y_2$-deficient mice. RNA was extracted from aortic arches from $P2Y_2^{-/-}$/low-density lipoprotein receptor (LDLR)$^{-/-}$ (n=7) and $P2Y_2^{+/+}$/LDLR$^{-/-}$ (n=8) mice fed a high cholesterol diet (HCD). mRNA concentrations of vascular cell adhesion molecule (VCAM)-1, intercellular adhesion molecule (ICAM)-1, keratinocyte-derived chemokine (KC), RANTES (regulated on activation normal T-cell–expressed and secreted), MCP-1 (monocyte chemoattractant protein-1), and interleukin (IL)-10 were determined with real-time polymerase chain reaction, and results were referred to GAPDH as housekeeping gene (**A**). Aortic roots of $P2Y_2^{-/-}$/LDLR$^{-/-}$ (n=7) and $P2Y_2^{+/+}$/LDLR$^{-/-}$ were stained for cell adhesion, integrins, and selectins immunohistologically and analyzed for expression (**B**). Ki-67 and caspase-3 expressions of smooth muscle cells were determined with immunohistochemistry in atherosclerotic lesions (**C**). Expression of surface integrins and cell adhesion molecules was measured by fluorescence-activated cell sorter ex vivo on peritoneal-derived monocytes and aortic endothelial cells after in vivo intraperitoneal stimulation with 500 nmol ATP (**D**). LFA indicates lymphocyte function–associated antigen 1.

ATP atherosclerosis study and of the intravital microscopy, atherosclerotic lesions from $P2Y_2$-deficient mice contained less macrophages. Moreover, the plaque of $P2Y_2$-deficient mice contained more SMCs, an effect that is most likely relative because of the reduced macrophages because we did not find differences for markers of apoptosis or proliferation in vivo. This plaque composition is associated with a more stable plaque phenotype in humans.[43] Remarkably, we found no differences in collagen content, which we observed in ATP-stimulated LDLR$^{-/-}$ mice. ATP binds to all P2X receptors and different P2Y receptors. Because administration of ATP to $P2Y_2$-deficient animals increased atherosclerosis only slightly compared with a significant increase in $P2Y_2$-competent animals, we conclude that $P2Y_2$ plays an important role in ATP-mediated atherogenesis.

To further clarify the mechanisms of $P2Y_2$ in atherosclerosis, we extracted RNA from $P2Y_2$-competent and $P2Y_2$-deficient atherosclerotic lesions. We observe a downregulation of the adhesion molecules VCAM-1, ICAM-1, and P-selectin in vivo. Particularly, VCAM-1 is known to induce leukocyte adhesion and atherosclerosis, whereas the role of ICAM-1 in atherosclerosis is controversial.[44–46] Stimulation with ATP induced VCAM-1 and E-selectin expression in aortic endothelial cell of wild-type mice, but did not alter the expression of ICAM-1 and P-selectin. However, in $P2Y_2$-deficient mice, VCAM-1 expression was rarely changed indicating a crucial role of endothelial VCAM-1 in the ATP–$P2Y_2$-mediated vascular inflammation. Integrin expression on monocytes remained unaffected by ATP stimulation. These experiments provide evidence that extracellular ATP attracts leukocytes to the endothelium directly via $P2Y_2$.

In summary, the present study shows that (1) extracellular ATP induces leukocyte and monocyte recruitment, (2) extracellular ATP is increased released in atherosclerosis and enhances atherosclerosis via $P2Y_2$, and (3) leukocyte adhesion

and atherosclerosis is reduced in $P2Y_2$-deficient mice by a downregulation of cell adhesion molecules. We conclude that inhibition of the ATP–$P2Y_2$ axis could represent a potentially fruitful target in the treatment of atherosclerotic disease and vascular inflammation.

Sources of Funding

This present research work is supported by internal funding of the Heart Center, University of Freiburg to A. Zirlik as well as by Deutsche Forschungsgemeinschaft Grant ID7/4-1 and a grant by the Boehringer-Ingelheim Foundation to M. Idzko.

Disclosures

None.

References

1. Brown GC. Control of respiration and ATP synthesis in mammalian mitochondria and cells. *Biochem J*. 1992;284(pt 1):1–13.
2. Idzko M, Ferrari D, Eltzschig HK. Nucleotide signalling during inflammation. *Nature*. 2014;509:310–317. doi: 10.1038/nature13085.
3. Junger WG. Immune cell regulation by autocrine purinergic signalling. *Nat Rev Immunol*. 2011;11:201–212. doi: 10.1038/nri2938.
4. Eltzschig HK, Eckle T, Mager A, Küper N, Karcher C, Weissmüller T, Boengler K, Schulz R, Robson SC, Colgan SP. ATP release from activated neutrophils occurs via connexin 43 and modulates adenosine-dependent endothelial cell function. *Circ Res*. 2006;99:1100–1108. doi: 10.1161/01.RES.0000250174.31269.70.
5. Khakh BS, Burnstock G. The double life of ATP. *Sci Am*. 2009;301:84–90, 92.
6. Eltzschig HK, Sitkovsky MV, Robson SC. Purinergic signaling during inflammation. *N Engl J Med*. 2012;367:2322–2333. doi: 10.1056/NEJMra1205750.
7. Schenk U, Westendorf AM, Radaelli E, Casati A, Ferro M, Fumagalli M, Verderio C, Buer J, Scanziani E, Grassi F. Purinergic control of T cell activation by ATP released through pannexin-1 hemichannels. *Sci Signal*. 2008;1:ra6. doi: 10.1126/scisignal.1160583.
8. Burnstock G. Pathophysiology and therapeutic potential of purinergic signaling. *Pharmacol Rev*. 2006;58:58–86. doi: 10.1124/pr.58.1.5.
9. Khakh BS, North RA. P2X receptors as cell-surface ATP sensors in health and disease. *Nature*. 2006;442:527–532. doi: 10.1038/nature04886.
10. Cicko S, Lucattelli M, Müller T, et al. Purinergic receptor inhibition prevents the development of smoke-induced lung injury and emphysema. *J Immunol*. 2010;185:688–697. doi: 10.4049/jimmunol.0904042.
11. Wilhelm K, Ganesan J, Müller T, et al. Graft-versus-host disease is enhanced by extracellular ATP activating P2X7R. *Nat Med*. 2010;16:1434–1438. doi: 10.1038/nm.2242.
12. Mizumoto N, Mummert ME, Shalhevet D, Takashima A. Keratinocyte ATP release assay for testing skin-irritating potentials of structurally diverse chemicals. *J Invest Dermatol*. 2003;121:1066–1072. doi: 10.1046/j.1523-1747.2003.12558.x.
13. Idzko M, Ferrari D, Riegel AK, Eltzschig HK. Extracellular nucleotide and nucleoside signaling in vascular and blood disease. *Blood*. 2014;124:1029–1037. doi: 10.1182/blood-2013-09-402560.
14. Go AS, Mozaffarian D, Roger VL, et al.; American Heart Association Statistics Committee and Stroke Statistics Subcommittee. Heart disease and stroke statistics–2014 update: a report from the American Heart Association. *Circulation*. 2014;129:e28–e292. doi: 10.1161/01.cir.0000441139.02102.80.
15. Libby P. Inflammation in atherosclerosis. *Nature*. 2002;420:868–874. doi: 10.1038/nature01323.
16. Hansson GK, Libby P, Schönbeck U, Yan ZQ. Innate and adaptive immunity in the pathogenesis of atherosclerosis. *Circ Res*. 2002;91:281–291.
17. Hechler B, Freund M, Ravanat C, Magnenat S, Cazenave JP, Gachet C. Reduced atherosclerotic lesions in P2Y1/apolipoprotein E double-knockout mice: the contribution of non-hematopoietic-derived P2Y1 receptors. *Circulation*. 2008;118:754–763. doi: 10.1161/CIRCULATIONAHA.108.788927.
18. Zerr M, Hechler B, Freund M, Magnenat S, Lanois I, Cazenave JP, Léon C, Gachet C. Major contribution of the $P2Y_1$receptor in purinergic regulation of TNFα-induced vascular inflammation. *Circulation*. 2011;123:2404–2413. doi: 10.1161/CIRCULATIONAHA.110.002139.
19. Stachon P, Peikert A, Michel NA, et al. P2Y6 deficiency limits vascular inflammation and atherosclerosis in mice. *Arterioscler Thromb Vasc Biol*. 2014;34:2237–2245. doi: 10.1161/ATVBAHA.114.303585.
20. Wiviott SD, Braunwald E, McCabe CH, Montalescot G, Ruzyllo W, Gottlieb S, Neumann FJ, Ardissino D, De Servi S, Murphy SA, Riesmeyer J, Weerakkody G, Gibson CM, Antman EM; TRITON-TIMI 38 Investigators. Prasugrel versus clopidogrel in patients with acute coronary syndromes. *N Engl J Med*. 2007;357:2001–2015. doi: 10.1056/NEJMoa0706482.
21. Cannon CP, Harrington RA, James S, et al; PLATelet inhibition and patient Outcomes Investigators. Comparison of ticagrelor with clopidogrel in patients with a planned invasive strategy for acute coronary syndromes (PLATO): a randomised double-blind study. *Lancet*. 2010;375:283–293. doi: 10.1016/S0140-6736(09)62191-7.
22. Li D, Wang Y, Zhang L, Luo X, Li J, Chen X, Niu H, Wang K, Sun Y, Wang X, Yan Y, Chai W, Gartner TK, Liu J. Roles of purinergic receptor P2Y, G protein-coupled 12 in the development of atherosclerosis in apolipoprotein E-deficient mice. *Arterioscler Thromb Vasc Biol*. 2012;32:e81–e89. doi: 10.1161/ATVBAHA.111.239095.
23. Yashiro K, Matsumoto Y, Ihara H, Suzuki Y, Kondo K, Urano T, Umemura K. Involvement of platelet activation by P2Y12 receptor in the development of transplant arteriosclerosis in mice. *Transplantation*. 2009;87:660–667. doi: 10.1097/TP.0b013e318196305a.
24. Soltoff SP, Avraham H, Avraham S, Cantley LC. Activation of P2Y2 receptors by UTP and ATP stimulates mitogen-activated kinase activity through a pathway that involves related adhesion focal tyrosine kinase and protein kinase C. *J Biol Chem*. 1998;273:2653–2660.
25. Kawamura H, Kawamura T, Kanda Y, Kobayashi T, Abo T. Extracellular ATP-stimulated macrophages produce macrophage inflammatory protein-2 which is important for neutrophil migration. *Immunology*. 2012;136:448–458. doi: 10.1111/j.1365-2567.2012.03601.x.
26. Vanderstocken G, Bondue B, Horckmans M, Di Pietrantonio L, Robaye B, Boeynaems JM, Communi D. P2Y2 receptor regulates VCAM-1 membrane and soluble forms and eosinophil accumulation during lung inflammation. *J Immunol*. 2010;185:3702–3707. doi: 10.4049/jimmunol.0903908.
27. Müller T, Robaye B, Vieira RP, Ferrari D, Grimm M, Jakob T, Martin SF, Di Virgilio F, Boeynaems JM, Virchow JC, Idzko M. The purinergic receptor P2Y2 receptor mediates chemotaxis of dendritic cells and eosinophils in allergic lung inflammation. *Allergy*. 2010;65:1545–1553. doi: 10.1111/j.1398-9995.2010.02426.x.
28. Chen Y, Corriden R, Inoue Y, Yip L, Hashiguchi N, Zinkernagel A, Nizet V, Insel PA, Junger WG. ATP release guides neutrophil chemotaxis via P2Y2 and A3 receptors. *Science*. 2006;314:1792–1795. doi: 10.1126/science.1132559.
29. Weber C, Zernecke A, Libby P. The multifaceted contributions of leukocyte subsets to atherosclerosis: lessons from mouse models. *Nat Rev Immunol*. 2008;8:802–815. doi: 10.1038/nri2415.
30. Wolf D, Stachon P, Bode C, Zirlik A. Inflammatory mechanisms in atherosclerosis. *Hamostaseologie*. 2014;34:63–71. doi: 10.5482/HAMO-13-09-0050.
31. Kukulski F, Ben Yebdri F, Lecka J, Kauffenstein G, Lévesque SA, Martín-Satué M, Sévigny J. Extracellular ATP and P2 receptors are required for IL-8 to induce neutrophil migration. *Cytokine*. 2009;46:166–170. doi: 10.1016/j.cyto.2009.02.011.
32. Inoue Y, Chen Y, Hirsh MI, Yip L, Junger WG. A3 and P2Y2 receptors control the recruitment of neutrophils to the lungs in a mouse model of sepsis. *Shock*. 2008;30:173–177. doi: 10.1097/shk.0b013e318160dad4.
33. Idzko M, Hammad H, van Nimwegen M, Kool M, Willart MA, Muskens F, Hoogsteden HC, Luttmann W, Ferrari D, Di Virgilio F, Virchow JC Jr, Lambrecht BN. Extracellular ATP triggers and maintains asthmatic airway inflammation by activating dendritic cells. *Nat Med*. 2007;13:913–919. doi: 10.1038/nm1617.
34. Bours MJ, Swennen EL, Di Virgilio F, Cronstein BN, Dagnelie PC. Adenosine 5'-triphosphate and adenosine as endogenous signaling molecules in immunity and inflammation. *Pharmacol Ther*. 2006;112:358–404. doi: 10.1016/j.pharmthera.2005.04.013.
35. Seye CI, Kong Q, Erb L, Garrad RC, Krugh B, Wang M, Turner JT, Sturek M, González FA, Weisman GA. Functional P2Y2 nucleotide receptors mediate uridine 5'-triphosphate-induced intimal hyperplasia in collared rabbit carotid arteries. *Circulation*. 2002;106:2720–2726.
36. Riteau N, Gasse P, Fauconnier L, Gombault A, Couegnat M, Fick L, Kanellopoulos J, Quesniaux VF, Marchand-Adam S, Crestani B, Ryffel B, Couillin I. Extracellular ATP is a danger signal activating P2X7

receptor in lung inflammation and fibrosis. *Am J Respir Crit Care Med*. 2010;182:774–783. doi: 10.1164/rccm.201003-0359OC.
37. Künzli BM, Berberat PO, Giese T, Csizmadia E, Kaczmarek E, Baker C, Halaceli I, Büchler MW, Friess H, Robson SC. Upregulation of CD39/NTPDases and P2 receptors in human pancreatic disease. *Am J Physiol Gastrointest Liver Physiol*. 2007;292:G223–G230. doi: 10.1152/ajpgi.00259.2006.
38. Jacobson KA, Balasubramanian R, Deflorian F, Gao ZG. G protein-coupled adenosine (P1) and P2Y receptors: ligand design and receptor interactions. *Purinergic Signal*. 2012;8:419–436. doi: 10.1007/s11302-012-9294-7.
39. Stokes L, Surprenant A. Purinergic P2Y2 receptors induce increased MCP-1/CCL2 synthesis and release from rat alveolar and peritoneal macrophages. *J Immunol*. 2007;179:6016–6023.
40. Labasi JM, Petrushova N, Donovan C, McCurdy S, Lira P, Payette MM, Brissette W, Wicks JR, Audoly L, Gabel CA. Absence of the P2X7 receptor alters leukocyte function and attenuates an inflammatory response. *J Immunol*. 2002;168:6436–6445.
41. Ley K, Laudanna C, Cybulsky MI, Nourshargh S. Getting to the site of inflammation: the leukocyte adhesion cascade updated. *Nat Rev Immunol*. 2007;7:678–689. doi: 10.1038/nri2156.
42. Guns PJ, Hendrickx J, Van Assche T, Fransen P, Bult H. P2Y receptors and atherosclerosis in apolipoprotein E-deficient mice. *Br J Pharmacol*. 2010;159:326–336. doi: 10.1111/j.1476-5381.2009.00497.x.
43. Daugherty A. Mouse models of atherosclerosis. *Am J Med Sci*. 2002;323:3–10.
44. Cybulsky MI, Iiyama K, Li H, Zhu S, Chen M, Iiyama M, Davis V, Gutierrez-Ramos JC, Connelly PW, Milstone DS. A major role for VCAM-1, but not ICAM-1, in early atherosclerosis. *J Clin Invest*. 2001;107:1255–1262. doi: 10.1172/JCI11871.
45. Patel SS, Thiagarajan R, Willerson JT, Yeh ET. Inhibition of alpha4 integrin and ICAM-1 markedly attenuate macrophage homing to atherosclerotic plaques in ApoE-deficient mice. *Circulation*. 1998;97:75–81.
46. Galkina E, Ley K. Vascular adhesion molecules in atherosclerosis. *Arterioscler Thromb Vasc Biol*. 2007;27:2292–2301. doi: 10.1161/ATVBAHA.107.149179.

Highlights

- Atherosclerosis is a chronic inflammatory disease.
- Extracellular ATP and its P2Y receptors as danger signal have been functionally linked with other inflammatory diseases such as lung inflammation and graft versus host disease.
- Our data identify the ATP–$P2Y_2$ axis as an important modulator of murine vascular inflammation and atherosclerosis.
- The interaction between ATP and $P2Y_2$ stimulates the induction of cell adhesion molecules such as vascular cell adhesion molecule-1 and thereby increases leukocyte recruitment to the atherosclerotic vessel.
- Indeed, inhibition of $P2Y_2$ could be a fruitful strategy in the emerging anti-inflammatory treatment of atherosclerosis.

Arteriosclerosis, Thrombosis, and Vascular Biology

JOURNAL OF THE AMERICAN HEART ASSOCIATION

Extracellular ATP Induces Vascular Inflammation and Atherosclerosis via Purinergic Receptor Y_2 in Mice

Peter Stachon, Serjosha Geis, Alexander Peikert, Adrian Heidenreich, Nathaly Anto Michel, Fatih Ünal, Natalie Hoppe, Bianca Dufner, Lisa Schulte, Timoteo Marchini, Sanja Cicko, Korcan Ayata, Andreas Zech, Dennis Wolf, Ingo Hilgendorf, Florian Willecke, Jochen Reinöhl, Constantin von zur Mühlen, Christoph Bode, Marco Idzko and Andreas Zirlik

Arterioscler Thromb Vasc Biol. 2016;36:1577-1586; originally published online June 23, 2016;
doi: 10.1161/ATVBAHA.115.307397
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

Print ISSN: 1079-5642. Online ISSN: 1524-4636