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Knockout Mice Reveal a Role for $P2Y_6$ Receptor in Macrophages, Endothelial Cells, and Vascular Smooth Muscle Cells

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ABSTRACT

P2Y receptors are G-protein-coupled receptors activated by extracellular nucleotides. The $P2Y_6$ receptor is selectively activated by UDP, and its transcript has been detected in numerous organs, including the spleen, thymus, intestine, blood leukocytes, and aorta. To investigate the biological functions of this receptor, we generated $P2Y_6$-null mice by gene targeting. The $P2Y_6$ knockout (KO) mice are viable and are not distinguishable from the wild-type (WT) mice in terms of growth or fertility. In thioglycollate-elicited macrophages, the production of inositol phosphate in response to UDP stimulation was lost, indicating that $P2Y_6$ is the unique UDP-responsive receptor expressed by mouse macrophages. Furthermore, the amount of interleukin-6 and macrophage-inflammatory protein-2, but not tumor necrosis factor-α, released in response to lipopolysaccharide stimulation was significantly enhanced in the presence of UDP, and this effect was lost in the $P2Y_6$ KO macrophages. The endothelium-dependent relaxation of the aorta by UDP was abolished in KO $P2Y_6$ mice. The contractile effect of UDP on the aorta, observed when endothelial nitric-oxide synthase is blocked, was also abolished in $P2Y_6$-null mice. In conclusion, we generated $P2Y_6$-deficient mice and have shown that these mice have a defective response to UDP in macrophages, endothelial cells, and vascular smooth muscle cells. These observations might be relevant to several physiopathological conditions such as atherosclerosis or hypertension.

Extracellular nucleotides are intercellular messengers acting in an autocrine and/or paracrine fashion (Abbracchio et al., 2006). They are released in the extracellular space in response to different stimuli or conditions such as cell lysis, hypoxia, shear stress, or microbial components (Lazarowski et al., 2003, Boeynaems et al., 2005, Abbracchio et al., 2006).

Extracellular nucleotides exert their action by activating transmembrane receptors of the P2 family. On the basis of their molecular structure and signaling pathways, mammalian P2 receptors are subdivided into ionotropic $P2X_{1–7}$ and G-coupled P2Y ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$, and $P2Y_{14}$) receptors. The former are all activated by ATP, whereas the latter show a selectivity for ADP ($P2Y_1$, $P2Y_{12}$, and $P2Y_{13}$), ATP and UTP ($P2Y_2$ and rodent $P2Y_4$), UTP (human $P2Y_4$), UDP ($P2Y_6$), and UDP-glucose ($P2Y_{14}$) (Abbracchio et al., 2006).

An impressive number of in vitro or ex vivo studies have shown that extracellular nucleotides regulate the biology of many cells types, but the identity of the receptors involved could not be determined conclusively because of the lack of selective agonists or antagonists, and the physiological significance of these observations remains unclear. The inactivation of mouse P2Y genes has led to the identification of the physiological roles of several P2Y receptors, revealing the pharmacotherapeutic potential of these receptors. Indeed, it

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ABBREVIATIONS: IP_3, inositol-1,4,5-trisphosphate; KO, knockout; WT, wild type; IL, interleukin; PCR, polymerase chain reaction; bp, base pair(s); TNF-α, tumor necrosis factor-α; ERK1/2, extracellular signal-regulated kinases 1 and 2; L-NA, nitro-L-arginine; L-NAME, nitro-L-arginine methyl ester; RT-PCR, reverse transcriptase-polymerase chain reaction; eGFP, enhanced green fluorescent protein; IP, inositol phosphate; LPS, lipopolysaccharide; MAP, mitogen-activated protein; FRT, FLP recombinase target; kb, kilobase(s).

has been shown by this way that the $P2Y_2$ receptor mediates the ATP- and UTP-triggered stimulatory effect on chloride secretion in airway epithelium (Cressman et al., 1999). The study of $P2Y_4$-null mice has demonstrated that this receptor transduces the ATP and UTP effects on chloride and potassium secretion in intestinal epithelium (Robaye et al., 2003, Ghanem et al., 2005, Matos et al., 2005). Gene targeting has also contributed to demonstrate that ADP triggers platelet aggregation by stimulating both $P2Y_1$ and $P2Y_{12}$ receptors (Léon et al., 1999, Fabre et al., 1999, Foster et al., 2001). The study of $P2Y_{12}^{-/-}$ mice has also demonstrated the role of that receptor in microglial chemotaxis (Haynes et al., 2006).

$P2Y_6$ receptor activation by UDP results in the formation of inositol-1,4,5-trisphosphate (IP_3) and an increase in cytoplasmic Ca^{2+}. Northern blotting has revealed a rather broad distribution of $P2Y_6$ transcripts: spleen, thymus, placenta, intestine, and blood leukocytes in human (Communi et al., 1996), and lung, spleen, stomach, intestine, and aorta in rat (Chang et al., 1995). By in situ hybridization, $P2Y_6$ messengers have been detected in T cells infiltrating inflammatory bowel tissue. This expression is correlated with an UDP-induced Ca^{2+} mobilization in both activated $CD4^+$ and $CD8^+$ lymphocytes (Somers et al., 1998). $P2Y_6$ messengers also colocalize with activated microglia in vivo, and UDP facilitates the uptake of microspheres by these cells in a $P2Y_6$ receptor-dependent manner (Koizumi et al., 2007). As for the $P2Y_2$ and $P2Y_4$ receptors, $P2Y_6$ seems to control chloride secretion by rat colonic epithelium, mouse gallbladder epithelium, and nasal epithelium (Lazarowski et al., 1997; Cressman et al., 1999; Köttgen et al., 2003).

Several studies have linked $P2Y_6$ function to interleukin (IL)-8 expression. In human monocytic THP-1 cells and in 1321N1 astrocytoma cells transfected with $P2Y_6$, UDP stimulates the production of IL-8 (Warny et al., 2001). Furthermore, LPS-induced IL-8 release by monocytic cells is amplified by $P2Y_6$ (Warny et al., 2001; Kukulski et al., 2007).

The $P2Y_6$ receptor may also have an important role in vascular physiology. Indeed, its mRNA is detected in freshly isolated rat aorta and in rat aortic smooth muscle cells in primary culture (Chang et al., 1995). UDP has been shown to provoke the contraction of mouse mesenteric arteries, rat and human cerebral arteries, and mouse renal arteries (Vial and Evans, 2002; Malmsjö et al., 2003; Vonend et al., 2005). Furthermore, UDP is a growth factor for aortic smooth muscle cells in vitro (Hou et al., 2002). UDP induced an endothelium-dependent relaxation of the mouse aorta, which, like a similar action of UTP, was maintained in $P2Y_2^{-/-}$ mice, suggesting the possible involvement of the $P2Y_6$ receptor (Guns et al., 2005, 2006).

Because of the lack of pharmacological tools to unambiguously identify the physiological roles of the $P2Y_6$ receptor, we have generated a $P2Y_6$ gene-targeted null mouse strain. We have evaluated in these mice the cytokine production by peritoneal macrophages, and smooth muscle cell contraction and endothelium-dependent relaxation of the aorta in response to UDP.

Materials and Methods

Generation of $P2Y_6$ Mutant Mice. The targeting construct was designed to delete the coding exon of the mouse $P2Y_6$ gene. The $P2Y_6$ gene was cloned from a 129/Sv λ ZAP genomic library (obtained from S. Refetoff, University of Chicago, Chicago, IL). A XhoI restriction site and a LoxP site were introduced by PCR 207 bp upstream of the ATG. The eGFP sequence (from the pEGFP NI vector) (Clontech-Takara Bio Europe, Saint-Germain-en-Laye, France) was fused in C-terminal to $P2Y_6$ at a newly created BamHI site. A cassette containing the second LoxP site and a neomycin resistance cassette flanked by two FRT sites (pPKOEZ plasmid; Zhang et al., 2002) has been introduced 105 bp downstream to the polyadenylation signal, into a SacI restriction site that will be lost in the construct. The left arm is a 7-kb KpnI (endogenous)–XhoI (new) fragment. The right arm is a 2.6-kb NotI-SacII fragment amplified by PCR. The resulting 15-kb construct, cloned into pBluescript, was linearized with KpnI and introduced into RI ES cells by electroporation.

G418-resistant clones were screened by PCR using primers A forward (in the construct) and reverse (outside the targeting construct) (Table 1). Correctly targeted clones were fully characterized by PCR, Southern blot, and karyotype. One clone was used to produce chimeric animals by aggregation with CD1 morulae followed by implantation in pseudopregnant CD1 female mice. Chimeric male mice were mated to CD1 female mice to generate heterozygous $P2Y_6$-eGFP offspring. $P2Y_6$ KO mice were obtained by mating chimeric male with PGK-CRE female mice. Mice were kept in a conventional animal facility.

Homozygous $P2Y_6$-eGFP or KO mice were further characterized by Southern blot as illustrated in Fig. 1. The internal probe is a 830-bp fragment corresponding to the kanamycin resistance gene present in the pKOEZ plasmid. The external probe is an 800-bp PCR product, located approximately 5 kb downstream to the $P2Y_6$ STOP codon and amplified from mouse genomic DNA with the primers B forward and reverse (Table 1).

Isolation of Peritoneal Macrophages. Mice were injected intraperitoneally with 3 ml of 4% (w/v) thioglycollate (Difco, Detroit, MI). Four days later, mice were killed, and peritoneal cells were

TABLE 1
Sequences of primers used in cloning and in RT-PCR, and a list of primers and probes used for real-time quantitative RT-PCR

	Sequence (5′→3′)	Amplicon
		bp
A		
Forward	GCAGTTTCATTTGATGCTCG	3100
Reverse	GGTATTTAGCCCTAACCACGG	
B		
Forward	GAGGTCTAGGGTAGATCATTG	800
Reverse	AGGAGCTGAGAGTGGACAGC	
$P2Y_6$		
Forward	ACCTTAAGTCTGACTCCTGC	550
Reverse	GGTAGCGCTGGAAGCTAATG	
HPRT		
Forward	CCTGCTGGATTACATTAAAGCACTG	350
Reverse	GTCAAGGGCATATCCAACAACAAAC	
$P2Y_1$		
Forward	CAAGCAGAATGG AGACACGAGT	
Reverse	GCATAGATCATCTCAGGG ATGTCTT	
Probe	ACTCAGGAGCTAGGATCTCGTGCC TTCAC	93
$P2Y_2$		
Forward	ACCTCAAGAGCAGGAGCTGATC	
Reverse	GCCATTGATGGTGCTATTCCA	
Probe	CTGCTGCCATTGCCCTGGACCT	75
$P2Y_4$		
Forward	GGCCAGAAGAAGCAGCAGAA	
Reverse	GGCTGGGACCTAGTGATGTGA	
Probe	CCCAGCTTCCTTAGTCCAGTCCAGGG	145
$P2Y_6$		
Forward	AGCAAGGCGGCTCGTATG	
Reverse	TCTCCAGCACAGGGCAAGA	
Probe	CCTACTTGGCTGTGCGCTCCACG	129
β-actin		
Forward	GCTCTGGCTCCTAGCACCAT	
Reverse	GCCACCGATCCACACAGAGT	
Probe	ATCAAGATCATTGCTCCTCCTGAGCGC	75

harvested by washing with ice-cold phosphate-buffered saline. After centrifugation, cells were resuspended in RPMI medium containing 5% heat-inactivated fetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin and seeded into 6- or 12-well plates (for inositol phosphate measurement or cytokines production, respectively). After 2 h, nonadherent cells were removed by washing with warm phosphate-buffered saline. The cells were incubated in 1 ml of culture medium at 37°C in a humidified 5% CO_2 incubator and washed again after overnight culture.

Inositol Phosphate Measurement. Peritoneal cells were seeded at a density of 4×10^6 cells/well in six-well plates. After 24 h, macrophages were labeled with 5 μCi/ml [*myo*-D-2-^{3}H]inositol in RPMI containing 5% fetal calf serum and antibiotics. After 48 h, cells were incubated for 2 h in RPMI without serum. The cells were then incubated in the same medium supplemented with 10 mM LiCl for 20 min before the addition of the agonists. Cells were stimulated for 15 min with the various agonists. The incubation was stopped by removing the medium and the addition of 1 ml of an ice-cold 3% perchloric acid solution. Inositol phosphates were extracted and separated on Dowex AG1-X8 columns (Bio-Rad Laboratories, Nazareth Eke, Belgium) as described previously (Communi et al., 1995). [^{3}H] recovered in the soluble IP_1, IP_2, and IP_3 fractions is expressed as a percentage of the total radioactivity incorporated.

ERK1/2 Activation. Thioglycollate-recruited macrophages were seeded at a density of 0.5×10^6 cells/well in six-well plates in complete RPMI medium. After 2 and 24 h, nonadherent cells were removed by washing. The cells were incubated for 2 h in RPMI medium and then stimulated with 100 μM UDP (Sigma-Aldrich, Bornem, Belgium) for 2 min or TNF-α (30 ng/ml) for 15 min. The stimulation was stopped by removing the medium, and cells were lysed in 100 μl of Laemmli buffer. A same amount of protein for each condition was separated by SDS-polyacrylamide gel electrophoresis on 10% acrylamide gel. After transfer on polyvinylidene difluoride membrane (Bio-Rad Laboratories, Nazareth Eke, Belgium), activated ERK1/2 was detected using a phospho-p44/42 MAP kinase (Thr202/Tyr204) antibody (Cell Signaling, Leiden, the Netherlands) and the Western Lighting Chemiluminescence Reagent Plus as a revelation kit (PerkinElmer, Zaventem, Belgium).

Cytokines Production Measurement. Thioglycollate-recruited cells were seeded at a density of 0.5×10^6 cells/well in 12-well plates in complete RPMI medium. After 24 h, nonadherent cells were removed, and the macrophages were stimulated with UDP or LPS (O111:B4; Sigma-Aldrich) or both as described in Fig. 3. After 24 h, supernatants were collected for cytokine production measurement. TNF-α and IL-6 production were determined with the Ready-Set-Go enzyme-linked immunosorbent assay kits from eBioscience (Immu-

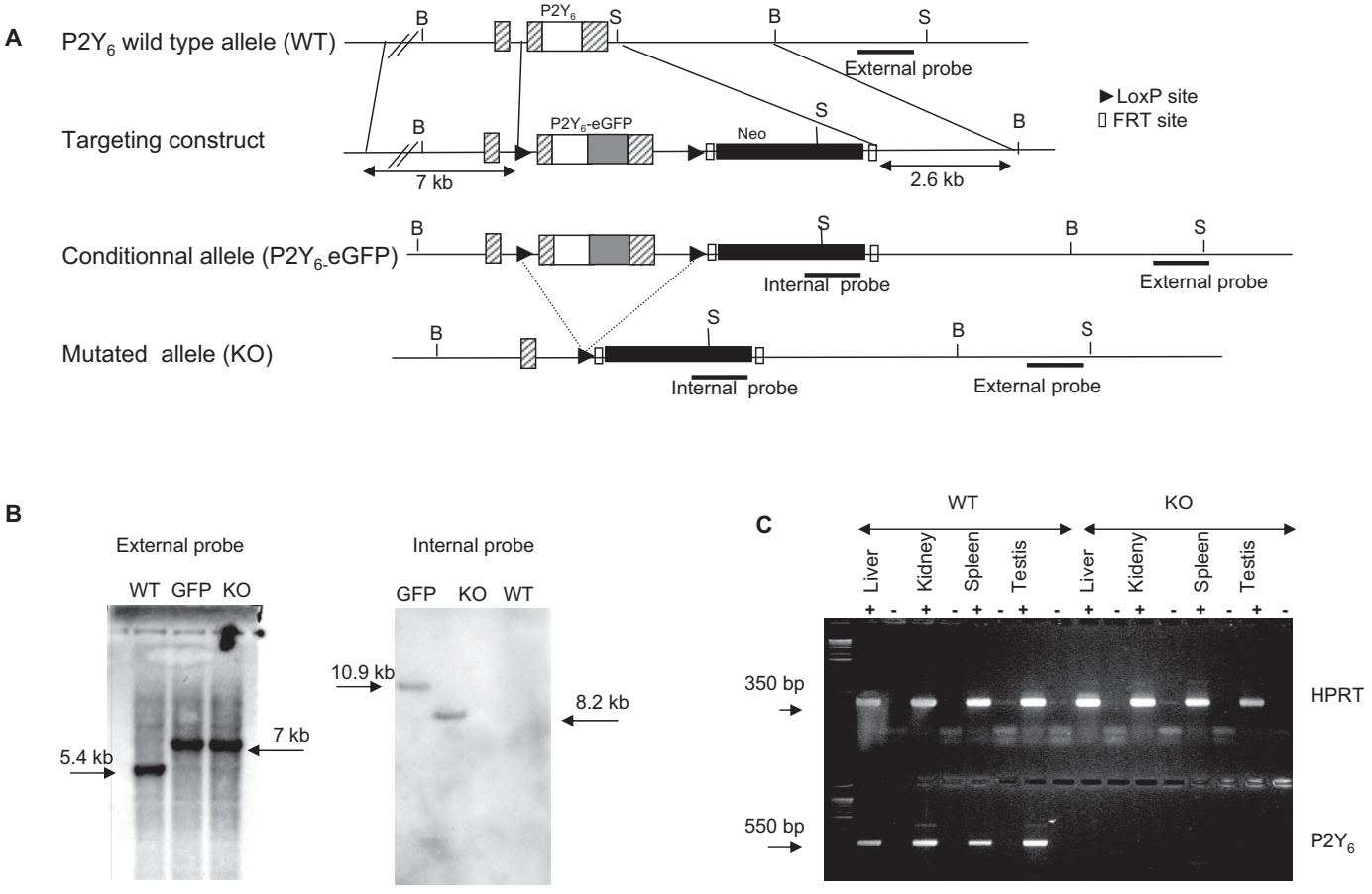

Fig. 1. Conditional deletion of the $P2Y_6$ gene. A, the $P2Y_6$ gene was fused in C-terminal to an eGFP sequence. The first LoxP site has been introduced into an intron 160 bp upstream of the unique $P2Y_6$ coding exon. A cassette containing the second LoxP site and a neomycin resistance cassette flanked by two FRT sites has been introduced 105 bp downstream to the polyadenylation signal, into a SacI site (S) that is destroyed. The cassette brings a new SacI site, which can be used to identify the different alleles by Southern blot. BstXI restriction sites are also represented (B). B, genomic DNA was prepared from WT, $P2Y_6$-eGFP, or KO homozygous mice and analyzed by Southern blot with SacI digestion for the external probe and BstXI digestion for the internal probe. The external probe detects a band of 5.4 kb for the WT locus and a band of 7 kb for the P2Y6-eGFP or KO locus, indicating homologous recombination. The internal probe detects a band of 10.9 kb for the $P2Y_6$-eGFP mice and a shorter band of 8.2 kb for the KO mice, confirming the elimination of the 2.7 fragment containing the $P2Y_6$-eGFP and 3′UTR sequences. C, detection of $P2Y_6$ mRNA in WT and KO mouse tissues by RT-PCR. RNA was prepared from liver, kidney, spleen, and testis and analyzed by RT-PCR (30 cycles). $P2Y_6$ RNA is present in the wild-type organs (PCR product of 550 bp) but absent in KO mice. + and − indicate plus reverse transcriptase or minus reverse transcriptase. Hypoxanthine-guanine phosphoribosyltransferase is used to control cDNA quality.

nosource, Halle-Zoersel, Belgium). MIP-2 production was measured with the mouse MIP-2 Duo-Set Elisa Kit (R&D Systems Europe Ltd., Oxon, United Kingdom).

Isolation of the Aorta. Mice (WT $n = 9$, KO $n = 9$) were anesthetized with sodium pentobarbital (75 mg/kg i.p.; Nembutal, Sanofi, Brussels, Belgium), and the aorta was carefully removed and cleaned of adherent tissue. The thoracic aorta was systematically divided into five 2-mm wide rings to study vasomotor function (Crauwels et al., 2003; Guns et al., 2005). The aortic arch was used for RNA isolation.

Vasomotor Studies. Rings were mounted between two parallel tungsten wire hooks in 10-ml organ baths with a Krebs-Ringer solution (37°C, continuously aerated with 95% O_2/5% CO_2, pH 7.4) containing 118 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, 0.025 mM CaEDTA, and 11.1 mM glucose. Tension was measured isometrically with a force transducer (Statham UC2; Gould, Cleveland, OH) connected to a data acquisition system (Moise 3; EMKA Technologies, Paris, France). Rings were gradually stretched until a stable loading tension of 16 mN to bring the segments to their optimal length-tension relationship. Indomethacin 10 μM was always present to avoid vasomotor interferences due to contractile prostanoids (Crauwels et al., 2003; Guns et al., 2005). Rings were first contracted with 50 mM KCl. After three washing steps, a cumulative concentration-response curve was made for phenylephrine (3 nM to 30 μM), and the EC_{50} value was assessed for each vessel segment. Thereafter, vessels were contracted with their individual EC_{50} values of phenylephrine followed by cumulative concentration-response of ATP, UTP, ADP, or UDP. After washout, rings were treated with 300 μM nitro-L-arginine (L-NA) and 300 μM nitro-L-arginine methyl ester (L-NAME), and contractile responses to each nucleotide were determined. A rotation system was used to generate vascular activity data from an average thoracic aorta segment.

RT-PCR Analyses. For classic RT-PCR analysis, total cellular RNA was prepared using the Tripure RNA extraction solution (Roche Applied Science, Vilvoorde, Belgium) followed by DNase I digestion (Fermentas, St. Leon-Rot, Germany). cDNA was prepared using the First Strand cDNA synthesis kit (Fermentas).

For real-time RT-PCR analysis of mouse aorta, the aortic arch was sliced with a sterile scalpel blade and put directly in lysis buffer containing guanidine thiocyanate (Stratagene, La Jolla, CA). Samples were then transferred onto an RNA-binding fiber matrix (Microprep kit; Stratagene) and treated with Rnase-free DNase I. Total RNA was eluted using Rnase- and DNase-free water at 60°C and stored at −80°C.

mRNA expression was evaluated on an ABI Prism 7300 (Applied Biosystems, Foster City, CA) (40 cycles of 15 s at 95°C and 1 min at 60°C) using the two-step reverse transcriptase-quantitative PCR core kit, primers, and probes (Table 1) from Eurogentec (Seraing, Belgium). mRNA expression of $P2Y_1$-, $P2Y_2$-, and $P2Y_6$ receptors was expressed relative to β-actin. The relative expression of P2Y receptors in WT mice was arbitrary set to 100%.

Results

$P2Y_6$ Gene Inactivation. We have designed a conditional targeting construct to delete the coding exon of the mouse $P2Y_6$ gene. The $P2Y_6$ gene was fused to the eGFP reporter gene, and the resulting fusion gene was flanked by two LoxP sites. A neomycin resistance cassette flanked by two FRT sites was introduced downstream to the polyadenylation signal. Left and right homology arms were, respectively, 7 and 2.7 kb (Fig. 1.A).

Correctly targeted ES cell clones were aggregated with CD1 morulae to produce chimeric male mice. These chimeras were mated to CD1 female mice to produce $P2Y_6$-eGFP heterozygous mice. To study $P2Y_6$ expression, homozygous $P2Y_6$-eGFP mice were produced (conditional allele). Unfortunately, we were not able to detect the eGFP protein, even by immunohistochemistry with an anti-green fluorescent protein antibody.

Mice with a deletion of the $P2Y_6$ gene were obtained by mating the chimeric male mice with female mice expressing the CRE recombinase under the control of a PGK promoter. The F1 mice were intercrossed to obtain wild-type, heterozygous, and null mice. The mutation was kept on a mixed CD1-129 sv background. Southern blots on liver DNA and RT-PCR on RNA extracted from various organs (spleen, testis, liver) confirmed the inactivation (Fig. 1, B and C).

All of the genotypes were obtained at the expected ratio (26% of WT and KO mice, 48% of heterozygous animal, $n = 143$). The $P2Y_6$-deficient mice are not distinguishable from wild-type mice. They are fertile, and their growth is identical with that of wild-type mice. Gross examination of internal organs did not reveal any abnormality.

Inositol Phosphate Production in Thioglycollate Elicited WT and KO Macrophages. As described in the Introduction, the stimulation of cultured macrophages with UDP is associated with IP_3-dependent Ca^{2+} mobilization and ERK1/2 pathway activation. To confirm the $P2Y_6$ gene inactivation and to identify the receptor mediating these UDP effects, we compared inositol phosphates production and ERK1/2 activation after UDP stimulation of thioglycollate-elicited macrophages isolated from WT and KO mice and maintained in culture.

By RT-PCR, we observed that $P2Y_2$ and $P2Y_6$ mRNA were expressed both in freshly isolated and cultured macrophages

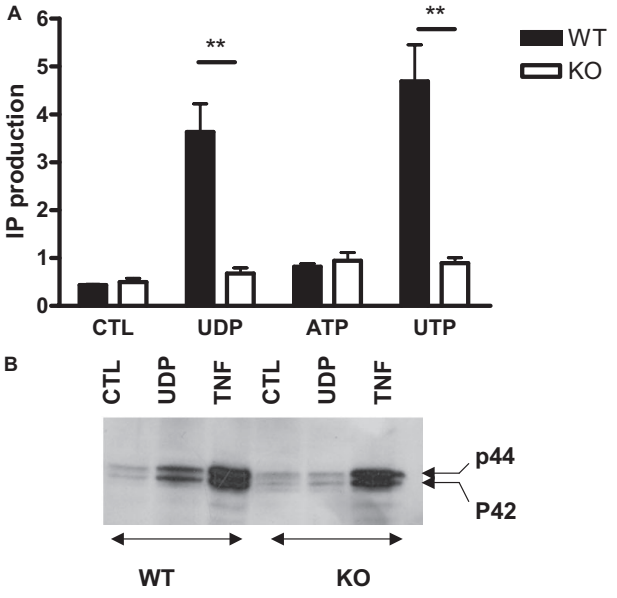

Fig. 2. Inositol phosphate production and ERK signaling in WT and KO thioglycollate-elicited macrophages. A, the peritoneal macrophages from WT and KO mice were labeled with [*myo*-^{3}H]inositol, and IP release was measured in response to a 15-min agonist stimulation. Values are means ± S.D. of five and four experiments (performed in triplicate), respectively, from WT or KO macrophages incubated in the absence of agonist (control: CTL) or in the presence of UDP 100 μM, ATP 100 μM, and UTP 100 μM. For each experimental condition, IP production is expressed as the percentage of [^{3}H] recovered in the soluble IP_1, IP_2, and IP_3 fractions relative to the total radioactivity incorporated. B, Western blot analysis using phospho-p44/42 MAP kinase antibody of WT and KO macrophages untreated or treated with UDP 100 μM for 2 min or TNF-α 30 ng/ml for 15 min.

and that cell culture triggered an increased expression of P2Y$_6$ mRNA, whereas that of P2Y$_2$ was not modified (data not shown). Macrophages isolated from P2Y$_6$ knockout mice were indistinguishable from wild-type cells, and an equivalent number of macrophages was recovered from the peritoneal cavity of both genotype mice.

As illustrated in Fig. 2A, WT macrophages activation by UDP resulted in a 9-fold increase in inositol phosphate (IP) production (control: 0.4 ± 0.0.01, n = 5; UDP 100 μM: 3.6 ± 0.6, n = 5). Macrophages isolated from P2Y$_6$ KO mice did not respond to UDP 100 μM (control: 0.5 ± 0.01, n = 4; UDP 100 μM: 0.7 ± 0.1, n = 4), whereas WT and KO macrophages responded equally to ATP 100 μM (WT: 0.8 ± 0.1, n = 5; KO: 0.9 ± 0.2, n = 3). WT macrophage activation by UTP 100 μM resulted in an 11-fold increase in IP production (control: 0.4 ± 0.1, n = 5; UTP: 4.7 ± 0.8, n = 5). In the KO macrophages, the UTP effect was greatly reduced, with a residual response comparable with the ATP effect (control: 0.5 ± 0.1, n = 4; UTP: 0.9 ± 0.2, n = 4).

UDP rapidly induced ERK1/2 activation in WT macrophages: phospho ERK1/2 was already detected in extracts from 2-min UDP-stimulated cells (Fig. 2B). This effect was transient because the level of ERK1/2 phosphorylation was almost identical with the level of the control condition after a 15-min incubation (data not shown). However, in KO macrophages, a 2-min UDP stimulation did not lead to any ERK1/2 activation because the level of phospho ERK1/2 was unchanged compared with this of control conditions (Fig. 2B). The stimulation with TNF-α induced ERK1/2 phosphorylation in both WT and KO macrophages. All of these results

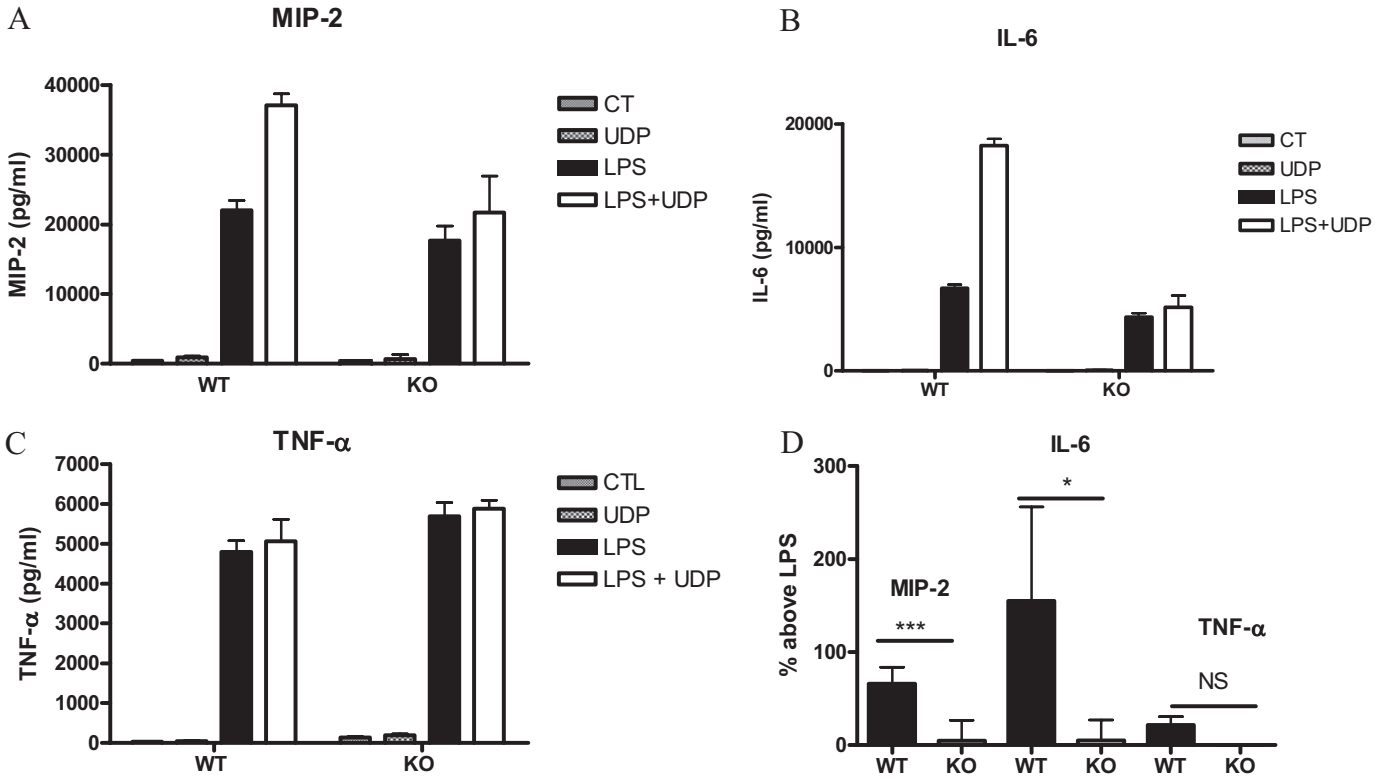

Fig. 3. Effect of P2Y$_6$ deletion on cytokines production by LPS-stimulated peritoneal macrophages. A to C, WT and KO thioglycollate-elicited macrophages were stimulated with 100 μM UDP, 10 ng/ml LPS, or 100 μM UDP + 10 ng/ml LPS for 24 h. MIP-2 (A), IL-6 (B), and TNF-α (C) production was measured in the culture supernatant by enzyme-linked immunosorbent assay. Data are mean ± S.D. of triplicates from one mouse. D, summary of results expressed as the percentage of stimulation above LPS. Data are means ± S.D. of eight WT and seven KO mice for MIP-2, four WT and five KO mice for IL-6, and three WT and three KO mice for TNF-α.

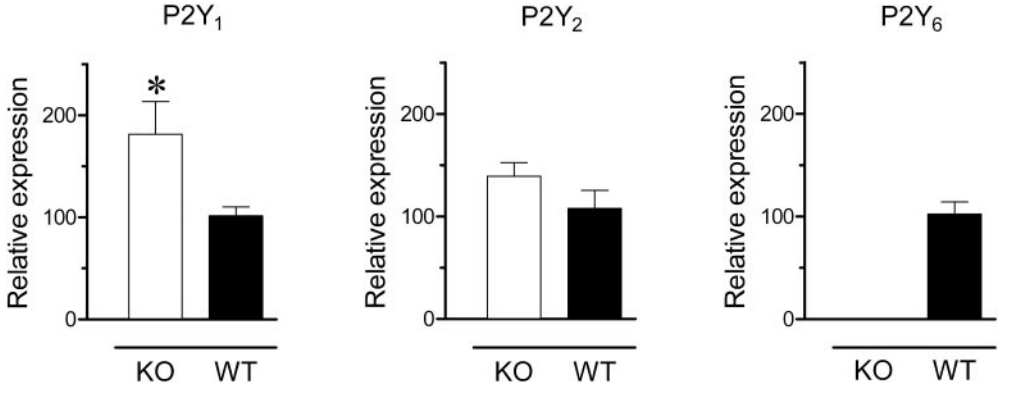

Fig. 4. Relative mRNA expression of P2Y$_1$ (left), P2Y$_2$ (middle), and P2Y$_6$ (right) receptors in aortic arches of WT and P2Y$_6$ receptor KO mice. The expression of the P2Y$_6$ receptor was not detectable, whereas mRNA of P2Y1$_1$ was increased in the aorta of KO mice. β-Actin was used as endogenous reference gene, and the mean value of the WT was set to 100%. Results are expressed as mean ± S.E.M., WT n = 5, KO n = 5. *, P = 0.027.

reveal that $P2Y_6$ is the unique UDP-responsive receptor expressed by thioglycollate-elicited macrophages and functionally confirm the loss of expression of the *P2Y*$_6$ gene.

Cytokine Production in Thioglycollate-Elicited WT and KO Macrophages. The $P2Y_6$ receptor is involved in IL-8 production by human monocytic cells (Warny et al., 2001; Cox et al., 2005; Kukulski et al., 2007). Chemokines with protein sequence homology to human IL-8 have not been identified in the mouse genome, and it is suggested that the mouse MIP-2 protein is a functional homolog of the human IL-8. Therefore, we have investigated the implication of the mouse $P2Y_6$ receptor in MIP-2 secretion. We also compared the production of two other proinflammatory cytokines, IL-6 and TNF-α, by mouse WT or KO peritoneal macrophages. Figure 3, A to C, illustrate typical results obtained from one mouse (each assay done in triplicate). Treatment of peritoneal macrophages with LPS (10 ng/ml) for 24 h resulted in strong MIP-2 (Fig. 3A), IL-6 (Fig. 3B), and TNF-α (Fig. 3C) production. Incubation with UDP 100 μM alone had no effect. For IL-6 and MIP-2 but not TNF-α, coincubation with LPS and UDP potentiated the response to LPS in WT mice but not in KO mice. Figure 3D summarizes the results obtained from several mice, and data are expressed as the percentage of stimulation above LPS. Coincubation of WT macrophages with LPS (10 ng/ml) and UDP (100 μM) significantly increased MIP-2 secretion (66 $\pm$ 6% above LPS $n = 8$), but this effect was lost in KO mice (4.5 $\pm$ 8.5% above LPS $n = 7$). Similar results were observed for IL-6 secretion: costimulation of WT cells with LPS and UDP increased the IL-6 production by 155 $\pm$ 50% above LPS, $n = 4$. This effect was lost in KO macrophages: 5 $\pm$ 10% above LPS, $n = 5$. Coincubation with UDP had no effect on TNF-α production (WT: 22 $\pm$ 9% above LPS, $n = 3$; KO: 2 $\pm$ 17% above LPS $n = 3$). These results confirm a role for $P2Y_6$ in the production of proinflammatory cytokines.

$P2Y_6$ Receptor Function in Endothelial and Vascular Smooth Muscle Cells of WT and KO Aorta: mRNA Expression and Vasomotor Responses. As expected, mRNA of $P2Y_6$ receptors was detectable in the aorta of WT mice, but not in KO mice (C_t value >40; Fig. 4). The relative expression of $P2Y_2$ receptors was not different between both strains, but $P2Y_1$ receptor mRNA was approximately 2-fold more abundant in the aorta of KO mice compared with WT. Force development evoked by 50 mM KCl (WT, 13.1 $\pm$ 0.5 mN; KO, 12.8 $\pm$ 0.6 mN) or phenylephrine (WT, 13.9 $\pm$ 0.2 mN; KO, 13.4 $\pm$ 0.4 mN), and sensitivity for phenylephrine (pD2: WT, 6.59 $\pm$ 0.04; KO, 6.57 $\pm$ 0.09) were very similar in both strains, indicating that contractile function was unaltered.

Endothelium-dependent relaxation was tested in the phenylephrine precontracted thoracic aorta of $P2Y_6$ knockout mice and WT mice (Fig. 5). In WT segments ATP, UTP, and UDP evoked complete relaxation, whereas ADP induced a partial response. Maximum responses induced by ATP (WT, 91.9 $\pm$ 2.7; KO, 86.6 $\pm$ 3.1% relaxation) or ADP (WT, 46.2 $\pm$ 11.8; KO, 50.2 $\pm$ 10.3% relaxation) and sensitivity to ATP (pD2: WT, 5.57 $\pm$ 0.07; KO, 5.44 $\pm$ 0.07) or ADP (pD2: WT, 6.29 $\pm$ 0.42; KO, 6.20 $\pm$ 0.31) were not different between the two strains. However, the curve of UTP was significantly shifted to the right (pD2: WT, 6.65 $\pm$ 0.07; KO, 6.08 $\pm$ 0.05, $P = 0.0004$), and the shift was even more pronounced for the $P2Y_6$-selective agonist UDP (pD2: WT, 6.66 $\pm$ 0.11; KO, <4.50).

To study P2Y receptor function on smooth muscle cells, endothelial nitric-oxide synthase was blocked by the combination of 300 μM L-NA and 300 μM L-NAME before the addition of nucleotides. ADP and ATP (up to 100 μM) induced very small constrictor responses that were not different between both strains. High concentrations of UTP, and particularly UDP, caused more substantial contractions in WT aorta rings that were completely absent in aorta rings from KO mice (Fig. 6).

Discussion

In this article we report the generation and the analysis of a $P2Y_6$ KO mouse strain. The gene targeting has been demonstrated by Southern blotting and the loss of expression by RT-PCR on RNA extracted from various tissues. Moreover we demonstrate that the $P2Y_6$ receptor is the unique receptor activated by UDP in terms of inositol phosphate production and ERK1/2 MAP kinase activation in thioglycollate-elicited peritoneal macrophages. It was shown recently that freshly isolated resident peritoneal macrophages are not responsive to UDP (del Rey et al., 2006). But it must be emphasized that the macrophages studied here were maintained in culture for at least 24 h. It is known that adherence induces macrophage activation, which seems to be associated with an increased expression of the $P2Y_6$ mRNA and a functional state of this receptor.

In cultured macrophages, UTP also triggered IP production. This effect was mainly due to the $P2Y_6$ receptor activation because it is largely reduced in KO macrophages. The remaining effect is most probably due to $P2Y_2$ stimulation because it is equivalent to the ATP response.

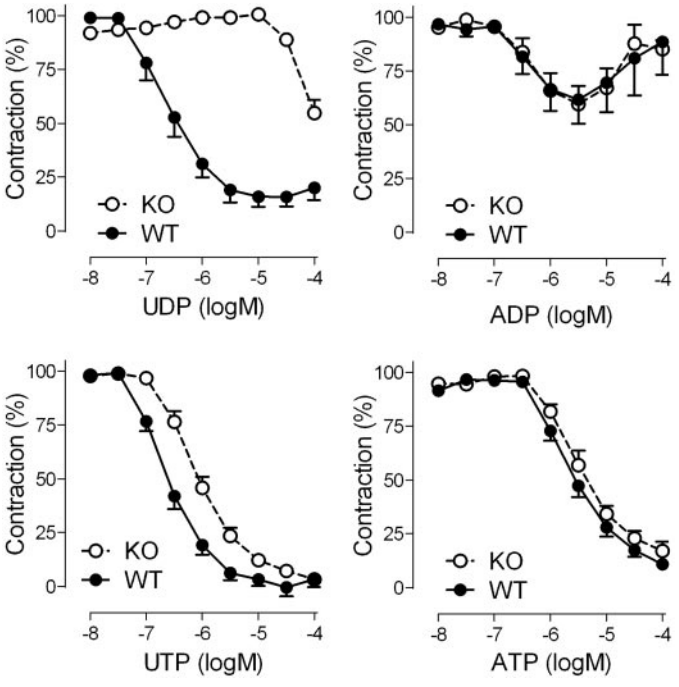

Fig. 5. Relaxation induced by UTP, UDP, ADP, and ATP in phenylephrine-constricted segments of the thoracic aorta of WT and $P2Y_6$ receptor KO mice. Nucleotides evoked complete (ATP, UTP, and UDP) or partial (ADP) endothelium-dependent relaxations in WT rings. The UDP and to a lesser extent UTP relaxation curves were displaced to the right in rings from KO mice. Results are expressed as mean $\pm$ S.E.M., WT $n = 5$, KO $n = 5$.

Extracellular nucleotides are known to activate monocytes and macrophages. In particular, UDP stimulates the secretion of IL-8 and other chemokines and cytokines from human monocytes (Warny et al., 2001; Cox et al., 2005; Kukulski et al., 2007). In mouse macrophages, UDP did not induce the production of MIP-2, the functional homolog of the human IL-8, but it amplified its production triggered by LPS. This was also true for the synthesis of IL-6. These effects were due to the activation of the P2Y$_6$ receptor because they were absent in KO macrophages. All of these results demonstrate the functional expression of P2Y$_6$ in those cells and the ability of UDP, via P2Y$_6$, to potentiate LPS-induced release of inflammatory mediators.

It has been known for a long time that P2Y$_1$ and P2Y$_2$ receptors are coexpressed on endothelial cells isolated from the aorta and other vessels and mediate endothelium-dependent relaxation and prostacyclin release in response to nucleotides (Motte et al., 1993; Communi et al., 1995; Buvinic et al., 2002). However, recent studies on the mouse aorta revealed in an unexpected way that although the endothelium-dependent relaxation by ATP was severely decreased in P2Y$_2^{-/-}$ mice, the relaxing effect of UTP was maintained, indicating the involvement of another receptor (Guns et al., 2006). This receptor might be P2Y$_6$ because UDP induced a relaxing effect on the mouse aorta with pharmacological features compatible with that receptor (Guns et al., 2005). Our data now demonstrate that hypothesis in a conclusive way, because the relaxation induced by UDP was abolished in P2Y$_6^{-/-}$ mice. The maintenance of a significant effect of UTP in the aorta of those mice can be explained by an action at the P2Y$_2$ receptor and suggests a small contribution of the P2Y$_6$ receptor in the UTP effect most probably due to the degradation of UTP into UDP. It is interesting that endothelium-dependent relaxation by UDP and UTP has been reported in isolated human pial arteries (Hardebo et al., 1987) and human left internal mammary artery segments (Wihlborg et al., 2003). Together with the recent reports that endothelium-dependent relaxation is impaired in P2X$_4^{-/-}$ (Yamamoto et al., 2006) and P2X$_1^{-/-}$ (Harrington et al., 2007) mice, our findings expand the range of P2 receptors functionally expressed on arterial endothelial cells.

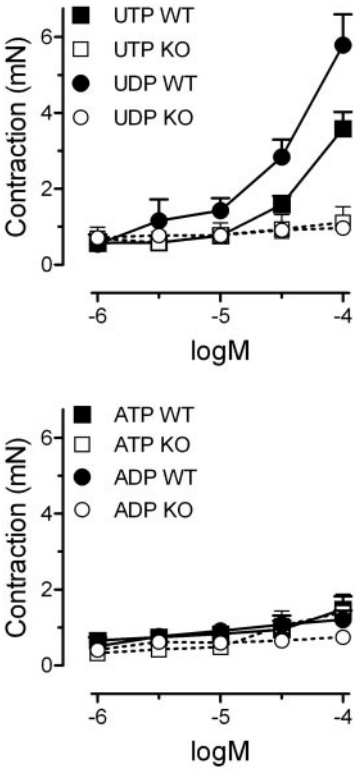

Fig. 6. Contractile responses induced by ATP, ADP, UTP, and UDP in aorta segments of WT and P2Y$_6$ receptor KO mice in the presence of 300 μM L-NA and 300 μM L-NAME. ATP and ADP were inactive, whereas UDP and UTP evoked contractions in WT rings, which were absent in rings from KO mice. Results are expressed as mean ± S.E.M., WT $n = 5$, KO $n = 5$.

The rat P2Y$_6$ receptor was cloned from an aortic smooth muscle cell cDNA library, and Northern blotting showed a high expression in these cells and in the aorta (Chang et al., 1995). Since then, several reports have shown contractile effects of UDP on various arteries, associated with the expression of P2Y$_6$ messengers (Hartley et al., 1998; Rubino et al., 1999; Lewis et al., 2000; Wang et al., 2002; Malmsjö et al., 2003). In P2X$_1^{-/-}$ mice, the contractile effect of ATP on mesenteric arteries was abolished, whereas the vasoconstriction by nerve stimulation was reduced by approximately 50% (Vial and Evans, 2002). The contractile effect of UTP and UDP was maintained, suggesting the involvement of the P2Y$_6$ receptor, although the pharmacology was atypical because UDP and UTP were almost equipotent. Our data on the aorta now demonstrate that the P2Y$_6$ receptor is indeed responsible for the contractile action of both UDP and UTP. These results confirm that P2Y$_6$ is expressed and functional in vascular smooth muscle cells.

In conclusion, we have generated P2Y$_6^{-/-}$ mice that are grossly normal in terms of growth, behavior, and reproduction. Our initial results show that this receptor is expressed in macrophages and aortic endothelial and smooth muscle cells. They demonstrate in a conclusive way previous suggestions that the P2Y$_6$ receptor is involved in both the direct contraction (Vial and Evans, 2002) and endothelium-dependent relaxation (Guns et al., 2006) of the aorta by UDP.

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