Increased atherosclerosis in P2Y$_{13}$/apolipoprotein E double-knockout mice: contribution of P2Y$_{13}$ to reverse cholesterol transport

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Aims
High-density lipoproteins (HDLs) protect against atherosclerosis mainly due to their function in hepatobiliary reverse cholesterol transport (RCT). This is a process whereby excess cholesterol from peripheral tissues is transported by HDL particles to the liver for further metabolism and biliary excretion. Hepatic uptake of HDL holoparticles involves the P2Y$_{13}$ receptor, independently of the selective cholesteryl ester uptake mediated by scavenger receptor class B, type I (SR-BI). Accordingly, P2Y$_{13}$-deficient mice (P2Y$_{13}^{-/-}$) have impaired RCT. This study assessed whether P2Y$_{13}$ deficiency would affect atherosclerotic development.

Methods and results
P2Y$_{13}^{-/-}$ mice were crossbred with atherosclerosis-prone apoE$^{-/-}$ mice. When 15 weeks old, P2Y$_{13}^{-/-}$/apoE$^{-/-}$ mice had more aortic sinus lesions than apoE$^{-/-}$ mice. Bone marrow transplantation showed that the absence of the P2Y$_{13}$ receptor in blood cells did not lead to significantly greater atherosclerotic plaque size formation compared with control apoE$^{-/-}$ reconstituted animals. Conversely, the absence of the P2Y$_{13}$ receptor, except in blood cells, resulted in lesion sizes similar to that in P2Y$_{13}^{-/-}$/apoE$^{-/-}$ reconstituted mice, pointing to a role for non-haematopoietic-derived P2Y$_{13}$. Unexpectedly, P2Y$_{13}^{-/-}$/apoE$^{-/-}$ mice displayed a lower HDL-cholesterol level than apoE$^{-/-}$ mice, which might be due to greater SR-BI expression in the liver. However, P2Y$_{13}$ deficiency in apoE$^{-/-}$ mice was translated into reduced biliary and faecal sterol excretion and impaired RCT from macrophage to faeces, suggesting that an alteration in hepatobiliary RCT could be solely responsible for the greater atherosclerosis observed.

Conclusion
The P2Y$_{13}$ receptor protects against atherosclerosis, primarily through its role in hepatobiliary RCT.

Keywords
High-density lipoprotein • Purinergic receptor • ATP synthase • Cholesterol • Bile acids

1. Introduction
Several large prospective studies have established that high-density lipoprotein (HDL) cholesterol is an independent negative risk factor for coronary artery disease and a major protective factor against atherosclerosis. The atheroprotective effects of HDL particles have been attributed to several mechanisms, which primarily reflect their ability to promote reverse cholesterol transport (RCT), a major pathway for removing excess cholesterol from the body.

The classic RCT pathway is a multistep process which involves (i) HDL-mediated efflux of excess cholesterol from extrahepatic cells, and particularly from atherosclerosis lipid laden macrophages in the arterial wall, (ii) HDL uptake by hepatocytes, and (iii) excretion of cholesterol from HDL into the bile and ultimately faeces, either directly or after metabolic conversion into bile acids (BAs).

Two pathways for HDL uptake by hepatocytes have been identified. The first involves the scavenger receptor class B, type I (SR-BI) that mediates selective uptake of cholesteryl ester (CE) from HDL by the liver. Two pathways for HDL uptake by hepatocytes have been identified. The first involves the scavenger receptor class B, type I (SR-BI) that mediates selective uptake of cholesteryl ester (CE) from HDL by the liver. The second involves an ecto-F$_1$-ATPase expressed at the plasma membrane of hepatocytes and contributes to the uptake of both protein and lipid moieties of the HDL particle, a process called HDL holoparticle endocytosis. More precisely, apolipoprotein A-I (apoA-I, the major
protein component of HDL) binds to the ecto-F_{1,ATPase} and stimulates the hydrolysis of extracellular ATP to ADP. The extracellular ADP generated then selectively activates the P2Y_{13} purinergic receptor, resulting in cytoskeleton reorganization and subsequent clathrin-dependent endocytosis of HDL holoproteins through a low-affinity receptor that has yet to be identified.

In addition to the hepatocytes, P2Y_{13} receptor mRNA is also expressed in neurons, osteoblasts, and immune cells such as monocytes, T cells, and dendritic cells. However, it does not seem to be involved in the immune response, at least not in immunological dendritic cell functions.

Consistent with the role of P2Y_{13} in HDL uptake by hepatocytes, hepatic HDL uptake, associated with reduced biliary sterol secretions and impaired macrophages-to-faeces RCT, was much lower in homozygous null P2Y_{13}-knockout (KO) mice (C57BL/6 P2Y_{13}^{-/-}) faeces than in C57BL/6 wild-type (WT) littermates.

Thus, because macrophages-to-faeces RCT is strongly associated with atherosclerosis, we wished to explore the potential involvement of P2Y_{13} receptor in the development of atherosclerosis. We recently observed that despite dramatically impaired RCT, P2Y_{13}^{-/-} mice fed a high cholesterol diet (1.25% cholesterol) have just as little lipid deposition in the aortic sinus as their WT littermates. However, this was not completely unexpected because lipoprotein metabolism in mice is different from that in humans, and WT mice have low levels of atherogenic apoB-lipoproteins, a limiting factor for cholesterol-induced atherogenesis.

In this context, the apolipoprotein E-deficient (apoE^{-/-}) mouse model has the advantage that it spontaneously develops atherosclerotic lesions. These lesions have many of the characteristics of those in humans with high levels of atherogenic apoB-lipoproteins and limited immune-mediated inflammatory responses at the early stage.

We therefore crossed apoE^{-/-} with P2Y_{13}^{-/-} mice to generate P2Y_{13}^{-/-}apoE^{-/-} double-KO mice (P2Y_{13}^{-/-}apoE^{-/-}). These animals were maintained on regular chow before collection of their hearts for analysis of the size and composition of atherosclerotic lesions. These lesions were bigger and more infiltrated with macrophages in P2Y_{13}^{-/-} and apoE^{-/-} compared with apoE^{-/-} mice. Bone marrow (BM) transplantation was performed to determine the relative contribution to the development of atherosclerosis of the P2Y_{13} receptors expressed on blood cells and in the rest of the body. The results exclude the involvement of blood cell P2Y_{13} receptors and confirm a role of the P2Y_{13} receptors in the liver since, compared with apoE^{-/-} mice, P2Y_{13}^{-/-}/apoE^{-/-} mice displayed profoundly impaired hepatobiliary secretion of sterols associated with reduced excretion of sterols in the faeces and reduced macrophages-to-faeces RCT.

2. Methods

2.1 Materials

All reagents (analytical grade) were from Sigma. Horseradish peroxidase (HRP)-conjugated anti-rabbit antibodies were purchased from Cell Signaling Technology (distributed by Ozyme, Saint-Quentin en Yvelines, France). The enhanced chemiluminescence system was purchased from Amersham Biosciences, Inc. (Orsay, France). 3H-cholesterol was purchased from PerkinElmer (Courtaboeuf, France).

2.2 Animals and diets

All animals used were male mice on the C57BL/6 genetic background. P2Y_{13}^-/apoE double-KO mice (P2Y_{13}^{-/-}/apoE^{-/-}) were generated by crossing P2Y_{13}^{-/-} with apoE^{-/-} mice (Jackson Laboratory, Bar Harbor, ME, USA). Littermate controls were from the breeding colony. Mice were caged in animal rooms with L : D 12 : 12 and ad libitum access to water and chow diet (#R04-10, SAFE, France) for 15 weeks. Heart tissue was taken from 15-week-old mice. All other procedures were carried out on 15-week-old mice unless otherwise stated. During experiments, mice were anesthetized using isoflurane (2%) and were euthanized using cervical dislocation.

All animal procedures were performed in accordance with the guidelines of the Committee on Animals of the Mid-Pyrénées Ethics Committee on Animal Experimentation and with the French Ministry of Agriculture license. Moreover, this investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health or the Directive 2010/63/EU of the European Parliament.

2.3 Atherosclerotic lesion characterization in the aortic sinus

ApoE^{-/-} and P2Y_{13}^{-/-}/apoE^{-/-} mice were killed, and their heart and ascending aorta removed. These tissues were washed in PBS and kept at 4°C for 24 h. Each heart was frozen on a cryostat mount with optimum cutting temperature compound (Tissue-Tek), and stored at −80°C. Aortic sinus sections were processed according to our standard method. For immunostaining, frozen sections from the aortic root were fixed in acetone/methanol, air-dried, and incubated with 10% relevant serum for 30 min. The specific primary antibody used was anti-remarkocyte/macrophage (clone MOMA-2, Serotec, Oxford, UK). The sections were then incubated with the corresponding secondary biotinylated antibodies (Dako) and visualized with an avidin–biotin–HRP complex (Vectastain ABC Kit, Vector Laboratories) and the 3′ dianamobenzidine peroxidase substrate kit (DakoCytomation, Glostrup, Denmark). Countercolouration was performed with Mayer’s haematoxylin. Irrelevant IgGs were used as a negative control. At least five sections per mouse were examined for each staining.

2.4 BM transplantation

To induce BM aplasia, apoE^{-/-} (apoE KO) and P2Y_{13}^{-/-}/apoE^{-/-} (double KO, dKO) 7-week-old male mice were exposed to a single dose of 9 Gy (2.8 Gy/min) total body irradiation using a BIOBEAM gamma irradiation device (Gamma-Service Medical GmbH) 1 day before the transplantation. We isolated BM cells from donor apoE KO and dKO mice, by flushing their femurs and tibias with sterile PBS. Single-cell suspensions were prepared by passing the cells through a 30 μm nylon gauze. Cells were counterspent and resuspended in sterile PBS. Irradiated recipients received 10^7 BM cells by intravenous injection into the orbital vein giving four groups:

(i) apoE^{-/-} recipient mice transplanted with apoE^{-/-} BM (KO > KO),
(ii) apoE^{-/-} recipient mice transplanted with apoE^{-/-}/P2Y_{13}^{-/-} BM (dKO > KO),
(iii) P2Y_{13}^{-/-}/apoE^{-/-} recipient mice transplanted with P2Y_{13}^{-/-}/apoE^{-/-} BM (dKO > dKO), and
(iv) P2Y_{13}^{-/-}/apoE^{-/-} recipient mice transplanted with apoE^{-/-} BM (KO > dKO).

Post transplantation, mice received chow diet for 8 weeks, and soaked with water and antibiotics in a standard fashion. Mice were euthanized and their hearts collected and processed as described in Section 2.3. Successful engraftment was confirmed by PCR.

2.5 Plasma lipoprotein analyses

Individual plasma samples were collected from apoE^{-/-} and P2Y_{13}^{-/-}/apoE^{-/-} mice that had received no food for 3 h. Total cholesterol and triglycerides (TGs) were measured with commercial kits (CHOD-PAP for cholesterol and GPO-PAP for TGs; BIOLABO SA, Maizié, France). Quantification of plasma lipoproteins was performed using an Ultimate 3000 HPLC system (Dionex, USA). About 10 μL of plasma was injected and lipoproteins were separated on Superose 6 10/300GL columns (GE Healthcare, USA) with PBS at pH 7.4 as mobile phase at a 0.5 mL/min flow rate. The column effluent was split equally into two lines by a microspltter 50:50, mixing with cholesterol and/or TG reagents (BIOLABO), thus achieving a simultaneous profile from single injection. The two enzymatic reagents were each
pumped at a rate of 0.1 mL/min using ISO-3100 analytical pumps (Dionex). Both enzymatic reactions proceeded at 40°C in a reactor coil (10 m × 0.5 mm, i.d.). Quantification was performed by measuring the percentage peak area of each lipoprotein (VLDL, LDL, and HDL) and by multiplying each percentage to the total amount of cholesterol and/or TGs measured in plasma samples.

### 2.6 Hepatic lipid analyses
Hepatic cholesterol and TGs were analysed, following Bligh and Dyer lipid extraction, by gas–liquid chromatography following standard methods.11

### 2.7 Biliary and faecal lipid analysis
Apoe−/− and P2Y13−/−/apoE−/− mice were starved for 3 h and were then anaesthetized by intraperitoneal injection of ketamine hydrochloride (100 mg/kg) and xylazine hydrochloride (15 mg/kg). At 11.00 a.m., the gall-bladder was cannulized and bile was collected for 30 min, after a stabilization time of 30 min. Bile flow was determined gravimetrically and output rates of BAs, cholesterol, and phospholipids were calculated by multiplying bile flow and concentration following our standard method.17 Faeces were collected for 48 h, lyophilized, and ground. Faecal neutral sterols and BA content were analysed after homogenization of 100 mg of faeces in 1 mL of purified water. For neutral sterols, the homogenates were analysed by gas–liquid chromatography, following Bligh and Dyer lipid extraction. For BAs, the faecal homogenates were acidified with 1 M acetic acid in 750 μL of methanol, then the mixture was centrifuged at 3500 × g for 10 min. The supernatant was then extracted by solid-phase extraction and finally eluted with methanol/water (90/10, v/v). The solution obtained was evaporated under vacuum and suspended in 100 μL of methanol. After centrifugation at 20 000 × g for 2 min, 25 μL of the supernatant was injected into the HPLC C18 column for analysis.

### 2.8 Analysis of hepatic gene expression by real-time quantitative PCR
Liver RNA isolation, reverse transcription, and real-time quantitative PCR analysis were performed by standard methods.17 The level of mRNA expression in apoE−/−/P2Y13−/− mice was calculated relative to the average of the housekeeping gene RPS29 and further normalized to the relative expression level in apoE−/− mice (eight mice per group).

### 2.9 Western blot
Individual mouse livers were homogenized using the Precellys® lysis kit in TNE buffer (Tris–HCl (50 mM), NaCl (100 mM), and EDTA (0.1 mM) 1% NP40) containing a protease inhibitor cocktail (leupeptin, aprotinin, and pepstatin A). Protein concentration was measured using the Bradford method (Biorad). Tissue homogenates (100 μg) were subjected to SDS–PAGE electrophoresis under reducing conditions, transferred onto PVDF membranes (Millipore), and analysed by immunoblotting according to standard protocols using the antibodies indicated. We used a rabbit mAb against mouse SR-B1 (clone EP1556Y) from Abcam and a mouse mAb against β-actin (clone AC-15) from Sigma. HRP-labelled secondary antibodies were from Sigma. Quantification was carried out using the Image J software.

### 2.10 In vivo macrophage-to-faeces RCT
The RCT assay was performed by standard methods.13 Briefly, thioglycollate-elicited mouse peritoneal macrophages, harvested from apoE−/− donor mice, were loaded for 24 h with 50 μg/mL of acetylated LDL and 5 μCi/mL of [3H]-cholesterol, then injected intraperitoneally in recipient mice (2 million dpm/mouse). Blood samples were taken 6, 24 and 48 h after macrophage injection, faeces were collected continuously for 48 h, and livers were harvested 48 h after macrophage injection and stored at −80°C until lipid extraction and radioactivity counting.13 All counts were expressed as a percentage of the administered tracer dose.

### 2.11 HDL functionality assays
HDL were isolated mouse plasma after precipitation of apoB-containing lipoproteins with polyethylene glycol-6000.18 The antioxidative property of HDL was assessed by measuring the capacity of HDL to inhibit the oxidation of native LDL.18,19 The anti-inflammatory property of HDL was evaluated on human umbilical vein endothelial cells (HUVECs) by measuring MCP-1 gene expression by standard methods.18 The efflux experiments were performed by measuring cholesterol efflux for 5 h from primary mouse peritoneal macrophages towards either plasma (1%, v/v) or apoB-depleted lipoproteins (2%, v/v) using standard methods.18

### 2.12 Cholesterol efflux towards HDL and apoA-I
HDLs were isolated from the plasma of normolipaemic healthy human donors by standard sequential ultracentrifugation of discontinuous KBr gradients.20 ApoA-I was isolated from HDL by ion-exchange chromatography following our established protocol.21 The cholesterol efflux assay was carried out on thioglycollate-elicited mouse peritoneal macrophages of apoE−/− and P2Y13−/−/apoE−/− mice. Cells were labelled for 24 h with 50 μg/mL of acetylated LDL and 5 μCi/mL of [3H]-cholesterol. The next day, the cells were washed with fresh media. They were then incubated with fresh DMEM (1 mg/mL of fatty acid free BSA) with free human apoA-I (10 μg/mL), or human HDL (100 μg/mL) at 37°C for 3 h. The medium was removed and clarified of cellular material, then cholesterol efflux was quantified by scintillation counting. The cell layers were lysed in 0.1 N NaOH, and cellular cholesterol content quantified by scintillation counting. Cholesterol efflux was expressed as the percentage effluxed [media counts/(media counts + cellular counts) × 100].

### 2.13 Statistical analysis
All results are presented as means ± SEM. Comparison between groups was made using the Mann–Whitney test for independent samples. Differences between more than two groups were analysed by two-way analysis of variance (ANOVA) followed by Bonferroni’s post hoc test if necessary. Outcomes of *P* < 0.05 were considered statistically significant. Analyses were performed using the GraphPad Prism 6 software.

### 3. Results

#### 3.1 P2Y13 receptor protects against atherosclerosis development on the apoE−/− background
We further investigated the effect of P2Y13 receptor deficiency in apoE−/− mice on atherosclerotic development at 15 weeks of age. We observed more lipid-rich atherosclerotic plaque deposition and macrophage content in the aortic arch of P2Y13−/−/apoE−/−, than in apoE−/− mice (Figure 1A and B). This indicates that the absence of P2Y13 receptor induced early stage atherosclerotic lesions.

To understand the contribution of the P2Y13 receptors in blood cells to the development of atherosclerosis, we performed BM transplantation. At the age of 7 weeks, male apoE−/− (apoE KO) and P2Y13−/−/apoE−/− (dKO) mice were sublethally irradiated and transplanted with BM of either genotype to give four groups of animals:

1. **apoE−/− recipient mice transplanted with apoE−/−** BM (KO > KO),
2. **apoE−/− recipient mice transplanted with P2Y13−/−/apoE−/−** BM (dKO > KO),
3. **P2Y13−/−/apoE−/−** recipient mice transplanted with P2Y13−/−/apoE−/− BM (dKO > dKO), and
4. **P2Y13−/−/apoE−/−** recipient mice transplanted with apoE−/− BM (KO > dKO),
Atherosclerotic lesions were then analyzed for each group, at the age of 15 weeks (Figure 1C). We first observed that the absence of the P2Y13 receptor in reconstituted dKO mice led to larger areas of lesion in the aortic sinus than in control KO > KO mice (19,008 ± 1919 μm² in KO > KO vs. 42,214 ± 3824 μm² in dKO > dKO; ***p < 0.001; Figure 1C). This is consistent with the greater atherosclerosis...
development in non-transplanted P2Y13<sup>−/−</sup>/apoE<sup>−/−</sup>, compared with apoE<sup>−/−</sup> mice (Figure 1A). The absence of the P2Y<sub>13</sub> receptor in blood cells (dKO > KO) did not result in a significant difference in lesion area compared with the KO > KO group (19 008 ± 1919 vs. 19 202 ± 1434 μm<sup>2</sup>, P = 0.93). Conversely, more atherosclerotic lesions were observed when P2Y13<sup>−/−</sup>/apoE<sup>−/−</sup> mice were transplanted with BM from apoE<sup>−/−</sup> mice (KO > dKO). The difference was similar in size to that in dKO > dKO mice (36 468 ± 3743 vs. 42 214 ± 3824 μm<sup>2</sup>, P = 0.31). These data, in combination, indicate that P2Y13 receptors from blood cells do not contribute to the development of atherosclerotic lesions in mice.

### 3.2 Altered lipoprotein levels in P2Y13<sup>−/−</sup>/apoE<sup>−/−</sup> mice in comparison with apoE<sup>−/−</sup> mice

P2Y<sub>13</sub>−/−/apoE<sup>−/−</sup> mice display ∼15% lower total cholesterol (TC) and 25% lower TGs in plasma than apoE<sup>−/−</sup> mice (both comparisons P < 0.05, n = 8 per group, Table 1). Plasma from these mice was then subjected to fast-protein liquid chromatography (FPLC) in order to analyse the lipid composition (total and free cholesterol, TG) of each lipoprotein class. The lower TC observed in P2Y13<sup>−/−</sup>/apoE<sup>−/−</sup> mice could be attributed mainly to lower cholesterol content within HDL (∼33 ± 6% compared with apoE<sup>−/−</sup> mice, P < 0.05) and to a lesser extent within LDL (∼13 ± 4% compared with apoE<sup>−/−</sup> mice, P < 0.05). Both free and esterified cholesterol were lower, since the free/total cholesterol ratio was not significantly different between lipoproteins from P2Y13<sup>−/−</sup>/apoE<sup>−/−</sup> and apoE<sup>−/−</sup> mice (Table 1). The cholesterol content in VLDL particles was slightly, but not significantly, lower in P2Y13<sup>−/−</sup>/apoE<sup>−/−</sup> mice, but their TG content was significantly less, reflecting the lower level of plasma TG. The TC profiling of FPLC-separated lipoprotein fractions from one representative mouse of each group shows that there was markedly less HDL-cholesterol (HDL-C) in P2Y13<sup>−/−</sup>/apoE<sup>−/−</sup> mice and moderately lower LDL-C (Figure 2). These profiles agree with the values in Table 1.

### 3.3 P2Y<sub>13</sub> deficiency in apoE<sup>−/−</sup> mice reduces biliary lipid secretions and faecal loss of cholesterol and BAs.

The P2Y<sub>13</sub> receptor promotes HDL holoparticle endocytosis in hepatocytes and hepatobiliary secretion of lipid. In line with this function is our observation that biliary flux and secretion of cholesterol, BA and phospholipids were significantly lower in P2Y13<sup>−/−</sup>/apoE<sup>−/−</sup> than in apoE<sup>−/−</sup> mice (Table 2).

Interestingly, this impaired secretion of lipid into the bile resulted in lower cholesterol and BA excretion from the body, as indicated by the lower cholesterol and BA content in the faeces of P2Y13<sup>−/−</sup>/apoE<sup>−/−</sup> compared with those of apoE<sup>−/−</sup> mice (Table 2). We then analysed faecal BA species and observed, in the faeces of P2Y13<sup>−/−</sup>/apoE<sup>−/−</sup> mice, significantly lower primary BA, muricholate (805.8 ± 94.9 vs. 509.1 ± 29.2 μg/g of faeces, P < 0.05), and cholate (157.6 ± 37.5 vs. 82.9 ± 10.7 μg/g of faeces, P < 0.05), and secondary BA, deoxycholate (611.0 ± 44.2 vs. 488.1 ± 40.9 μg/g of faeces), and lithocholate (30.1 ± 3.7 vs. 11.2 ± 2.6 μg/g of faeces, P < 0.05; Figure 3). We also analysed the hepatic lipid content. The level of esterified cholesterol was greater in the liver of P2Y13<sup>−/−</sup>/apoE<sup>−/−</sup> than in apoE<sup>−/−</sup> mice (36.72 ± 12.40%, P < 0.05), whereas hepatic TG content was lower (38.83 ± 7.55%, P < 0.05; Table 2).

### 3.4 Influence of P2Y<sub>13</sub> deficiency in apoE<sup>−/−</sup> mice on hepatic gene expression profiles

Because P2Y<sub>13</sub> deficiency in apoE<sup>−/−</sup> mice results in substantial changes in plasma, hepatic, and biliary lipids, we analysed hepatic gene expression...
of other HDL/apoA-I receptors involved in HDL uptake and biogenesis by the liver, namely SR-BI, ATP-binding cassette transporter A1 (ABCA1), and ABCG1. There was much higher mRNA expression for SR-BI in the liver of P2Y13+/apoE−/− than in those of apoE−/− mice (2.67 ± 0.29-fold, *P < 0.01), whereas mRNA levels of ABCA1 and ABCG1 were unchanged between the two genotypes (Figure 4A). The increase in SR-BI gene expression translated into up-regulation of SR-BI protein expression in the liver of P2Y13+/apoE−/− than in apoE−/− mice (4.14 ± 1.19-fold, *P < 0.05, Figure 4B). This suggests that the mice adjust hepatic SR-BI expression to compensate for the loss of functioning P2Y13 receptors and the associated lack of HDL holoparticle endocytosis.

3.5 P2Y13+/apoE−/− mice have impaired RCT from macrophages to faeces

We next measured the mobilization of 3[1H]-cholesterol from macrophages to plasma, liver, and faeces, a well-established method to evaluate RCT in vivo.23 P2Y13+/apoE−/− and apoE−/− recipient mice were injected in the peritoneum with 3[1H]-cholesterol-loaded macrophages from apoE−/− mice. The animals were killed 48 h after macrophage injection, and the radioactivity in plasma, liver, and faeces was quantified to evaluate the distribution of cholesterol along the RCT pathway. Plasma and hepatic 3[1H]-cholesterol counts were similar in P2Y13+/apoE−/− and apoE−/− mice (Figure 5A and B). However, the radioactivity found in faeces was significantly lower in P2Y13+/apoE−/− than in apoE−/− mice, both for radioactivity associated with BAs and for that associated with neutral sterols (−66.11 ± 3.17 and −47.17 ± 6.46%, respectively, Figure 5C). This reflects the association of P2Y13 receptor deficiency in apoE−/− mice with impaired RCT in vivo, as already known for P2Y13−/− mice compared with WT P2Y13+/+ mice.11

3.6 Unchanged functional properties of HDL particles from P2Y13+/apoE−/− mice

We analysed whether P2Y13 deficiency in apoE−/− mice modified the functionality of HDL particles. We observed that isolated HDL particles from P2Y13+/apoE−/− mice did not differ in either their anti-inflammatory or antioxidative properties from those in HDL from apoE−/− mice (Figure 6A and B). Plasma from P2Y13+/apoE−/− and apoE−/− mice displayed similar cholesterol efflux capacity, measured from 3[1H]-cholesterol-loaded bone marrow-derived macrophages (Figure 6C). Accordingly, there were identical efflux rates to HDL particles isolated from a fixed amount of plasma for both P2Y13+/apoE−/− and apoE−/− mice (Figure 6D). Since plasma HDL-C was lower in P2Y13+/apoE−/− than in apoE−/− mice, we also determined cholesterol efflux towards HDL after correcting for the cholesterol mass level present in the sample. This measure was also similar in P2Y13+/apoE−/− and apoE−/− mice (Figure 6E), indicating that P2Y13 deficiency does not modify cholesterol efflux capacity of HDL per se. In addition, we also observed similar cholesterol efflux to purified human apoA-I or HDL from macrophages of P2Y13+/apoE−/− and apoE−/− mice (see Supplementary material online, Figure S1), indicating that P2Y13 deficiency in macrophages does not influence cholesterol efflux capacity.

4. Discussion

We report here that the P2Y13 receptor plays a protective role in the development of early atherosclerotic lesions. At 15 weeks of age, the lesions formed in the aortic root of P2Y13+/apoE−/− mice were larger than those in apoE−/− mice. Macrophage content was also greater compared with apoE−/− animals. In addition to expression in hepatocytes, P2Y13 receptor mRNA is expressed in some haematopoietic...
cells such as monocytes, T cells, and dendritic cells that may be involved in atherosclerosis. Our BM transplantation experiments indicated that blood cell P2Y13 receptors are not involved in the development of atherosclerosis, suggesting that P2Y13 expression in circulating immune cells does not contribute to an immunoinflammatory response that would have increased atherosclerotic development. Since P2Y13 receptor mRNA is not expressed in vascular smooth muscle cells and endothelial cells (Cavelier et al.24 and not shown), the phenotype of our P2Y13−/−/apoE−/− mice might be explained by the effect of the P2Y13 deficiency on metabolic pathways involved in atherosclerosis. Accordingly, our data indicate that metabolic parameters responsible for accelerated atherosclerosis in P2Y13−/−/apoE−/− compared with apoE−/− mice include (i) lower hepatobiliary lipid secretion, (ii) lower cholesterol and BA excretion in faeces, and (iii) lower functional RCT from macrophages to faeces. The P2Y13−/−/apoE−/− mice display lower bile flow and secretion of all biliary lipids, associated with lower output of both neutral sterols and BAs in the faeces. Therefore, tracer recovery in faecal neutral sterols and BAs was also lower when RCT from macrophages to faeces is measured. It is interesting that these changes differ from the phenotype of SR-BI-deficient mice in which only biliary secretion of cholesterol is measured. It is interesting that these changes differ from the phenotype of SR-BI-deficient mice in which only biliary secretion of cholesterol is measured.

This metabolic phenotype of P2Y13−/−/apoE−/− mice is partially consistent with low HDL endocytosis into the liver in P2Y13 KO mice. This was also associated with impaired hepatobiliary RCT. However, dKO mice display an unexpected low level of HDL-C (−33 ± 6%, P < 0.05) and to a lesser extent of LDL-C (−13 ± 4%, P < 0.05) (Table 1). However, given the role of P2Y13 receptors in HDL endocytosis by hepatocytes, inactivation of this receptor would be expected to produce high rather than low HDL-C levels. One explanation of this discrepancy is that P2Y13 receptor deficiency in apoE−/− mice leads to compensatory changes in the hepatic expression of SR-BI. And, indeed, protein expression was up-regulated in the liver of P2Y13−/−/apoE−/− mice. These observations are concordant with the fact that mice with liver-specific overexpression of SR-BI transgene (SR-BI-Tg Liver) display low HDL-C plasma level and high hepatic cholesterol content. In addition, due to the ability of SR-BI to bind LDL and VLDL and to promote the clearance of these lipoproteins, SR-BI-Tg Liver mice display low plasma levels of LDL-C, VLDL-C, and TG. These changes resemble those occurring in P2Y13−/−/apoE−/− mice, which suggests that the low levels of LDL-C and VLDL-TG in these mice could also be related to high expression of SR-BI in the liver. The regulatory mechanisms responsible for the up-regulation of SR-BI in P2Y13−/−/apoE−/− mice will require further investigation, although it is more likely to be transcriptional rather than post-transcriptional given that both mRNA and protein expression levels of SR-BI were high but that...
mRNA expression levels of PDZK1, a post-transcriptional regulator of SR-BI, were unchanged (not shown).

It is of interest that mice overexpressing SR-BI in the liver have high biliary cholesterol secretion and macrophage-RCT, which translates into a low incidence of atherosclerosis. However, we observed here that P2Y13−/−/apoE−/− mice display a totally reversed phenotype. Thus, the up-regulation of SR-BI in P2Y13−/−/apoE−/− mice, besides reducing the HDL-C level, fails to compensate in vivo for the adverse effects of P2Y13 receptor deficiency on RCT and atherosclerosis. Furthermore, it is possible that SR-BI activity in P2Y13−/−/apoE−/− mice is less up-regulated than is the expression of the corresponding protein since apoE−/− mice display low SR-BI activity in promoting selective uptake of CE from HDL. This is in line with data from animal models for other RCT targets: the association between HDL-C levels and macrophage-specific RCT is thus not straightforward, indicating that HDL-C levels should be used with caution as a surrogate for predicting fluxes through the RCT pathway.

Overall, our data argue for a central role for the P2Y13 receptor, which in our model cannot be substituted for by SR-BI, in the process of bile lipid secretion and of RCT and document an anti-atherogenic function of the P2Y13 receptor.

Our results also confirm the interesting strict relationship of P2Y13 receptor activity to the function of HDL in hepatobiliary RCT. The strictness of this relationship is clear because no other HDL properties, such as anti-inflammatory or antioxidative properties, nor the HDL capacity to elicit efflux from cholesterol-loaded macrophages, differed between HDL from P2Y13−/−/apoE−/− and that from apoE−/− mice. Likewise, these properties do not differ between HDL from P2Y13−/− and that from WT P2Y13+/+ mice, suggesting that P2Y13 receptor does not contribute to the protective effect of HDL on immunoinflammatory responses.

Recently, the effects of P2Y13 receptor activation in mice were investigated in two independent studies using two P2Y13 receptor agonists, namely Cangrelor (The Medicine Company, NJ, USA) and CT1007900.

Figure 5 Lack of P2Y13 receptor in apoE−/− mice reduces in vivo macrophage-to-faeces RCT. Two million 3H-cholesterol-labelled peritoneal macrophages from apoE−/− donor mice were injected intraperitoneally in 15-week-old apoE−/− (light grey) and P2Y13−/−/apoE−/− (dark grey) mice. (A) 3H-cholesterol appearance in plasma 48 h after macrophage administration. (B) 3H-cholesterol tracer recovery within liver 48 h after macrophage injection. (C) 3H-cholesterol appearance in faeces collected continuously from 0 to 48 h after macrophage injection. Data are expressed as percent cpm injected ± SEM; n = 6 mice per group. Statistical significance was determined by using the Mann–Whitney test for independent samples. *P < 0.05 compared with apoE−/− values.

Figure 6 Lack of the P2Y13 receptor in apoE−/− mice does not affect HDL functionality. HDL function was determined as protection of HUVECs against inflammation (A), protection of LDL against oxidation (B), and cholesterol efflux (C–E). Data are presented as means ± SEM, n = 8 mice per group. TBARS: thiobarbituric acid reactive substances.
in apoE cates that P2Y13 receptor protects against the development of atherosclerotic development.

In conclusion, our study demonstrates in vivo that the P2Y13 receptor is a key component of the hepatobiliary RCT process and clearly indicates that P2Y13 receptor protects against the development of atherosclerosis, at least in mice. In the context of recent failures of clinical trials of agents developed to raise HDL-C levels, failures that have introduced considerable controversy to this field, our study supports the concept that future HDL therapies need to target HDL functions, for instance by improving HDL particle flux to the liver, rather than simply increasing HDL-C. Thus, involvement of the P2Y13 receptor in HDL-mediated RCT makes it an attractive target for therapeutic intervention against atherosclerotic lesion development.

Supplementary material
Supplementary material is available at Cardiovascular Research online.

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