P2Y₂ Nucleotide Receptor Is a Regulator of the Formation of Cardiac Adipose Tissue and Its Fat-Associated Lymphoid Clusters

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The formation of pericardial adipose tissue (PAT) and its regulatory function in cardiac inflammation are not well understood. We investigated the potential role of the ubiquitous ATP/UTP nucleotide receptor P2Y₂ in the PAT by using P2Y₂-null mice. We observed that P2Y₂-null mice displayed a lower mass of PAT and a reduced density of its fat-associated lymphoid clusters (FALCs) and, more particularly, B cells. Loss of P2Y₂ receptor in pericardial preadipocytes decreased their adipogenic differentiation and maturation abilities *in vitro*. Gene profiling identified P2Y₂ target genes in PAT linked to immunomodulation. These data led to the identification of an increase of M2c anti-inflammatory macrophages correlated with increased apoptosis of B lymphocytes in P2Y₂-null pericardial fat. In addition, follicular helper T cells, which contribute to B cell expansion in germinal centers, were dramatically decreased. The effect of P2Y₂ loss was also investigated after ischemia-mediated expansion of FALCs in a model of myocardial infarct. Loss of P2Y₂ led to reduced expansion of B and neutrophil populations in these clusters, whereas density of M2c anti-inflammatory macrophages was increased. Our study defines the P2Y₂ nucleotide receptor as a regulator of the formation and inflammatory status of pericardial fat. The P2Y₂ receptor could represent a therapeutic target in the regulation of PAT function before and during cardiac ischemia.

Keywords: adipogenesis, adipose stem cells, cardiac fat, extracellular nucleotides, P2Y receptors, fat-associated lymphoid cluster

Introduction

PERICARDIAL ADIPOSE TISSUE (PAT) comprised epicardial adipose tissue (EAT) and paracardial adipose tissue (AT) [1]. The two deposits surround the heart, with the EAT in direct contact with the myocardium and the paracardial located over the parietal pericardium [2]. The EAT and paracardial AT are usually present in humans and most mammals, and despite a previous uncertainty about the presence of EAT in rodents, this has recently been demonstrated [3]. Adipokines are secreted by PAT and act on the myocardium in an endocrine or paracrine way. Depending on the AT microenvironment and inflammatory state, they can exert a protective anti-inflammatory effect or, on the contrary, exacerbate inflammation [4,5]. In particular pathological conditions, PAT has been linked to atherosclerosis, coronary artery disease, and metabolic syndrome [6–9].

Moro et al. have pointed out the link between AT and cardiovascular diseases and described the presence of lymphoid structures in AT, called fat-associated lymphoid clusters (FALCs) [10]. FALCs share some similarities with the omental milky spots: They both lack a fibrous capsule and the B and T lymphocytes that mainly compose them are in direct contact with the adipocytes, with no organized division be-

tween the cell populations [10]. FALCs are found in almost every AT, and PAT displays one of the highest density of FALCs [11]. These clusters contain B1, B2, and T lymphocytes as well as myeloid and other innate immune cell populations [12].

B cells have a role in myocardial infarction (MI), regulating the recruitment and proliferation of monocytes and the dendritic cell population, affecting the MI outcome [13,14]. The inflammatory response generated after MI is an essential part of both the repair and remodeling processes that take place in the myocardium, and a spatiotemporal dysregulation can lead to adverse prognosis [15]. Therefore, a complete understanding of the regulation of cardiac inflammatory signals is critical to design effective therapies and the role of PAT in cardiac inflammation needs to be investigated.

MI and hypoxic conditions, mechanical stretching, and stimulation by catecholamines are associated with a release of extracellular nucleotides by cardiac endothelial cells and cardiomyocytes [16,17]. Extracellular nucleotides are known to regulate inflammation, mediating the release of inflammatory cytokines and leukocyte recruitment [18,19]. Diverse roles in the modulation of cardiac function and cardioprotection have been described for P2Y receptors [20,21]. In particular, P2Y₂

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receptor, a ubiquitous receptor for ATP and UTP [22], is a mediator of UTP cardioprotective effect toward hypoxic damage in vitro [23]; whereas in vivo P2Y₂ activation reduces post-ischemic myocardial damages [23,24]. P2Y₂ also mediates neutrophil chemotaxis [25], inflammation [26,27] and is involved in the differentiation of mesenchymal stem cells [28,29]. PAT is a reservoir of adipose-derived stem cells (ASCs), which are able to differentiate into bone, fat, and cartilage. Nucleotide receptors are known regulators of the differentiation process of mesenchymal stem cells [30,31]. Recently, P2Y₄, a UTP and ATP receptor, was reported as a negative regulator of adipogenic differentiation of ASCs from cardiac AT [32]. The P2Y₂ receptor was never considered for its role in AT.

Due to the connection with the heart and the protective role of AT, already described during atherosclerosis [33], the discovery of the AT modulator is one of the promising approaches to improve therapy and regenerative medicine after MI. In this study, we investigated the role of the P2Y₂ receptor in the formation of the PAT and FALCs by using P2Y₂-null mice. We investigated the involvement of this nucleotide receptor in basal PAT and also in the ischemia-mediated expansion of these FALCs in a model of MI to reveal a potential role in the regulation of the inflammatory response after ischemia.

Methods

Animals and ethics statement

P2Y₂-null C57BL/6 mice were a generous gift from the laboratory of Dr. B. Koller. All mice used in this study were authorized by the Animal Care Use and Review committee of the Free University of Brussels.

Isolation and differentiation of cardiac ASCs

Cardiac ASCs (cASCs) were isolated from the stromal vascular fraction of PAT. Pericardial tissue was minced and incubated in collagenase A solution (2.5 g/L collagenase A, 50 µg/mL DNase; both Roche, Basel, Switzerland) at 37°C for 45 min. The resulting suspension was filtered through a nylon mesh (40 µm) and fractionated into mature adipocytes and stromal vascular fraction by centrifugation at 500g for 5 min. The lysis of red blood cells into the stromal vascular fraction was performed by ACK lysis buffer, and the ASCs were selected thanks to their adhesion to plastic.

ASCs were cultured for 3 days in proliferation medium [Dulbecco's modified Eagle's medium:F-12, (Gibco™, ThermoFisher Scientific, MA), containing 3% newborn calf serum, 1% penicillin/streptomycin] and for 4 or 7 days in adipogenic differentiation medium (proliferation medium supplemented with 50 µM indomethacin, 1 µM dexamethasone, 0.5 mM isobutylmethylxanthine, and 5 µg/mL insulin). The number of differentiated cells was measured by Oil Red O staining (Sigma-Aldrich, St. Louis, MO). The percentage of differentiated adipocytes refers to the ratio between the number of cells with lipid droplets (stained in red by Oil red O) and the total number of cells in the field (nucleus stained in blue by Hoechst).

Ischemia in vivo experiments: left anterior descending ligation

P2Y₂-null and wild type (WT) mice aged from 11 to 13 weeks were used in this study. MI was induced by permanent

ligation of the left anterior descending (LAD) coronary artery. Mice were anesthetized with midazolam (5 mg/kg), medetomidin (0.5 mg/kg), and fentanyl (0.05 mg/kg); intubated; and ventilated with a MiniVent mouse ventilator (Harvard Apparatus). Left thoracotomy was performed in the fourth left intercostal space, and the pericardium was carefully incised to maintain the integrity of the pericardial AT. The chest wall and skin were closed with 5-0 nylon sutures (Ethicon, Somerville, NJ). After surgery, naloxone (1.2 mg/kg), flumazenil (0.5 mg/kg), and atipamezol (2.5 mg/kg) were injected to reverse the effect of anesthesia. Postoperative analgesia (buprenorphine, 0.1 mg/kg) was given for the first 12 h after surgery. Twenty-four hours after LAD ligation, the PAT, the heart, and the spleen were collected; washed in cold phosphate-buffered saline (PBS); dried; weighted; and stored at -80°C for mRNA or processed for flow cytometry analysis.

Hematoxylin/eosin staining

Tissues were collected from P2Y₂^{-/-} and WT mice aged from 11 to 13 weeks, fixed in paraformaldehyde 4% for 4 h, rinsed, and paraffin-embedded (Histokinette®). PAT sections were cut at 6-µm thickness, placed on pre-coated slices (VWR international, Heverlee, Belgium), and stained in hematoxylin/eosin (H/E). Pictures of H/E staining were acquired by using Zeiss AxioImager Z1 (LiMiF; Light Microscopy Facility, Erasme, Belgium). The surface of adipocytes and FALCs was measured by using Fiji software.

Flow cytometry

The PAT of mice at basal state or subjected to LAD was collected after the sacrifice of the animal and perfusion with PBS to remove peripheral cells. The PAT was then finely minced and digested in collagenase A solution (2.5 g/L collagenase A, 50 µg/mL DNase; both Roche) at 37°C for 45 min. The remaining red blood cells were lysed by using ACK lysis buffer. The spleen was triturated through a 100-µm nylon mesh strainer and centrifuged at 15 min at 500g, 4°C. The resulting single-cell suspension was rinsed, resuspended in PBS supplemented with 3% fetal bovine serum and CD16/CD32 Fc-block (BD Pharmingen; clone 2.4G2), and stained with a mixture of fluorochrome-conjugated antibodies for 45 min on ice. Antibodies used were CD45 (clone 30F11), CD11b (clone M1/70), Ly6G (clone 1A8), F4/80 (clone BM8), KI-67 (clone SolA15), CD19 (clone 6D5), CD3 (clone 17A2), CXCR5 (clone L138D7) (all from BioLegend, San Diego, CA), and MertK (AF591; R&D). To obtain an absolute quantification of the cell number in the tissue, we used the CountBright™ Absolute Counting Beads (Thermo Fisher Scientific). Data were acquired on a Fortessa (BD Biosciences), and analysis was performed with FlowJo software (Ashland).

RNA sequencing

RNA sequencing experiments were performed on a pool of RNA from the PAT of three WT and P2Y₂^{-/-} mice at basal conditions. RNA was extracted from freshly harvested PAT by using the RNeasy Mini Kit (Qiagen) after cells had been lysed in TRI Reagent Solution (Invitrogen). One microgram per fifty microliter of RNA was engaged, and the quality was checked by using a Bioanalyzer 2100 (Agilent Technologies). cDNA libraries were obtained by using the TruSeq Stranded

mRNA Library Prep kit (NuGen) following the manufacturer's recommendations. The multiplex libraries (18 pM) were loaded on flow cells, and sequences were produced by using a HiSeq PE Cluster Kit v4 and TruSeq SBS Kit v3-HS from a HiSeq 1500 (Illumina). Approximately 25 million paired-end reads per sample were mapped against the mouse reference genome (GRCm38.p4/mm10) by using STAR software to generate read alignments for each sample. Annotations Mus_musculus GRC38.87.gtf were obtained from ftp.Ensembl.org. After transcript assembling, gene level counts were obtained by using HTSeq. Genes with ratio (fold change) >0.5 and a fold change $P2Y_2^{-/-}/WT >2$ or <0.5 were considered. Gene Ontology enrichment analyses was performed with DAVID software. Enriched biological processes were selected by the software to have a significant ($P < 0.05$) modified Fisher exact P -value, or EASE score.

Quantitative reverse transcription-polymerase chain reaction experiments

Total mRNA was extracted through homogenization of the tissue in a glass-teflon tissue grinder in TRIzol reagent (Life Technologies, Groningen, The Netherlands), followed by purification with the RNeasy kit column (Qiagen, Hilden, Germany) from ATs of $P2Y_2^{-/-}$ and WT mice. mRNA was reverse transcribed by using random hexamers and Superscript II Reverse Transcriptase (ThermoFischer Scientific). Reverse transcription-polymerase chain reaction amplification mixtures contained 10 ng template cDNA. Reactions were run on a 7500 Fast Real Time PCR System (Applied Biosystems, Foster City, CA). PCR data were normalized for each gene to the RPL32 housekeeping gene.

Statistics

Data were expressed as mean $\pm$ standard error of mean (SEM) for in vitro and in vivo studies. Endpoint comparisons with two groups were performed by using an unpaired two-tailed Student's t test or Mann-Whitney test for parametric and nonparametric data, respectively (Prism Software, version 6; GraphPad, CA). For parallel repeated-measures studies, two-way analysis of variance was used with Bonferroni post hoc evaluations to determine the significance for individual time points (Prism Software; GraphPad). For all studies, P value was considered as $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

Results

Decreased PAT mass in $P2Y_2$ -deficient mice

The quantification of the PAT of $P2Y_2^{-/-}$ and WT male mice aged between 11 and 13 weeks identified a reduction of its mass in the $P2Y_2^{-/-}$ mice compared with the WT mice (6.9 ± 0.4 mg vs. 8.5 ± 0.4 mg, respectively, mean $\pm$ SEM, $**P < 0.01$, Fig. 1A, top left). This reduction was still significant after normalization to body weight ($0.0293 \pm 0.0015\%$ vs. $0.0356 \pm 0.0016\%$, mean $\pm$ SEM, $**P < 0.01$, Fig. 1A, bottom left). A comparable difference was also observed in female mice PAT (9.4 ± 0.4 mg in the $P2Y_2^{-/-}$ vs. 11.4 ± 0.5 mg in the WT, mean $\pm$ SEM, $**P < 0.01$, Fig. 1A, center). No reduction of adipose tissue mass was observed for the gonadal adipose tissue (GAT) of both male (Fig. 1A, right) and female mice (data not shown), although we de-

tected the expression of $P2Y_2$ receptor in both adipose tissue deposits after quantitative polymerase chain reaction analysis (Fig. 1B). The difference in PAT is not to be attributable to an atrophy of $P2Y_2^{-/-}$ cardiac adipocytes (Fig. 1C).

$P2Y_2$ receptor negatively regulates adipogenic differentiation of pericardial ASCs

To test whether $P2Y_2$ has a role in modulating adipogenic differentiation, we evaluated the effect of its loss on the differentiation of cASCs derived from the PAT of $P2Y_2^{-/-}$ and WT mice. The cASCs were isolated from the stromal vascular fraction of digested PAT and after a proliferation period of 3 days submitted to an adipogenic predifferentiation protocol during 4 and 7 days. The differentiation into preadipocytes was characterized by the presence of small lipid droplets stained with Oil Red O and was significantly lower for cASCs derived from $P2Y_2^{-/-}$ PAT compared with the WT ($14.41 \pm 1.48\%$ vs. $28.08 \pm 2.00\%$ of differentiated cells after 4 days and $13.71 \pm 3.44\%$ vs. $46.13 \pm 4.42\%$ of differentiated cells after 7 days, mean $\pm$ SEM, $*P < 0.05$, Fig. 2A, B).

Reduced size of FALCs in the PAT of $P2Y_2$ -deficient mice

In the PAT, the large majority of leukocytes is concentrated in secondary lymphoid structures called FALCs, as shown by H/E staining of paraffin slides of PAT (Fig. 3A). To evaluate the impact of $P2Y_2$ on the formation and composition of FALCs in PAT, we quantified the mean area of these clusters in $P2Y_2^{-/-}$ and WT PAT. We observed that the mean size of FALCs was smaller in $P2Y_2^{-/-}$ mice ($9,356 \pm 727 \mu m^2$ vs. $13,421 \pm 1,602 \mu m^2$, mean $\pm$ SEM, $*P < 0.05$, Fig. 3A). Flow cytometry analyses of digested PAT confirmed a reduced number of CD45+ cells in the $P2Y_2^{-/-}$ PAT compared with the WT. These data were normalized to the mass of PAT to avoid differences due to the reduced mass of the tissue in $P2Y_2^{-/-}$ mice (Fig. 3B).

The analyses of leukocyte populations in FALCs by flow cytometry highlighted a reduced presence of CD19+ B220+ B cells in $P2Y_2^{-/-}$ PAT compared with WT (Fig. 3B) whereas other cells such as T cells, neutrophils, and monocytes/macrophages were not affected by the loss of $P2Y_2^{-/-}$ (not shown). We observed no difference between B cell quantification in spleen, blood, and bone marrow of WT and $P2Y_2^{-/-}$ mice (Fig. 3C). Reduced leukocyte number in $P2Y_2^{-/-}$ PAT was not correlated to a reduced proliferation of CD45+ cells inside the tissue (Fig. 3D), as tested by flow cytometry by using intracellular proliferation marker Ki67. Instead, flow cytometry analysis with annexin V/propidium iodide apoptosis kit revealed increased early apoptosis of B cells in $P2Y_2^{-/-}$ PAT (Fig. 3E).

Regulation of anti-inflammatory genes, M2c macrophages, and follicular helper T cell populations in $P2Y_2^{-/-}$ PAT

To study the genes regulated by $P2Y_2$ in PAT, we performed RNAseq experiments on a pool of RNA from PAT of three $P2Y_2^{-/-}$ and WT mice. A comparison of gene expression between $P2Y_2^{-/-}$ and WT mice showed that 958 genes were differentially regulated (fold change >2 or <0.5 ; only genes with CPM >0.5 in at least one of the two

FIG. 1. Decreased PAT mass in P2Y₂-deficient mice. (A) Absolute (top row) and normalized on body weight (bottom row) quantification of PAT and GAT of P2Y₂^{+/+} and P2Y₂^{-/-} male (n=27 for PAT and n=10 for GAT) and female (n=24) mice aged between 11 and 13 weeks. (B) mRNA level quantification of P2Y₂ receptor in PAT and GAT of P2Y₂^{+/+} mice (n=3). (C) Adipocyte area measurement performed on hematoxylin/eosin paraffin-embedded PAT sections of P2Y₂^{+/+} and P2Y₂^{-/-} mice (magnification 200×, scale bar=50 μm). Each point is a mean of at least 100 adipocytes from two to three different fields (n=6 mice). Data represent mean ± SEM. **P < 0.01; ns, not significant. PAT, pericardial adipose tissue; GAT, gonadal adipose tissue; SEM, standard error of mean.

conditions were considered) (Fig. 4A). A gene enrichment analysis with DAVID software on the RNAseq data from P2Y₂^{-/-} versus WT PAT revealed that several biological processes linked to immune response, inflammation, and chemotaxis were enriched (Fig. 4B). Among these genes,

MERTK was upregulated in the P2Y₂^{-/-} PAT, together with cytokines such as interleukin (IL)-10 secreted inter alia by MerTK+ M2c macrophages (Fig. 4C). To investigate a potential polarization of macrophages versus the M2c subtype in the PAT of P2Y₂^{-/-} mice, we performed flow cytometry

FIG. 2. P2Y₂ receptor negatively regulates adipogenic differentiation of pericardial ASCs. (A) Immunofluorescence staining with Oil Red O (red) and Hoechst (blue) of pericardial ASCs after 4 or 7 days of adipogenic differentiation. Magnification 200× (scale bar=50 μm). (B) Percentage of P2Y₂^{+/+} and P2Y₂^{-/-} pericardial ASCs differentiated in adipocytes after culture in control (CTRL) medium or 4 and 7 days in adipogenic differentiation medium. Differentiated cells are identified by the presence of lipid droplets stained with Oil Red O (n=3 independent cultures). Data represent mean ± SEM. *P < 0.05; **P < 0.01. ASC, adipose-derived stem cell. Color images are available online.

FIG. 3. Reduced number of leukocytes and dimensions of PAT FALCs in P2Y₂-deficient mice. **(A)** Representative hematoxylin/eosin staining of paraffin-embedded PAT sections (magnification 50 $\times$, scale bar=500 μm). Enlargements in *squares* show single FALCs (magnification 200 $\times$, scale bar=50 μm). Measurement of average area (*right*) of FALCs performed on hematoxylin/eosin paraffin-embedded PAT sections of P2Y₂^{+/+} and P2Y₂^{-/-} mice. Each point is a mean of five slides of the tissue taken at a distance of at least 100 μm from each other ($n=5$ mice). **(B)** Flow cytometry gating strategy and quantification of CD45⁺ leukocytes and CD19⁺ B220⁺ B cells in PAT in basal conditions. The number of cells is normalized on the mass of the PAT ($n=50$ for basal and 16 for MI). **(C)** Number of B cells in spleen ($n=12$), blood ($n=7$; cells for 1 mL of peripheral blood), and bone marrow (BM) ($n=11$; cells for BM of one femur) of WT and P2Y₂ KO mice. **(D)** Percentage of proliferating CD45⁺ leukocytes in pericardial AT, at steady state ($n=7$), obtained with intracellular flow cytometry staining of Ki67. **(E)** Percentage of apoptotic B cells in pericardial AT, based on flow cytometry analysis of Annexin V and propidium iodide staining for identification of early and late apoptotic cells ($n=5$). Results are pooled from three independent experiments. Mean values $\pm$ SEM are shown. * $P < 0.05$; ** $P < 0.01$. FALC, fat-associated lymphoid cluster; MI, myocardial infarction; AT, adipose tissue; WT, wild type. Color images are available online.

analysis on digested PAT. The percentage of MerTK⁺ macrophages was increased in P2Y₂^{-/-} PAT compared with the WT ($79.2 \pm 2.6\%$ vs. $65.8 \pm 2.7\%$, mean $\pm$ SEM, ** $P < 0.01$, Fig. 4D). In the spleen, we observed no difference in MerTK⁺ macrophages between WT and P2Y₂^{-/-} mice (Fig. 4D). To further investigate the B cell defect in P2Y₂^{-/-} PAT, we also quantified T follicular helper cells, known to participate in B cell expansion in the germinal center of secondary lymphoid structures. The number of CD45⁺ CD19⁻ CD11b⁻ CD3⁺ CXCR5⁺ cells, identified as T follicular helper, was quantified via flow cytometry on digested PAT and normalized on the mass of the tissue. The number of T follicular helper cells was significantly reduced in the P2Y₂^{-/-} mice compared with

the WT in the PAT ($144 \times 10^3 \pm 36 \times 10^3$ cells/g PAT vs. $459 \times 10^3 \pm 62 \times 10^3$ cells/g PAT, mean $\pm$ SEM, ** $P < 0.01$, Fig. 4E) but not in the spleen (Fig. 4E).

Reduced expansion of FALCs in P2Y₂-deficient mice after MI

To evaluate FALCs expansion in the PAT after MI, we performed permanent ligation of the LAD artery on male mice aged between 11 and 13 weeks. We used flow cytometry on digested PAT to quantify the number of leukocytes after infarction and compare them with nonischemic mice. We observed a 2.5-fold increase of CD45⁺ cells in WT mice after LAD compared with

FIG. 4. Regulation of inflammatory genes, M2c macrophages, and follicular helper T cell populations in P2Y₂^{-/-} PAT. (A) MA plot (M:log ratio and A:mean average) of RNAseq experiment data performed on PAT of P2Y₂^{-/-} mice versus WT. Every dot represents a gene, plotted after its logarithmic average expression in the two samples (x axis) and the logarithmic fold change of expression P2Y₂^{-/-}/WT (y axis). Only genes with ratio (fold change) >0.5 in at least one on the two conditions were considered. (B) Selection of biological processes revealed as enriched for differentially expressed genes between P2Y₂^{-/-} and WT PAT RNA after a Gene Ontology enrichment analyses performed with DAVID software. Only genes with ratio (fold change) >0.5 and a fold change >2 or <0.5 were considered. The modified Fisher Exact *P*-value, or EASE score, was reported by the software and indicated here as logarithmic *P*-value. (C) Logarithmic fold change of some inflammatory genes differentially expressed in P2Y₂^{-/-} PAT versus WT. (D) Gating strategy and flow cytometry quantification of CD45⁺ B220⁻ Ly6G⁻ CD11b⁺ F4/80⁺ MerTK⁺ macrophages (as percentage of macrophages) in PAT (*n*=5) and in spleen (*n*=5). (E) Flow cytometry quantification of CD45⁺ CD19⁻ CD11b⁻ CD3⁺ CXCR5⁺ T follicular helper cells in PAT (*n*=8) and in spleen (*n*=12). Mean ± SEM is shown. ***P*<0.01.

basal conditions ($4.2 \times 10^7 \pm 0.5 \times 10^7$ vs. $1.6 \times 10^7 \pm 0.2 \times 10^7$, mean ± SEM, ****P*<0.001, Fig. 5B). The reduced number of leukocytes in the P2Y₂^{-/-} after MI compared with the WT was mainly due to a lower expansion of CD19⁺ B cells ($4.9 \times 10^6 \pm 0.9 \times 10^6$ vs. $10.2 \times 10^6 \pm 1.2 \times 10^6$, mean ± SEM, **P*<0.05, Fig. 5C) and a smaller increase after LAD of the neutrophils (Ly6G⁺ cells) (Fig. 5E) and monocyte/macrophage (CD45⁺ CD19⁻ Ly6G⁻ CD3⁻ CD11b⁺) populations (Fig. 5F). The number of T cells (CD3⁺ cells) did not significantly differ between P2Y₂^{-/-} and WT PAT after infarction (Fig. 5D). Despite the decrease of monocytes/macrophages in P2Y₂-null mice, analysis of the different populations of polarized macrophages revealed an expansion of MerTK⁺ macrophages in PAT after MI. This increase of MerTK⁺ macrophages is two times more pronounced in P2Y₂ KO mice (Fig. 5G).

Discussion

Human EAT modulates cardiac functions in a paracrine and endocrine way through secretion of adipokines from adipocytes and resident leukocytes [6]. In pathological conditions such as obesity and coronary artery disease, the cardioprotective effect of EAT is lost due to a decreased secretion of anti-inflammatory adipokines. Currently, the volume of EAT is a well-established marker of visceral adiposity and an independent predictor of coronary artery disease [34,35]. In view of the established link between cardiac fat volume and cardiovascular disease risk, the identification

of regulators of the formation and functions of cardiac adipose tissue appears exceptionally important [36,37]. PAT is defined as the fat deposit lining the left ventricle of the mouse heart, down to the apex and encompasses both paracardial and EATs [1]. The presence of the latter in mice was finally discovered in 2015 by Yamaguchi et al. [3]. In this study, we investigated the role of the nucleotide receptor P2Y₂ in the formation of murine PAT.

We initially observed a reduced mass of PAT in mice deficient for P2Y₂ nucleotide receptor compared with the WT controls. No decrease was observed in the total body weight or in the mass of GAT in P2Y₂-null mice compared with WT mice. The specificity of the mass defect in cardiac fat is intriguing knowing the ubiquitous P2Y₂ receptor expression. The potential role of P2Y₂ receptor in the formation and function of cardiac adipose tissue was then investigated. First, we evaluated the effect of P2Y₂ loss on adipogenic differentiation of ASCs isolated from P2Y₂-null and WT PAT. We observed a significant negative effect of P2Y₂ loss on lipid droplets formation already during the first days of cASCs differentiation, and ultimately a strong decrease of pre-adipocyte maturation. The involvement of P2Y₂ in adipogenic differentiation of cASCs in vitro could be correlated with the reduced cardiac fat mass of P2Y₂-null mice.

Notably, our laboratory previously described an opposite, positive effect of P2Y₄ loss on PAT mass and cardiac adipogenesis [32]. The opposite effect of P2Y₂ and P2Y₄ loss on cardiac fat formation was unexpected. Both nucleotide

FIG. 5. Reduced number of B cells, neutrophils, and monocytes/macrophages and increased number of MerTK+ macrophages in P2Y₂-null PAT after MI. **(A)** Gating strategy for the flow cytometry quantification of CD45+ leukocytes in PAT at basal state and 24 h after MI. **(B)** Flow cytometry quantification of CD45+ leukocytes, **(C)** CD19+ B220+ B cells, **(D)** CD3+ T cells, **(E)** Ly6G+ neutrophils, and **(F)** CD19- Ly6G- CD3- CD11b+ monocytes/macrophages in PAT of WT and P2Y₂^{-/-} mice at steady state ($n=11-20$) and after 24 h of MI ($n=9-13$). The number of cells is normalized on the mass of the PAT. **(G)** Quantification of MerTK+ macrophages, expressed as fold change on WT mice without infarction ($n=3$). Mean $\pm$ SEM is shown. * $P<0.05$; ** $P<0.01$; *** $P<0.001$; ns, not significant. Color images are available online.

receptors share comparable pharmacological responses to their common ligands, UTP and ATP, but they have a different time course of expression during ASC adipogenic differentiation [32]. This difference, the diversity in tissues expression and in coupling with downstream G proteins [38], could explain the opposite effect on preadipocyte differentiation. However, further experiments are needed to ultimately discern the contribution of these two receptors in the adipogenic process.

P2Y₂ is a known regulator of inflammation and immune response. It is involved, between others, in neutrophil chemotaxis [25], asthma-mediated infiltration of eosinophils [39], and dendritic and T cell accumulation in lungs of pneumonia virus-infected mice [27]. We, therefore, investigated whether its loss would affect the formation of FALCs in PAT. FALCs are nonclassical secondary lymphoid structures where the majority of adipose tissue leukocytes are grouped and that react to inflammatory insults, organizing a fast immune response. We demonstrated that P2Y₂ loss is correlated with reduced pericardial FALCs size, confirmed by a reduced number of CD45+ leukocytes in PAT. This reduction was mainly ascribable to a reduced number of B cells, which represent the main cell population of FALCs. Moreover, a higher percentage of B cells was found to be in early apoptosis stage in P2Y₂-null mice compared with WT.

The results of RNAseq gene expression analysis on PAT showed that P2Y₂ loss was correlated with an increased

expression of tyrosine kinases receptor MerTK in PAT of P2Y₂-null mice, reflecting polarization of macrophages in the anti-inflammatory M2c subtype. M2c macrophages secrete potent anti-inflammatory mediators such as IL-10. The expression of MerTK on phagocytes facilitates their clearance of apoptotic cells and its deficiency is cause of long-term accumulation of apoptotic cells in the germinal centers of secondary lymphoid organs [40]. MerTK deficiency has also been linked to an increased activation and proliferation of total and germinal center B cells and an enhanced CD4+ T helper cell activation and differentiation into effector cells, including T follicular helper cells [40].

Despite germinal center structures, typical of secondary lymphoid organs, not having been observed in FALCs [10], Bénézech et al. demonstrated that B cells in FALCs undergo germinal center differentiation into plasma cells and germinal center-like B cells during peritoneal immune challenges [11]. Taken together, these observations suggest that an increased expression of MerTK in FALCs of P2Y₂-null mice could negatively affect the differentiation and proliferation of B cells during adaptive immune responses. The reduced number of B cells found in P2Y₂-null mice PAT could be then linked to the increased expression of MerTK on P2Y₂-null macrophages. Moreover, T follicular helper cell number was significantly reduced in P2Y₂-null mice PAT compared with WT controls, which could also be linked to M2c polarisation.

FALCs react to inflammatory and immunological challenges in body serous cavities by rapidly increasing their number and size [11]. B cell proliferation and maturation into antibodies-secreting plasma cells occur at the site of immune insult [41]. It has recently been demonstrated that the inflammatory process observed after MI in the heart is partially regulated by FALCs and B cells of PAT [13]. The inflammatory phase is a fundamental and necessary stage of the reparative process after MI. However, its resolution is also crucial for proper heart repair and remodeling. An excessively prolonged or intense inflammatory phase can lead to sustained tissue damage and improper healing, ultimately leading to a poor clinical outcome. To achieve the perfect balance between the beneficial and the detrimental effects of inflammation, a fine regulation of the inflammatory signals and cells is necessary [42].

We used, as an *in vivo* model of MI, the permanent ligation of the LAD artery. Our data support that impaired number and composition of pericardial FALCs at basal conditions could be amplified in an ischemic situation and affect the regulation of the post-ischemic myocardial inflammation. The comparison between ischemic and nonischemic WT mice showed, as expected, an expansion of the number of leukocytes in pericardial FALCs after 24h, corresponding to the beginning of the inflammatory phase. P2Y₂-null mice displayed an impaired expansion of B cells after MI and a smaller increase of neutrophils and monocytes/macrophages. Further, ischemic mice showed an expansion of MerTK+ anti-inflammatory macrophages, which was two times higher in P2Y₂-null mice than in WT mice. This macrophage subtype coordinates the clearance of apoptotic cardiomyocytes and neutrophils in the affected myocardium. Indeed, after an acute phase mainly driven by pro-inflammatory macrophages, these cells are substituted by reparative macrophages that support cardiac wound healing by promoting myofibroblast accumulation, collagen deposition, and angiogenesis. Wan et al. demonstrated that precise clearance of apoptotic cells is mandatory for favorable myocardial infarct healing, whereas failed resolution may lead to heart failure [43]. Extracellular nucleotides released after an ischemic event have an impact on the regulation of the inflammatory process, acting on a broad variety of leukocytes in both the myocardium and cardiac fat. Cell-specific P2Y₂ knockout would be needed to evaluate the respective contribution of each cell type involved in UTP effect on cardiac inflammation.

All the actors involved in myocardial healing, including cardiac adipose tissue, have to be taken into consideration when addressing therapies aimed at modulating post-ischemic inflammation. Until now, the cardioprotective properties of PAT were preferably attributed to the regulation of factors such as adiponectin, hepatocyte growth factor, or vascular endothelial growth. It was previously demonstrated that UTP-pretreatment of WT mice before LAD reduces infarct size [44]. This cardioprotective effect of UTP was lost in P2Y₂-null mice [24] and attributed to the UTP-mediated protection of cardiomyocytes from hypoxic stress [45]. The role of P2Y₂ in cardiac fat and FALCs investigated in this study could contribute to the previously observed UTP cardioprotective effect.

P2Y₂ is defined in this study as a regulator of cardiac adipogenesis and leukocyte populations constituting the lymphoid clusters inside the cardiac fat. These findings not only bring a new key element to the study of the complex role

of P2Y₂ receptor in inflammation but also highlight how the identification of regulators of cardiac fat and FALCs formation constitute a major interest in the study of cardioprotective mechanisms. The identification of P2Y₂ as a positive regulator of cardiac fat mass and FALCs formation could have interesting applications in the field of cardiovascular diseases.

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