Circulation
JOURNAL OF THE AMERICAN HEART ASSOCIATION

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Long-Term Heart Transplant Survival by Targeting the Ionotropic Purinergic Receptor P2X7

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Background—Heart transplantation is a lifesaving procedure for patients with end-stage heart failure. Despite much effort and advances in the field, current immunosuppressive regimens are still associated with poor long-term cardiac allograft outcomes, and with the development of complications, including infections and malignancies, as well. The development of a novel, short-term, and effective immunomodulatory protocol will thus be an important achievement. The purine ATP, released during cell damage/activation, is sensed by the ionotropic purinergic receptor P2X7 (P2X7R) on lymphocytes and regulates T-cell activation. Novel clinical-grade P2X7R inhibitors are available, rendering the targeting of P2X7R a potential therapy in cardiac transplantation.

Methods and Results—We analyzed P2X7R expression in patients and mice and P2X7R targeting in murine recipients in the context of cardiac transplantation. Our data demonstrate that P2X7R is specifically upregulated in graft-infiltrating lymphocytes in cardiac-transplanted humans and mice. Short-term P2X7R targeting with periodate-oxidized ATP promotes long-term cardiac transplant survival in 80% of murine recipients of a fully mismatched allograft. Long-term survival of cardiac transplants was associated with reduced T-cell activation, T-helper cell 1/T-helper cell 17 differentiation, and inhibition of STAT3 phosphorylation in T cells, thus leading to a reduced transplant infiltrate and coronaropathy. In vitro genetic upregulation of the P2X7R pathway was also shown to stimulate T-helper cell 1/T-helper cell 17 cell generation. Finally, P2X7R targeting halted the progression of coronaropathy in a murine model of chronic rejection as well.

Conclusions—P2X7R targeting is a novel clinically relevant strategy to prolong cardiac transplant survival. (***Circulation***. 2013;127:463-475.)

Key Words: immunology ■ P2X7R ■ rejection ■ transplantation

Heart transplantation is a lifesaving procedure for patients with end-stage heart failure[1,2]; however, despite much improvement in surgical approaches and patient management, the late outcome of graft survival has not improved in the past decade.[2,3] Although current immunosuppressive regimens have brought about a drastic reduction in acute rejection episodes,[3] the rate of chronic rejection leading to cardiac transplant loss has not improved, and the risk of immunosuppression-related graft toxicity, cancer, infections, and coronary allograft vasculopathy remains high.[2–4] Thus, to improve transplantation outcomes and lessen the need for life-long immunosuppression, it is critical to continue the development of novel immunomodulatory strategies to promote stable graft acceptance.[5] The alloimmune response involves complex immunologic interactions between inflammatory mechanisms, which cause graft rejection, and anti-inflammatory mechanisms, which promote tolerance of the graft.[6] Blocking pathogenic and upregulating tolerogenic mechanisms has been shown to result in enhanced graft survival.[7] To achieve complete activation of T cells during the alloimmune response,

delivery of signal 1 (engagement of the T-cell receptor [TCR] with the major histocompatibility complex-peptide complex on antigen-presenting cells) and signal 2 (interaction of costimulatory molecules) is required.[8,9] However, other players, including innate immunity and inflammation, have been recently recognized to exert a major role.[10,11] The purine ATP, released during cell damage/inflammation, may serve as 1 such signal. ATP is present at high concentrations within the cells,[12] and it is thus released following cell damage or death[13]; moreover, ATP can be secreted by immune cells following activation.[14,15] ATP is abundant at sites of inflammation and is sensed by ionotropic purinergic P2X receptors (7 receptors named P2X1R to P2X7R, or P2XsR).[16–18] P2XsR have been associated in vitro to T-cell activation and interleukin (IL)-2 production[14,19–21] and in vivo to graft-versus-host disease.[18] Recently, ATP has been shown to play a major role in T-helper cell (Th17) differentiation[22] and regulatory T cell (Treg) inhibition,[23] and T-helper cell 1 (Th1) and Th17 immune responses have been associated with organ rejection.[24,25] Indeed, natural ATP/P2XsR inhibitors with the ability to hydrolyze ATP are expressed on the cellular plasma membrane (eg, the ectonucleotidases CD39 and CD73).[26] Novel P2X7R inhibitors are available for human use, including periodate-oxidized ATP (oATP), CE224,535, AZD9056, and GSK1482160, thereby rendering P2X7R targeting a potential path to be tested in transplantation.[27] oATP, a small Schiff base molecule, is an irreversible antagonist of P2X7R owing to the selective modification of lysine residues that occurs in the vicinity of the ATP-binding site[28]; oATP has also been proposed to exert additional inhibitory effect on the other purinergic receptors.[20] We aim to unveil the largely unknown role of P2X7R in heart transplantation and to target the ionotropic purinergic receptor P2X7R to achieve tolerance toward cardiac transplants.

Materials and Methods

Immunologic Methods can be found in the online-only Data Supplement Materials.

Patients

Cardiac samples were obtained from the right side of the interventricular septum of cardiac transplant recipients at Niguarda Ca' Granda Hospital, Milan, Italy. Samples were formalin-fixed, paraffin-embedded, sectioned, and histologically graded by a cardiac pathologist according to the 2005 International Society for Heart and Lung Transplantation criteria.[29] Characteristics of patients and the immunosuppressive regimen[30] are depicted in the Table.

Mice

C57BL/6, BALB/c, C57BL/6 P2X7−/−, B6.C-H2bm12 (bm12), and C57BL/6 Rag1−/− mice of various ages were obtained from the Jackson Laboratory. ABM TCR-Tg mice have been described previously[31] and were maintained as a breeding colony in our animal facility. All mice were cared for and used in accordance with institutional guidelines. Protocols were approved by the Harvard Medical School Animal Care and Use Committee.

Interventional Studies

Mice were injected intraperitoneally with oATP (Medestea srl) 250 μg/d intraperitoneally for 14 days and Colivelin (Tocris, Minneapolis, MN) 7

Table.   Patient Characteristics

Year of transplantation	2011
Number of transplantations	23
Sex (M/F)	16/7
Age, y	47±15
End-stage heart failure etiology, n (%)	
Postischemic	6 (26.9)
Dilated CMP	11 (47.8)
Valvular CMP	2 (8.7)
Retransplantation	1 (4.3)
Hypertrophic CMP	1 (4.3)
Postcorrection congenital CMP	1 (4.3)
CMP associated with Steinert myotonic dystrophy syndrome	1 (4.3)
Initial immunosuppressive regimen*	
Induction	ATG
Maintenance	Csa+methylprednisolone+mycophenolate mofetil
Acute graft rejection episodes,† n (%)	10 (43.8)
Mortality, n (%)	
Acute rejection 3R (50 d after transplantation)‡	1 (4.3)
Multiorgan failure (70 d after transplantation)	1 (4.3)
Donor	
Age, y	45±13
Ischemia, min	185±53

Data are expressed as mean±SD. CMP indicates cardiomyopathy; ATG, thymoglobulin; and Csa, cyclosporine; P2X7R, purinergic receptor P2X7.

*Immunosuppressive regimen was subsequently tuned according to patient conditions.[30]

†Requiring intravenous steroids treatment.

‡Assessed for P2X7R expression.

nmol/L per day for 7 days. In vitro assays were performed in the presence of varying concentrations of oATP, NF-449 (P2X1R inhibitor), and 5-BDBD (P2X4R inhibitor) from Tocris Bioscience (Bristol, UK)

Heart Transplantation

Vascularized cardiac allografts were transplanted intra-abdominally by using microsurgical techniques as described by Corry et al.[32] Rejection was determined as complete cessation of cardiac contractility and was confirmed by direct visualization.

Histology and Immunohistochemistry

Immunohistochemistry was performed with 5-μm-thick formalin-fixed, paraffin-embedded tissue section. Photomicrographs (400×) were taken by using an Olympus BX41 microscope (Center Valley, PA). The following primary antibodies were used: anti-Mac2, anti-CD3 (Cell Marque, CA), anti-CD20, anti CD68 (Dako, Glostrup, Denmark), and anti-P2X7 (Alomone Labs, Jerusalem, Israel). As isotype control for anti-P2X7R, normal rabbit immunoglobulin G (R&D Systems, MN) was used. Graft histology was evaluated by an expert pathologist and was quantified as follows: (i) heart coronary vasculopathy: 0, normal arteries; 1, mild arterial wall infiltration; 2, heavy arterial wall infiltration with partial luminal occlusion; 3, complete luminal occlusion; and (ii) heart cell infiltrate: 0, no cell infiltrate; 1, mild cell infiltrate; 2, medium cell infiltrate; 3, heavy cell infiltrate.

Confocal Microscopy

Immunofluorescence samples were observed by using a confocal system (LSM 510 Meta scan head integrated with the Axiovert 200 mol/L inverted microscope; Carl Zeiss, Jena, Germany) with a 63× oil objective. Images were acquired in multitrack mode with the use of consecutive and independent optical pathways.

Quantitative Real-Time Polymerase Chain Reaction

RNA was purified by using an RNeasy kit (Qiagen, CA) and was reverse-transcribed into cDNA by using Superscript III (Invitrogen, CA). Transcripts were amplified using a 7300 Real-Time polymerase chain reaction (PCR) System (Applied Biosystems, CA); primers were obtained from Applied Biosystems. Real-time PCR products were normalized to copies of GAPDH.

Flow Cytometry and Intracellular Cytokine Staining

Annexin V Apoptosis Detection Kit, anti-mouse CD4, CD8, CD25, CD44, CD62L, interferon-γ (IFN-γ), IL-17, and FoxP3, were purchased from BD Biosciences and eBioscience (San Diego, CA), anti-mouse P2X7 was purchased from Alomone Labs.[33]

Phoshoprotein Determination

Cells were lysed by using 30 µL of lysis buffer provided with the Millipore MILLIPLEX MAP kit (Millipore, MA) supplemented with protease inhibitor. The Millipore MILLIPLEX MAP Cell Signaling Assay and the Luminex xMAP platform were used according to the manufacturer's protocol (Millipore).

Western Blot

Total protein concentration in cell lysates was measured by using a Bradford assay (Bio-rad, CA). Proteins were resolved on an sodium dodecyl sulfate polyacrylamide gel via electrophoresis and were transferred to a nitrocellulose membrane and then incubated with primary rabbit anti-P2X1, anti-P2X4, and anti-P2X7 receptor antibodies (Alomone Labs), anti-T-bet/Tbx21 and anti-ROR-γ (Abcam, MA), anti-Stat3 and anti-Phospho Stat3 (Ser727) (Cell Signaling, MA), and GAPDH (Cell Signaling), as well. Densitometric quantification of band intensity was analyzed by using ImageJ software and was normalized to GAPDH-loading control expression.

Statistical Analysis

Data are expressed as means±standard error of mean. Statistical differences in survival times were determined by the use of Kaplan-Meier curves, followed by the log-rank test. Analyses were performed in SAS (SAS Institute, NC, v. 9.3). All data were transformed to their base 10 logarithms for their statistical evaluation. The Levene test was used to test homogeneity of variances. Whenever unequal variances existed in the comparison of 3 groups or more, Welch analysis of variance with appropriate degrees of freedom (≥2) was used to compare the groups. Subsequently, post hoc analysis of Welch *t* test with Bonferroni correction was applied. In the 2-group comparison, Levene test and Welch *t* test were used. *P*<0.05 was considered significant. Graphs were generated by using GraphPad Prism version 5.0 (GraphPad Software).

Results

P2X7R Is Induced in Cardiac Transplants During the Alloimmune Response

We evaluated the expression of P2XsR in cardiac transplants in vivo. Hearts from BALB/c (H-2^d) mice were heterotopically transplanted into C57BL/6 (H-2^b) mice, and cardiac transplants and splenocytes were harvested at day 7 after transplantation (the average time for cardiac transplant

rejection). Expression of the P2XsR was compared with baseline values (ie, untransplanted BALB/c hearts or naïve C57BL/6 splenocytes) by real-time PCR. Upregulation of P2X1R (14-fold increase), P2X4R (4-fold increase), and P2X7R (9-fold increase) receptors was observed in the cardiac transplants (Figure 1A, 1D, and 1G). Conversely, no induction of P2XsR was observed in splenocytes of cardiac-transplanted mice (data not shown). To dissect the specific effect of the alloimmune response from effects of ischemia-reperfusion injury and nonspecific inflammation, we compared P2XsR expression in allografts and syngrafts (C57BL/6 hearts into C57BL/6 recipients). P2X1R, P2X4R, and P2X7R were also upregulated in syngeneic heart transplants in comparison with baseline (Figure 1A, 1D, and 1G); however, only P2X7R was significantly increased in allogeneic in comparison with syngeneic transplants (Figure 1G). P2X2R, P2X3R, P2X5R, and P2X6R (Figure 1B, 1C, 1E, and 1F) expression was unchanged in both cardiac syngrafts and allografts in comparison with baseline. We then assessed which cell type was responsible for the P2X7R upregulation observed with the use of immunohistochemical analysis. P2X7R staining was negative in untransplanted BALB/c hearts (data not shown), whereas clear positive staining was evident in the mononuclear immune cell infiltrate of cardiac transplants harvested at day 7 (Figure 1H1 and 1H2); cardiomyocytes and other parenchymal cells appeared to be negative for P2X7R staining. We also confirmed complete overlap between CD3$^+$ T cells and P2X7R expression via immunofluorescence and confocal microscopy (Figure 1I1 through 1I3). In addition, we evaluated whether P2X7R upregulation was a phenomenon present in patients (Table). When we analyzed the graft infiltrate in patients experiencing acute heart rejection, clear expression of P2X7R was observed (Figure 1J1 and 1J2), and confocal imaging confirmed that P2X7R staining overlapped with CD3 staining (Figure 1K1 through 1K3). No colocalization was evident between P2X7R expression and CD20$^+$ B cells (L) or CD68$^+$ macrophages (M).

In Vivo Short-term P2X7R Targeting Prevents Cardiac Transplant Rejection, Abrogates the Th1/Th17 Immune Response, and Reduces Effector T Cells in Mice

We next tested the effect of P2X7R targeting in preventing cardiac transplant rejection by using the P2X7R inhibitor oATP. Untreated C57BL/6 mice transplanted with BALB/c hearts (fully mismatched) invariably rejected grafts within 7 days (mean survival time of 7 days; Figure 2A). P2X7R targeting with the use of the short-term treatment of oATP (250 mg oATP intraperitoneally daily for 14 days) induced long-term cardiac transplant survival (>100 days) in 80% of recipients (Figure 2A). To evaluate the effect of P2X7R targeting on the immune system of cardiac-transplanted mice, splenocytes were harvested at days 7 and 100 posttransplantation and were challenged with BALB/c-irradiated splenocytes in an enzyme-linked immunosorbent spot (ELISPOT) assay. Reduced numbers of IFN-γ–producing cells (Figure 2B) and increased numbers of IL-4–producing cells (Figure 2C) were observed in oATP-treated in comparison with untreated mice at day 7; a stronger effect
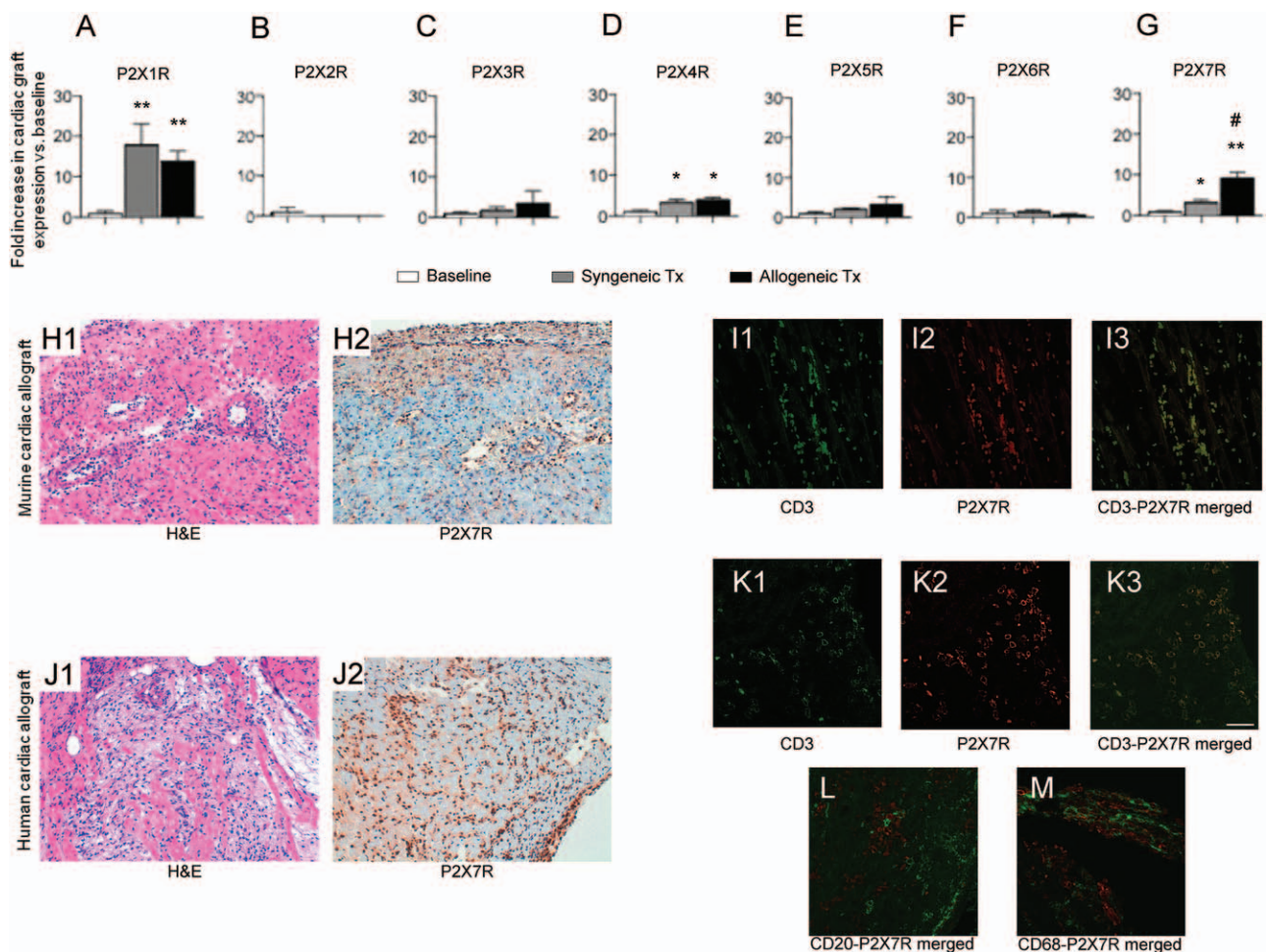
Figure 1. Syngeneic C57BL/6 (Syngeneic Tx) and allogeneic BALB/c cardiac transplants (Allogeneic Tx) were harvested from C57BL/6 recipient mice 7 days posttransplantation, and P2XsR mRNA levels were compared with untransplanted BALB/c hearts (Baseline). An upregulation of P2X1R (**A**), P2X4R (**D**), and P2X7R (**G**) expression in both syngrafts and allografts was evident in comparison with baseline (n=3, *P<0.05, **P<0.01); greater P2X7R upregulation was observed in allogeneic than in syngeneic heart transplants (n=3, #P<0.05; **G**). No upregulation for P2X2R (**B**), P2X3R (**C**), P2X5R (**E**), and P2X6R (**F**) was observed. Substantial infiltration was observed at day 7 after transplantation in allografts (**H1**). Immunohistochemistry revealed that mononuclear infiltrating cells were positive for P2X7R expression (**H2**), whereas myocytes, endothelial cells, and fibroblast showed no expression (**H2**). Immunofluorescence confirmed complete overlapping between CD3$^+$ T cells and P2X7R expression (**I1** through **I3**). P2X7R was expressed in lymphocytes infiltrating human cardiac transplants undergoing acute rejection as well (**J1** and **J2**). Complete overlapping between CD3$^+$ T cells and P2X7R was also demonstrated (**K1** through **K3**), whereas no colocalization was observed between P2X7R (red; **L** and **M**) and CD20$^+$ B cells (green, **L**) or CD68$^+$ macrophages (green, **M**). P2X1R indicates purinergic receptor P2X1; P2X2R, purinergic receptor P2X2; P2X3R, purinergic receptor P2X3; P2X4R, purinergic receptor P2X4; P2X5R, purinergic receptor P2X5; P2X6R, purinergic receptor P2X6; P2X7R, purinergic receptor P2X7; and H&E, hematoxylin and eosin.

was observed at day 100 in oATP-treated mice (Figure 2B and 2C). The percentages of peripheral CD4$^+$ effector T cells (CD4$^+$CD44^{hi}CD62L^{low}, or CD4$^+$ Teffs), CD8$^+$ effector T cells (CD8$^+$CD44^{hi}CD62L^{low}, or CD8$^+$ Teffs), regulatory T cells (CD4$^+$CD25$^+$FoxP3$^+$, or Tregs), and Th17 cells (CD4$^+$IL17$^+$) were quantified in cardiac-transplanted mice by flow cytometric analysis. The percentages of CD4$^+$ Teffs (Figure 2D) and CD8$^+$ Teffs (Figure 2E) were reduced by in vivo short-term P2X7R targeting in mice at days 7 and 100 posttransplantation in comparison with untreated mice. The percentage of Th17 cells was similarly reduced (Figure 2F). No differences in the percentage of Tregs were observed between treated and untreated mice at day 7, but an increase was observed at day 100 (Figure 2G). Thus, we show that short-term P2X7R targeting reshapes the immune system and induces hyporesponsiveness toward donor antigens.

In Vivo Short-term P2X7R Targeting Induces Anergy Toward Donor-Derived Alloantigens but Preserves Immunocompetence in Cardiac-Transplanted Mice

To assess whether long-term cardiac transplant survival was associated with active regulation toward alloantigens or with reduced immunocompetence, hearts from BALB/c mice were transplanted into immunodeficient C57BL/6 Rag$^{-/-}$ mice together with splenocytes obtained from cardiac transplant recipients. Graft rejection was observed within 20 days in mice adoptively transferred with naïve C57BL/6 splenocytes, and no protection was observed when splenocytes obtained from mice with long-term graft function were coadoptively transferred with naïve splenocytes (Figure 2H). Conversely, prolonged graft survival >50 days was observed when splenocytes obtained from cardiac-transplanted mice with long-term
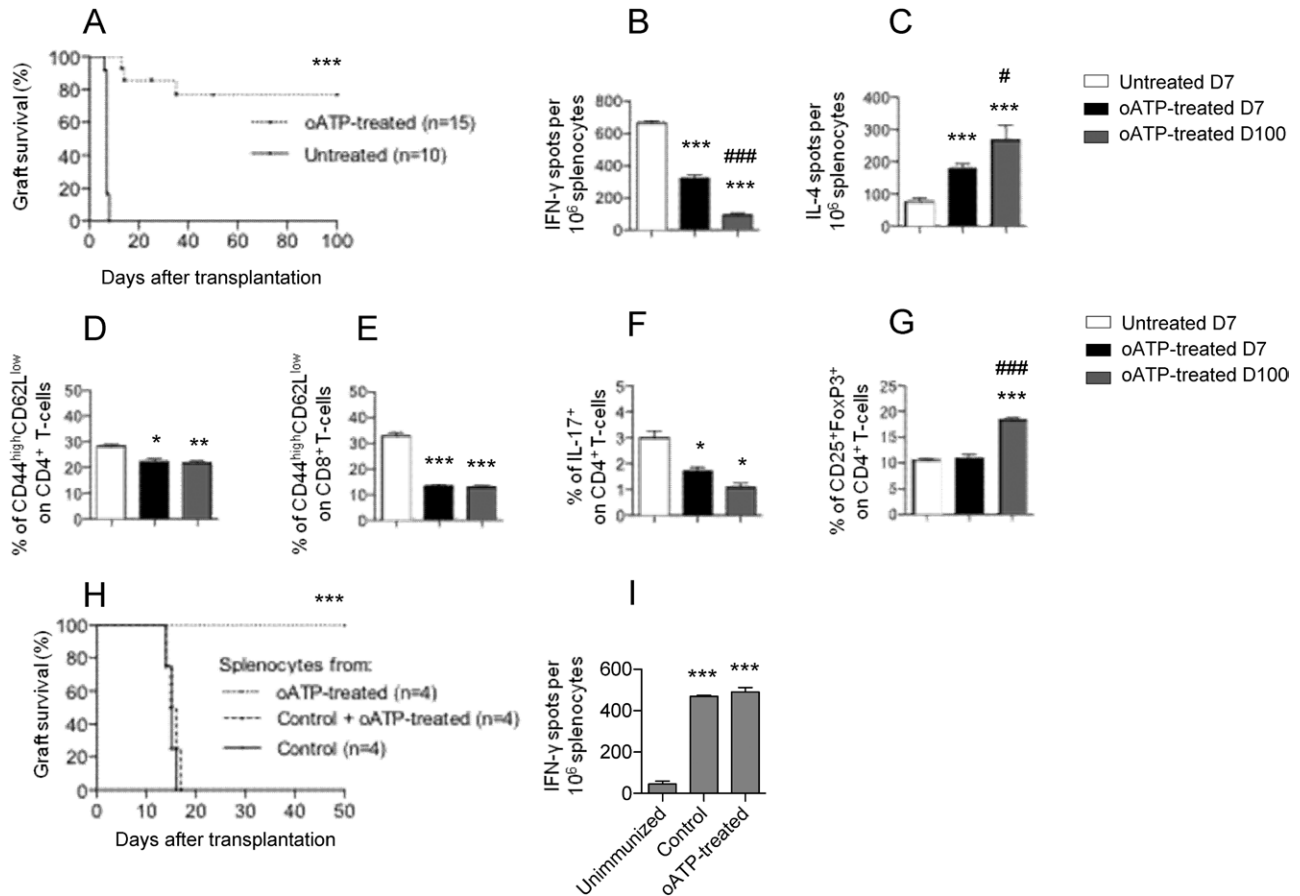
Figure 2. P2X7R inhibition with oATP in the BALB/c into C57BL/6 cardiac transplantation model prevented transplant rejection in 80% of transplants (n=15, ***$P<0.001$ versus Untreated; **A**). When recipient splenocytes were rechallenged with donor splenocytes in an ELISPOT assay at day 7 after transplantation, they displayed fewer IFN-γ–producing cells (n=5, ***$P<0.001$ versus Untreated day 7; **B**) and more IL-4–producing cells (n=5, ***$P<0.001$ versus Untreated day 7; **C**) with further polarization at day 100 (n=5, IFN-γ: ###$P<0.001$; IL-4: #$P<0.05$ versus Untreated and oATP-treated day 7; **B** and **C**). A significant reduction in the number of CD4+ Teffs (n=5, *$P<0.05$, **$P<0.01$ versus Untreated day 7; **D**), CD8+ Teffs (n=5, ***$P<0.001$ versus Untreated day 7; **E**), and IL-17+CD4+ T cells (n=5, *$P<0.05$ versus Untreated day 7; **F**) was observed at days 7 and 100 in oATP-treated mice. No differences in the percentages of peripheral Tregs were observed between oATP-treated and untreated mice at day 7, whereas an increase in the Treg population was seen at day 100 (n=5, ***$P<0.001$ versus Untreated day 7, ###$P<0.001$ versus oATP-treated day 7; **G**). BALB/c cardiac transplant rejection was observed in C57BL/6 Rag−/− mice adoptively transferred with naïve C57BL/6 splenocytes alone (Controls) or in combination with splenocytes from oATP-treated mice, whereas prolonged graft survival was observed when splenocytes from oATP-treated mice were transferred alone (n=4, ***$P<0.001$ versus Controls; **H**). Immunocompetence was preserved in oATP-treated mice as assessed by in vitro ovalbumin response after in vivo immunization (n=3, ***$P<0.001$ versus Unimmunized; **I**). ELISPOT indicates enzyme-linked immunosorbent spot; IFN-γ, interferon γ; oATP, periodate-oxidized ATP; IL-4, interleukin 4; and IL-17, interleukin 17.

graft function were adoptively transferred (Figure 2H). These data suggest that in vivo short-term P2X7R targeting is more likely to induce anergy toward graft antigens than to induce active regulation. We then tested the immunocompetence of treated mice at day 100 after transplantation. Naïve C57BL/6 mice or oATP-treated mice were immunized with ovalbumin, and splenocytes were rechallenged in vitro 7 days later; no differences in numbers of IFN-γ–producing cells were observed (Figure 2I). These data demonstrate that in vivo short-term P2X7R targeting promotes anergy toward graft antigens while maintaining immunocompetence.

In Vivo Short-term P2X7R Targeting Reduces Infiltration and Th1/Th17 Transcripts in Cardiac Transplants

To further investigate the effect of in vivo short-term P2X7R targeting on the antigraft response, we analyzed cardiac transplant infiltrate in oATP-treated and untreated mice. Histological analysis performed at day 7 posttransplantation revealed a reduced CD3+ T cell infiltrate in treated (Figure 3B1 and 3B2) in comparison with untreated mice (Figure 3A1 and 3A2), and semiquantitative analysis confirmed a reduction in degree of infiltration and of coronary vasculopathy (Figure 3C and 3D). To investigate the effect of oATP treatment on Th1 and Th17 cells infiltrating the cardiac transplant, we analyzed allograft mRNA expression of T-bet (a Th1 cell marker) and of ROR-γ (a Th17 cell marker); both markers appeared substantially reduced in oATP-treated mice (Figure 3E and 3F), but no difference in the Th2 transcript GATA3 was observed (data not shown). At day 100, cardiac allografts were well preserved and free from infiltration in oATP-treated mice (Figure 3G1 and 3G2). Thus, pathological analysis confirms that P2X7R targeting preserves cardiac transplant morphology.
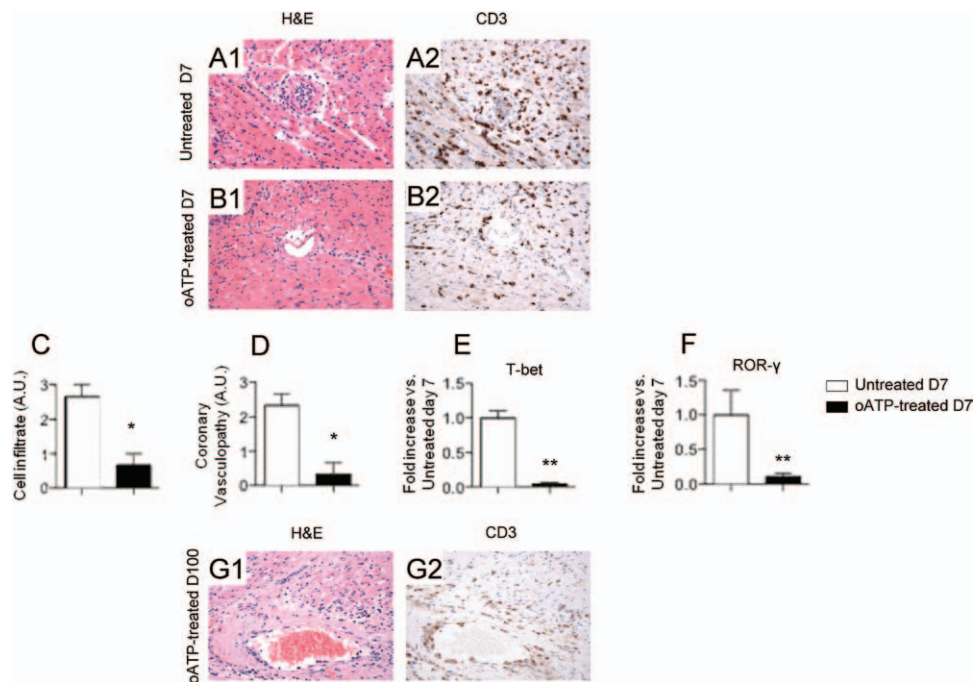
Figure 3. Histological analysis of cardiac transplants at day 7 after transplantation revealed preserved graft parenchyma, reduced artery lumen occlusion, and fewer numbers of CD3+ graft-infiltrating cells in oATP-treated (**B1** and **B2**) in comparison with untreated mice (**A1** and **A2**). Semiquantification of graft infiltrate (n=5, *P<0.05 versus Untreated day 7; **C**) and coronary vasculopathy (n=5, *P<0.05 versus Untreated day 7; **D**) confirmed the protection conferred by oATP treatment. T-bet (a Th1 marker) (n=3, **P<0.01 versus Untreated day 7; **E**) and ROR-γ (a Th17 marker) (n=3, **P<0.01, versus Untreated day 7; **F**) transcripts were reduced in oATP-treated in comparison with untreated mice. Preserved parenchyma, mild coronary vasculopathy, and very few CD3+ lymphocytes and MAC2+ macrophages were observed at 100 days after transplantation in oATP-treated mice with long-term functioning cardiac transplants (**G1** and **G2**). oATP indicates periodate-oxidized ATP; and Th, T-helper cell.

In Vivo Short-term P2X7R Targeting Inhibits the Expansion of Alloantigen-specific T Cells

To address whether the inhibition of the effector T-cell compartment is related to reduced priming and expansion of alloreactive T cells or to their increased apoptosis, we tracked alloreactive-specific T cells in a transgenic model of cardiac transplantation. Bm12 hearts were transplanted into C57BL/6 Rag^{-/-} mice, and 3×10^6 ABM CD4+ TCR-Tg T cells (specific for bm12 major histocompatibility complex class II antigens) were subsequently adoptively transferred into cardiac transplant recipients.[31] Seven days posttransplantation, reduced numbers of ABM CD4+ TCR-Tg T cells were evident in oATP-treated in comparison with untreated mice (Figure 4A). A marked reduction in the numbers of Teffs and Th17 cells within the Tg population was also observed in oATP-treated in comparison with untreated mice (Figure 4B and 4C, respectively). We then examined whether the reduced number of alloantigen-specific CD4+ T cells was due to reduced proliferation or to increased apoptosis. A decrease in ABM CD4+ TCR-Tg T-cell proliferation, as assessed by the dilution of the intracellular dye carboxyfluorescein diacetate succinimidyl ester, was observed in oATP-treated (Figure 4E) in comparison with untreated mice (Figure 4D), without substantial differences in TCR-Tg T-cell apoptosis 4 days following adoptive transfer (Figure 4F and 4G). The results obtained demonstrate that the inhibition of the effector cell compartment on oATP treatment is not mediated

by a significant increase in apoptosis, but rather by decreased proliferation.

P2X7R is Required for oATP to Prolong Cardiac Transplant Survival

oATP has been proposed to exert secondary immunomodulatory mechanisms, primarily related to the inhibition of the remaining P2XsR.[20] To verify that the effect on the prevention of cardiac transplant rejection was predominantly due to P2X7R inhibition, we made use of the P2X7R^{-/-} C57BL/6 mouse model. BALB/c hearts were transplanted into P2X7R^{-/-} mice, and cardiac transplant survival was compared with that of wild-type recipients. A significant prolongation of cardiac transplant survival was observed in P2X7R^{-/-} recipients, confirming the role of P2X7R in allograft rejection (Figure 5A). The ability of oATP to prolong cardiac transplant survival was severely altered in P2X7R^{-/-} mice, suggesting that, in the context of cardiac alloimmune response, oATP acts mainly, although not exclusively, through P2X7R (Figure 5A). Moreover, these data suggest that compensatory mechanisms exist in P2X7R^{-/-} mice; indeed, ATP has been shown to signal through other P2XsR, in particular, through P2X1R and P2X4R in the context of T-cell activation and immune function.[21] We thus analyzed CD4+ T cells obtained from P2X7R^{-/-} mice by Western blot, and an upregulation of P2X1R and P2X4R was observed in comparison with wild-type mice (Figure 5B through 5D). Given these data,
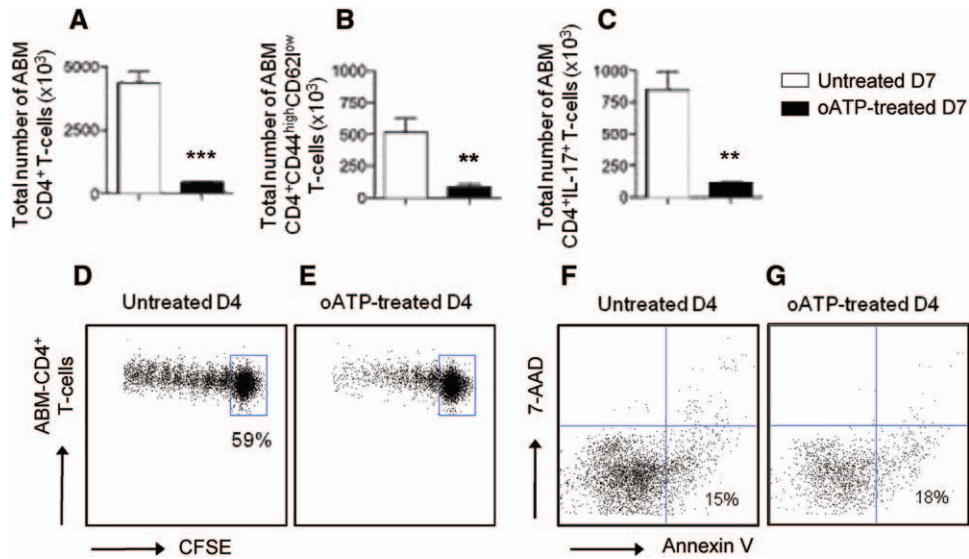
Figure 4. ABM TCR-Tg CD4+ T cells were injected intraperitoneally into bm12 cardiac-transplanted C57BL/6 Rag−/− mice. Reduced numbers of ABM TCR-Tg CD4+ T cells were observed after 7 days in the spleens of oATP-treated in comparison with untreated mice (n=3, ***P<0.001 versus Untreated day 7; **A**). A significant reduction in the numbers of CD4+ Teffs (n=3, **P<0.01 versus Untreated day 7; **B**) and Th17 cells (n=3, **P<0.01 versus Untreated day 7; **C**) was also evident. ABM TCR-Tg CD4+ T cells were then labeled with the intracellular dye CFSE before injection and were harvested after 4 days; significant inhibition of proliferation was observed in oATP-treated mice (**E**) in comparison with untreated mice (**D**). Data are representative of 3 experiments. Similar levels of apoptosis (percentage of AnnexinV+7AAD− cells) of ABM TCR-Tg CD4+ T cells were observed in untreated and oATP-treated mice (**F** and **G**, respectively, representative of 3 experiments). CFSE indicates carboxyfluorescein diacetate succinimidyl ester; oATP, periodate-oxidized ATP; and TCR, T-cell receptor.

we hypothesize that the upregulation of P2X1R and P2X4R may partially compensate for P2X7R function in our model. Moreover, analysis of T-cell populations in P2X7R−/− mice revealed higher percentages of Teff and Treg cells (Figure 5E

and 5F, respectively), thus demonstrating that genetic deletion of P2X7R and the compensatory upregulation of other P2XsR exert profound and complex effects on T-cell activation and homeostasis.
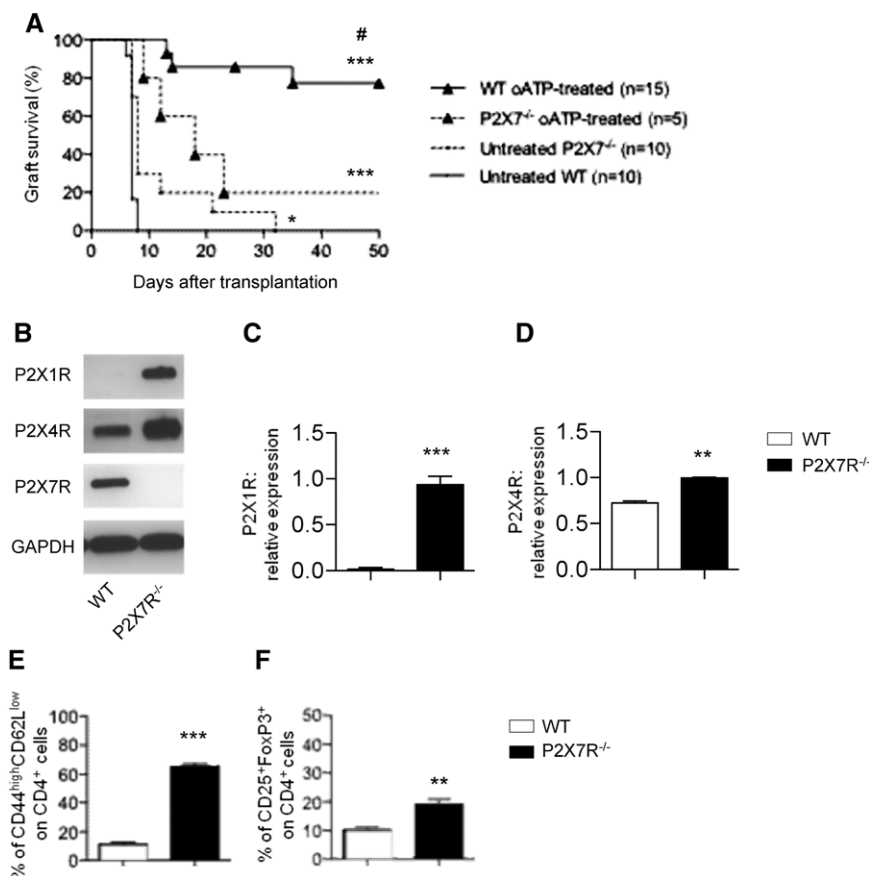

Figure 5. BALB/c hearts were transplanted into WT or P2X7R−/− C57BL/6 mice; graft survival prolongation was observed in P2X7R−/− recipients (*P<0.05 versus WT untreated; **A**); oATP treatment was less effective in prolonging cardiac transplant survival in P2X7R−/− mice in comparison with WT (***P<0.001 versus WT Untreated; #P<0.05 versus all; **A**). Western blot analysis of CD4+ T cells revealed higher P2X1R and P2X4R expression in P2X7R−/− than in WT mice (n=3, **P<0.01; ***P<0.001 versus WT; **B** through **D**). Greater numbers of CD4+ T effector (n=3; ***P<0.001 versus WT; **E**) and T regulatory cells (n=3; **P<0.01 versus WT; **F**) were present in P2X7R−/− than in WT mice. WT indicates wild type; oATP, periodate-oxidized ATP; P2X1R, purinergic receptor P2X1; P2X4R, purinergic receptor P2X4; and P2X7R, purinergic receptor P2X7.

In Vitro P2X7R Targeting Inhibits CD4+ T-Cell Activation and Th1/Th17 Differentiation

To address the mechanisms underlying P2X7R targeting-mediated inhibition of cardiac transplant rejection, we analyzed the effect of P2X7R targeting on T-cell activation and Th1/Th17 differentiation in vitro. P2X7R was expressed on CD4+ and CD8+ T cells isolated from splenocytes, as assessed by Western blotting and real-time PCR, with higher expression levels observed in CD4+ T cells (Figure 6A and 6B). We then tested the effect of in vitro P2X7R targeting with oATP during CD4+ T-cell activation. First, in an ELISPOT assay, naïve CD4+ T cells were stimulated with 0.5 µg/mL anti-CD3-Ig and anti-CD28-Ig for 24 hours, and, when 100 µM oATP was added to cultures, the number of IFN-γ–producing CD4+ T cells was significantly reduced in comparison with controls (Figure 6C). Second, in a T-cell proliferation assay, carboxyfluorescein diacetate succinimidyl ester–labeled naïve CD4+ T cells were stimulated for 96 hours

with 0.5 µg/mL anti-CD3-Ig and anti-CD28-Ig, and treatment with 100 µM oATP diminished CD4+ T cell proliferation (Figure 6D, lower) in comparison with controls (Figure 6D, upper). Expression levels of the P2XsR were not influenced by the presence of oATP during anti-CD3-Ig/anti-CD28-Ig stimulation (online-only Data Supplement Figure IA). To assess whether oATP is specific for P2X7R or whether it also induces its effect through the inhibition of other purinergic receptors (eg, P2X1R or P2X4R, which have been involved in immune function),[21] we combined 100 µM oATP with 50 µM P2X1R (NF-449) and 50µM P2X4R (5-BDBD) inhibitors; a further suppression of anti-CD3-Ig/anti-CD28-Ig–mediated IFN-γ production was obtained (Figure 6E). These data demonstrate that the concentration of oATP is not (at least not completely) blocking P2X1R and P2X4R. We next tested the effect of in vitro P2X7R targeting during a Th1/Th17 generation and differentiation assay. A small percentage of naïve Th0 CD4+ T cells (CD4+CD25−) were shown to express
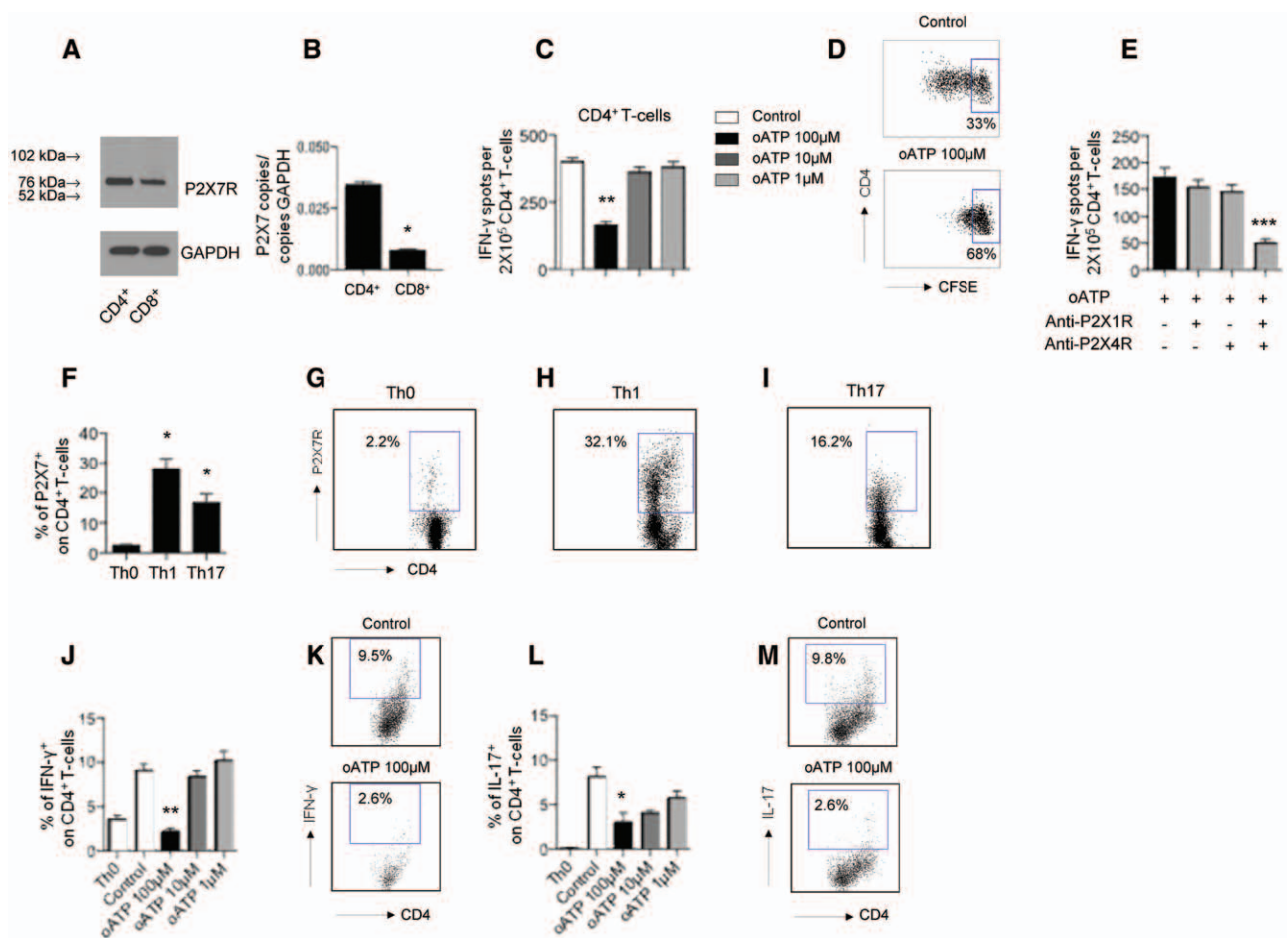

Figure 6. Greater P2X7R expression was observed in CD4+ in comparison with CD8+ T cells, as assessed by Western blot (**A**) and real-time PCR analysis (**B**) (n=3, *P<0.05 versus CD4+ T cells). Anti-CD3-Ig/anti-CD28-Ig-stimulated CD4+ T cells were used in an ELISPOT assay in conjunction with oATP treatment, and a reduction in IFN-γ–producing CD4+ T cells was observed (n=5, **P<0.01 versus Control; **C**). CFSE dilution analysis revealed a lesser number of proliferating CD4+ T cells (**D, lower**; representative of 3 different experiments) in the presence of oATP compared to control (**D, upper**; representative of 3 different experiments). In an ELISPOT assay, IFN-γ production in CD4+ T cells was further inhibited when anti-P2X1R and anti-P2X4R were added (n=5, ***P<0.001 versus oATP; **E**). Although naïve Th0 cells displayed marginal P2X7R expression, when they were differentiated into Th1 or Th17 cells, an upregulation of P2X7R in these cells was observed (n=3, *P<0.05 versus naïve Th0; **F** through **I**). In our Th1/Th17 differentiation assay, reduced numbers of IFN-γ+ (n=4, **P<0.01 versus Control [no oATP]; **J** and **K**) and IL-17+ (n=4, *P<0.05 versus Control; **L** and **M**) CD4+ T cells were generated in the presence of oATP. PCR indicates polymerase chain reaction; IFN-γ, interferon γ; ELISPOT, enzyme-linked immunosorbent spot; CFSE, carboxyfluorescein diacetate succinimidyl ester; P2X1R, purinergic receptor P2X1; P2X4R, purinergic receptor P2X4; P2X7R, purinergic receptor P2X7; IFN-γ, interferon γ; oATP, periodate-oxidized ATP; IL-17, interleukin 17; and Th, T-helper cell.

P2X7R (Figure 6F and 6G); however, when Th0 CD4+ T cells were activated and differentiated in the appropriate cytokine milieu into Th1 or Th17 cells, an upregulation of P2X7R was observed (Figure 6F, 6H, and 6I). P2X7R targeting with oATP inhibited Th0 conversion into both Th1 (Figure 6J and 6K) and Th17 (Figure 6L and 6M), as assessed by the percentage of IFN-γ+ and IL-17+ cells, respectively. We show, therefore, that P2X7R targeting through oATP suppresses activation, proliferation, and Th1/Th17 differentiation of CD4+ T cells. We then further investigated the role of P2X7R on T-cell activation and Th1/Th17 differentiation by genetic upregulation of P2X7R. P2X7R cDNA was transduced by using a pMY-IRES-GFP retroviral vector into CD4+ T cells (pmY-P2X7R CD4+ T cells), and upregulation of P2X7R expression was confirmed by Western blot (online-only Data Supplement Figure IIA). pmY-P2X7R CD4+ T cells were then challenged with anti-CD3-Ig/anti-CD28-Ig stimulation in an ELISPOT assay. A greater number of IFN-γ–producing cells was observed in pmY-P2X7R CD4+ T cells in comparison with cells transduced with the empty vector (pmY-mock CD4+ T cells) (online-only Data Supplement Figure IIB). In a Th1/Th17 generation assay, pmY-P2X7R CD4+ T cells seemed to display increased differentiation capacity in comparison with pmY-mock CD4+ T cells, as assessed by T-bet (online-only Data Supplement Figure IIC and IID) and ROR-γ expression (online-only Data Supplement Figure IIE and IIF).

In Vitro and In Vivo P2X7R Targeting Inhibits STAT3 Phosphorylation

We next assessed which molecular pathway during T-cell activation was preferentially inhibited by the targeting of P2X7R. Several key components of T-cell receptor signaling involved in the alloimmune response[34] were examined, and the phosphorylation kinetics following in vitro anti-CD3-Ig/anti-CD28-Ig–mediated CD4+ T-cell activation was evaluated by using the Luminex assay.[35] Peak phosphorylation for the analyzed pathways was observed at 30 minutes for JNK/SAPK1 and at 60 minutes for p70/S6, STAT3, and STAT5; a second peak was seen for STAT5 at 24 hours (Figure 7A). A significant inhibition of activation-induced STAT3 phosphorylation was observed when oATP was added to the culture (Figure 7E), whereas the phosphorylation of JNK/SAPK1, p70/S6, or STAT5 in CD4+ T cells was unaffected (Figure 7B through 7D, respectively). We then confirmed by Western blot that oATP dose-dependently inhibits STAT3 phosphorylation (Figure 7F). To demonstrate that the effect of oATP is STAT3-phosphorylation-inhibition dependent, we tested the effect of Colivelin treatment, a peptide that promotes STAT3 phosphorylation[36,37] in an add-back experiment. We first confirmed that Colivelin (Col) was able to restore the activation-induced phosphorylation of STAT3 in a dose-dependent manner in oATP-treated CD4+ T cells by Western blotting (Figure 7G). Moreover, in an ELISPOT assay, in which naïve CD4+ T cells were stimulated with 0.5 µg/mL anti-CD3-Ig and anti-CD28-Ig, treatment with Colivelin was shown to significantly revert oATP-mediated suppression of IFN-γ–producing cells (Figure 7H). The effect of oATP and Colivelin on STAT3 phosphorylation and T-cell function was also evaluated following cardiac transplantation. BALB/c hearts were transplanted into C57BL/6 mice,

and STAT3 phosphorylation was assessed by Western blot in CD4+ T cells isolated from splenocytes of mice 7 days after transplantation. oATP-treated mice displayed reduced levels of phosphorylated STAT3 in comparison with untreated mice, and, paralleling the results obtained in vitro, the use of Colivelin was able to reestablish STAT3 phosphorylation in CD4+ T cells of oATP-treated mice (Figure 7I and 7J). From a functional point of view, the use of Colivelin greatly abrogated the effect of oATP on allograft survival (Figure 7K). These data demonstrate that P2X7R signaling is crucial for T-cell activation and Th1/Th17 generation and that this effect is STAT3 dependent.

In Vivo Short-term P2X7R Targeting Prevents Coronary Vasculopathy in a Model of Chronic Heart Cardiac Transplant Rejection

To evaluate the importance of P2X7R signaling and targeting in a clinically relevant setting, we tested the effect of oATP treatment in a model of cardiac transplant chronic rejection (bm12 donors to C57BL/6 recipients). In this model, C57BL/6 mice do not acutely reject bm12 cardiac allografts, but transplanted hearts develop transplant-associated coronary vasculopathy. Cardiac allograft pathology was assessed 40 days after transplantation of bm12 hearts into C57BL/6 mice. Advanced coronary vasculopathy and severe lymphocyte and macrophage interstitial and vascular infiltration were observed in untreated mice (Figure 8A1 through 8A4) in comparison with oATP-treated mice (Figure 8B1 through 8B4), which displayed only mild cellular infiltration and the absence of coronaropathy, as well (Figure 8C and 8D, respectively). oATP-treated mice also showed a reduced number of IFN-γ–producing cells (Figure 8E) and an increased number of IL-4–producing cells (Figure 8F) in an ELISPOT assay when splenocytes were challenged with donor antigens.

Discussion

The introduction of novel immunosuppressive drugs has led to significant improvement in short-term cardiac transplant survival rates but has been unable to significantly improve allograft survival in the long-term[1,2] Furthermore, current immunosuppressive regimens are associated with multiple complications, including cancer, opportunistic infections, diabetes mellitus, kidney failure, and hypertension.[2–4] To improve transplantation outcomes, it is therefore critical to continue the development of novel strategies to lessen the need for lifelong immunosuppression and to achieve stable graft acceptance. We thus studied the role of the ionotropic purinergic P2X receptors (a family of receptors with largely unknown function in the alloimmune response) in cardiac transplant rejection and tolerance.

Aside from signal 1 (TCR engagement) and signal 2 (costimulatory molecule interaction), soluble signals have also been recognized as major players in T-cell activation; we predicted that ATP would exert a fundamental role in the context of allograft organ rejection, in particular, in the first phase after transplantation when it could be released in marked amounts by necrotic or ischemic cells. Among all P2XsR, our data show that P2X7R is specifically upregulated during the alloantigen response in the graft and is expressed by virtually
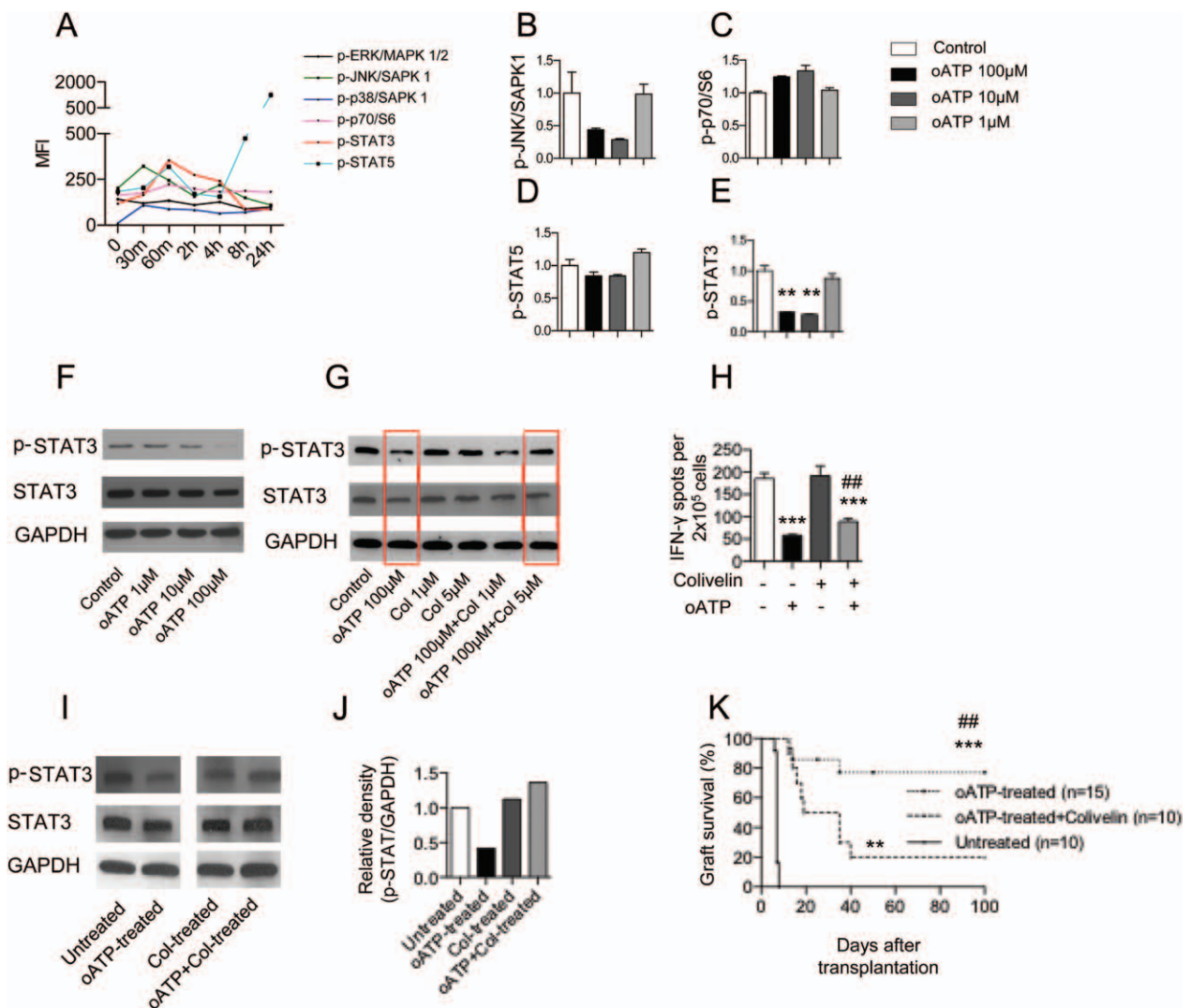
Figure 7. Peak phosphorylation (assayed by Luminex) of JNK/SAPK1 was evident at 30 minutes after anti-CD3-Ig and anti-CD28-Ig stimulation in CD4+ T cells, whereas peaks in p70/S6, STAT3, and STAT5 phosphorylation were evident at 60 minutes; a second peak of phosphorylation for STAT5 was evident at 24 hours (**A**) (shown as MFI [mean fluorescence intensity]). P2X7R targeting via oATP treatment inhibited activation-induced STAT3 phosphorylation (n=3, **P<0.01 versus Control, **E**), whereas target of P2X7R had no effect on phosphorylation of JNK/SAPK1 (**B**), p70/S6 (**C**), or STAT5 (**D**). Western blot analysis confirmed reduced STAT3 phosphorylation dose dependently with oATP treatment (**F**; representative of 3 different experiments). Colivelin (Col) prevented oATP-mediated inhibition of STAT3 phosphorylation (**G**) and rescued CD4+ T cells from oATP-mediated suppression of IFN-γ production (n=8, ***P<0.001 versus Colivelin[–]/oATP[–]; ##P<0.01 versus Colivelin[–]/oATP[+]; **H**). In our allogeneic model of cardiac transplantation, oATP treatment inhibited STAT3 phosphorylation in CD4+ T cells (**I** and **J**). Colivelin restored STAT3 phosphorylation on CD4+ T cells (**I** and **J**) and significantly impaired the prolongation of cardiac transplant survival associated with oATP treatment (**K**; **P<0.01 and ***P<0.001 versus Untreated; ##P<0.01 versus oATP+Colivelin). oATP indicates periodate-oxidized ATP; and IFN-γ, interferon γ.

all T cells infiltrating the graft. On the contrary, P2X1R and P2X4R are similarly increased in both syngrafts and allografts, and their upregulation may thus be more related to ischemia reperfusion injury and to posttransplant inflammation than specifically to the alloantigen response. It is noteworthy that we found P2X7R to be upregulated in T cells infiltrating cardiac transplants during clinical acute rejection, confirming the relevance of the P2X7R pathway in patients undergoing heart transplantation. In vivo, short-term P2X7R targeting with oATP robustly promoted long-term cardiac transplant survival in 80% of the recipients in a fully mismatched model of heart transplantation; moreover, oATP was able to inhibit the development of coronary vasculopathy in a chronic model of cardiac rejection. oATP was initially and primarily described as

a P2X7R inhibitor,[28] and, although recent articles have pointed to a broader effect of oATP as an inhibitor of other purinergic receptors,[20] oATP remains to date the most well-characterized and effective drug to target in vivo P2X7R.[38,39] The relative ineffectiveness of oATP treatment in P2X7R−/− mice appears to confirm that, although additional inhibition of other purinergic receptor may be present, there is a more restricted P2X7R-mediated effect in our model; moreover, our in vitro data show that, at the concentration used, oATP does not (at least not completely) inhibit other P2XsR. P2X7R targeting is associated with a reduction in Teff and Th17 cells, which are known to be highly relevant to the alloimmune response, and we observed a reduction in the Th1/Th17 lymphocytic infiltrate. Inhibition of the antigraft effector T-cell compartment (Th1 and Th17) may
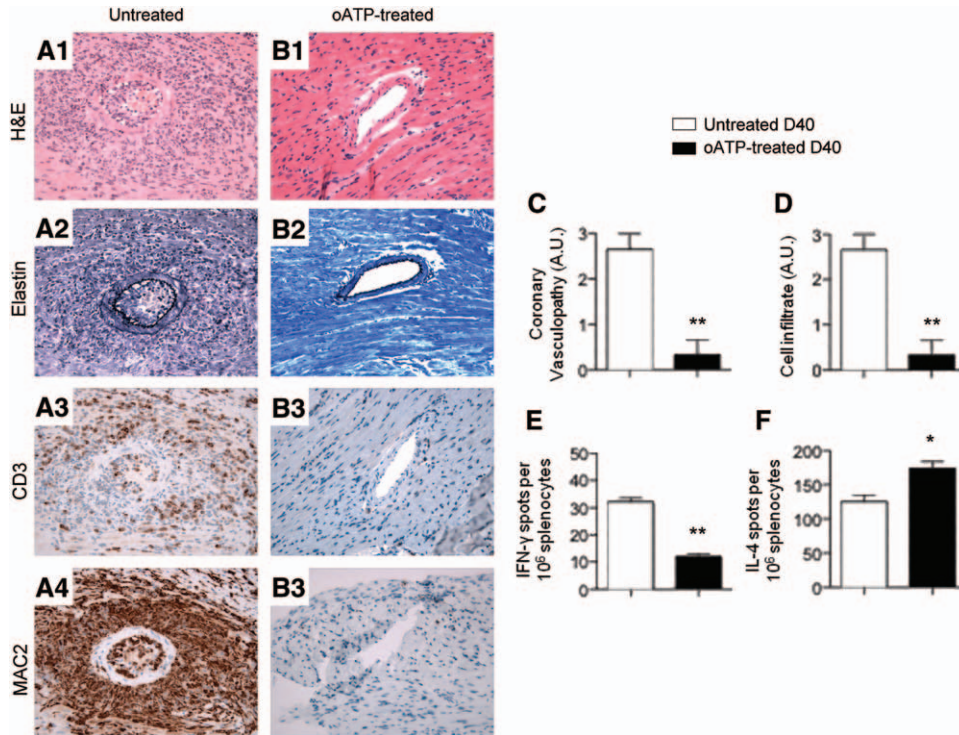
Figure 8. Bm12 hearts were transplanted in C57BL/6 recipients, and histological analysis of the transplants was performed at day 40 after transplantation. Hematoxylin and eosin (H&E) and elastin staining showed advanced intimal proliferation and vascular occlusion of the arteries in untreated mice (**A1** and **A2**), whereas preserved vessel morphology was observed in oATP-treated mice (**B1** and **B2**). Massive vascular and parenchymal T cell (**A3**) and macrophage (**A4**) infiltration was seen in untreated mice, but not in oATP-treated mice (**B3** and **B4**). Semiquantification of coronary vasculopathy (**C**) and infiltration (**D**) confirmed that cardiac transplants were protected by oATP treatment (n=3; **P<0.01 versus Untreated day 40). Results of an ELISPOT assay showed reduced numbers of IFN-γ–producing cells (**E**) and increased numbers of IL-4–producing cells (**F**) among recipient splenocytes challenged with donor antigens in oATP-treated mice (n=3; *P<0.05; **P<0.01 versus Untreated day 40). ELISPOT indicates enzyme-linked immunosorbent spot; oATP, periodate-oxidized ATP; IFN-γ, interferon γ; and IL-4, interleukin 4.

thus be responsible for the observed long-term graft function. Although P2X7R inhibition has been related to a Th17-to-Treg shift in in vitro assays[23] and in a model of inflammatory bowel disease,[14] flow cytometric analysis of treated mice does not support a clear expansion of the Treg compartment (observed only later after treatment) in our model. In contrast, our adoptive transfer experiments show an inhibition of the antigraft effector T-cell compartment and demonstrate, as well, a state of anergy toward graft antigens in treated mice, which is likely responsible for the observed long-term graft survival. Anergy toward alloantigens does not seem to be related to a state of chronic immunosuppression, as demonstrated by the preserved anti-ovalbumin response after immunization, which is particularly applicable to the clinical setting. From a molecular point of view, targeting P2X7R appears to be a result of activation-mediated STAT3 phosphorylation, given the reduced level of phosphorylated STAT3 following oATP treatment. Furthermore, Colivelin treatment, which restores STAT3 phosphorylation, reverts, although not completely, the effect of in vitro and in vivo oATP treatment, showing that other pathways may be affected by oATP.[14,18] STAT3 is a well-known transcription factor with multiple roles in mediating T-cell activation and proliferation.[22,40,41] Our in vitro and in vivo data point to a novel axis in T-cell activation, which links ATP-P2X7R-STAT3 and activation of the antigraft effector T-cell compartment (Th1 and Th17 cells), which can be

inhibited by the use of oATP. The centrality of this pathway at the crossroads of T-cell activation and differentiation is further demonstrated by the transduction of P2X7R on T cells and the associated effects of greater activation and Th1/Th17 differentiation. The P2X7R pathway in T cells is highly important in the context of cardiac transplant rejection and may synergize with the P2X7R pathway in antigen-presenting cells[18] to promote graft rejection. Willhem and colleagues have recently described a role for P2X7R in graft-versus-host disease[18] by using the P2X7R−/− model; in comparison, (i) we tested a pharmacological inhibitor of P2X7R, which is more clinically relevant; (ii) we tested the effect of P2X7R targeting in 2 different models of solid-organ transplantation (ie, the acute and the chronic rejection model); (iii) we demonstrated a direct effect of P2X7R inhibition on T cells, which is independent of the inhibitory effect on antigen-presenting cell function; (iv) we showed that the effect of P2X7R targeting is mediated by an inhibition of the T-cell effector compartment more than by an expansion of the regulatory T-cell compartment; (v) we highlight the necessity of exercising some caution in the analysis of data by using P2X7R−/− mice given that the upregulation of other P2XsR (in the knock-out mouse, but not during pharmacological targeting) may partially obscure P2X7R function. On the NIH clinical trials web site (http://www.clinicaltrials.gov), nearly 80 clinical trials are registered to promote tolerance in transplantation. None of these trials

is specifically aimed or designed to target P2X7R-mediated alloimmunity, yet our results clearly indicate the need for novel therapeutic options for transplantation. We envision P2X7R-mediated immunity as a novel pathway for cardiac transplant rejection. Indeed, a P2XR-targeting strategy has considerable translational potential, because novel anti-P2X7R inhibitors are available for clinical use (eg, oATP, CE 224,535, AZD9056, and GSK1482160). Short-term P2X7R targeting at the time of transplantation may prove to be valuable in the clinical setting by promoting cardiac transplant acceptance, lessening the need for chronic immunosuppression, and preventing the development of coronary graft vasculopathy.

Disclosures

None.

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CLINICAL PERSPECTIVE

Heart transplantation is a lifesaving procedure for patients with end-stage heart failure and provides a better survival and quality of life in comparison with medical treatment or device-based therapies. Recently, advances in immunosuppression and improved clinical care have enhanced the early survival of patients with cardiac transplants. However, >20% of patients do not survive beyond 3 years, and those who survive are afflicted with the long-term complications associated with chronic immunosuppression (eg, posttransplant diabetes mellitus, coronary vasculopathy, nephropathy, infections, and malignancies). To improve transplantation outcomes and lessen the need for life-long immunosuppression, it is crucial to continue the development of novel immunomodulatory strategies. Short-term targeting of the purinergic receptor P2X7 (P2X7R) may provide a novel therapeutic opportunity. Soon after transplantation, purine ATP is released by necrotic cells and causes the activation of graft-infiltrating T cells, which sense ATP by the highly expressed receptor P2X7R. Our data show that short-term P2X7R targeting with periodate-oxidized ATP robustly promotes, in a fully-mismatched model of acute rejection, long-term cardiac transplant survival in 80% of recipients, with preservation of immunocompetence. Moreover, periodate-oxidized ATP inhibits the development of coronary vasculopathy in a model of chronic rejection. Nearly 80 clinical trials are registered on the National Institutes of Health web site to promote long-term graft function, and none of these is designed to target P2X7R-mediated alloimmunity. Because novel P2X7R inhibitors are available for clinical use (eg, periodate-oxidized ATP, CE-224,535, AZD9056, and GSK1482160), short-term P2X7R targeting may have considerable translational potential by promoting cardiac transplant acceptance, thereby lessening the need for chronic immunosuppression and preventing the development of coronary vasculopathy.

ONLINE SUPPLEMENTAL MATERIAL

IMMUNOLOGICAL METHODS

Adoptive transfer of C57BL/6 cells

$20x10^6$ splenocytes obtained from naïve C57BL/6 mice or from oATP-treated transplanted mice at day 100 after transplantation, or $10x10^6$ obtained from naïve C57BL/6 mice and $10x10^6$ obtained from oATP-treated mice, were injected into BALB/c cardiac-transplanted C57BL/6 Rag1$^{-/-}$ mice, and survival was assessed as described.

Adoptive transfer of TCR-Tg cells

$3x10^6$ ABM TCR-Tg CD4$^+$ T cells were injected into bm12 cardiac-transplanted C57BL/6 Rag1$^{-/-}$ mice, and splenocytes were harvested 7 days post-transplant. The total numbers of ABM TCR-Tg CD4$^+$ T cells, Teffs, and Th17 cells were assessed.

Cell purification and CFSE labeling

CD4$^+$, CD8$^+$, and CD4$^+$CD25$^-$ T cells were isolated from splenocytes using MACS microbeads (Miltenyi Biotec, Auburn, CA). CFSE labeling was performed using the Vybrant® CFDA SE Cell Tracer Kit (Invitrogen, Carlsbad, CA).

ELISPOT

An ELISPOT assay was used to measure the number of IFN-γ-/IL-4-producing cells (BD Biosciences, San Jose, CA). Allogeneic splenocytes (BALB/c) or anti-CD3- and anti-CD28-Ig (BD Biosciences, 0.5 μg/ml each) were used to stimulate $1x10^6$ responder splenocytes or $2x10^5$ T cells (C57BL/6)[1]. Spots were counted using an Immunospot analyzer (Cellular Technology Ltd., Cleveland, OH). In a similar experiment, expression levels of P2XsR were assessed by quantitative real-time PCR.

Ovalbumin immunization

Ovalbumin peptide (Sigma Aldrich, St. Louis, MO) emulsified in complete Freund's adjuvant (Sigma Aldrich) was injected once (100 μg/mouse i.p.). Splenocytes were collected and used in *in vitro* assays after rechallenge with 1 μmol/L ovalbumin peptide for 24 h[2].

T-cell differentiation assay

Naive sorted CD4^{+}CD25^{-} T cells (Th0) were isolated and activated for 5 days with 1 μg/ml of plate-bound anti-CD3 and anti-CD28 antibodies (BD Biosciences). Cultures were supplemented with either 5 ng/ml mIFN-γ and 10 μg/ml of anti-IL-4 mAb for Th1 differentiation, or with 10 μg/ml of anti-IFN-γ mAb and anti-IL-4 mAb, 5 ng/ml of mTGF-β and 20 ng/ml of rIL-6 for Th17 differentiation. Proteins and antibodies were purchased from R&D Systems (Minneapolis, MN) and BD Biosciences.

P2X7R transfection of T cells

The hP2X7R cDNA construct (kindly provided by Dr. Gary Buell [Ares-Serono Research Laboratories, Geneva, Switzerland] and Dr. Francesco Di Virglio [University of Ferrara, Ferrara, Italy]) was inserted into the pMY-IRES-GFP plasmid (Cell BioLabs, Inc., San Diego, CA). The Platinum-E Retroviral Packaging cell line was transfected with the pMY-IRES-GFP plasmid expressing P2X7R using Lipofectamine 2000 (Invitrogen), and supernatant was collected. T cells were activated in the presence of Mouse T-Activator CD3/CD28 Dynabeads (Invitrogen), and viral supernatant was added.

SUPPLEMENTAL FIGURE LEGEND

Figure S1. P2XsR expression was tested in CD4^{+} T cells after 24 hours of anti-CD3-Ig/anti-CD28-Ig stimulation with or without 100μM oATP. No differences in the

expression levels of P2X1R, P2X3R, P2X4R, P2X5R, or P2X7R were observed (n=3, A), while levels of P2X2R and P2X6R remained undetectable.

Figure S2. Greater P2X7R expression was observed in pmY-P2X7R-transfected T cells compared to pmY-mock-transfected T cells (A). Anti-CD3-Ig/anti-CD28-Ig-stimulated untransfected (Control), pmY-P2X7R- and pmY-mock-transfected CD4$^+$ T cells were tested in an ELISPOT assay. An increased number of IFN-γ-producing CD4$^+$ T cells was observed in pmY-P2X7R-transfected cells (n=5, ***p<0.001 vs. Control and pmY-mock-transfected; B). In our Th1/Th17 differentiation assay, untransfected pmY-P2X7R- and pmY-mock-transfected naïve Th0 cells were assessed for T-bet and ROR-γ expression. Higher T-bet expression (C, quantified in D, representative of 3 different experiments) and ROR-γ expression (E, quantified in F, representative of 3 different experiments) were observed by western blotting of pmY-P2X7R-transfected cells.

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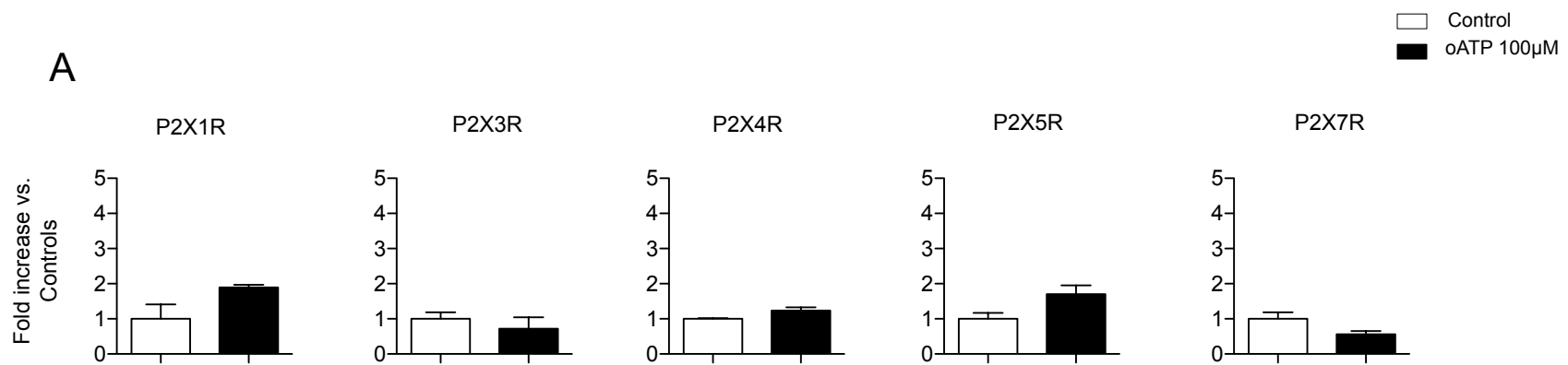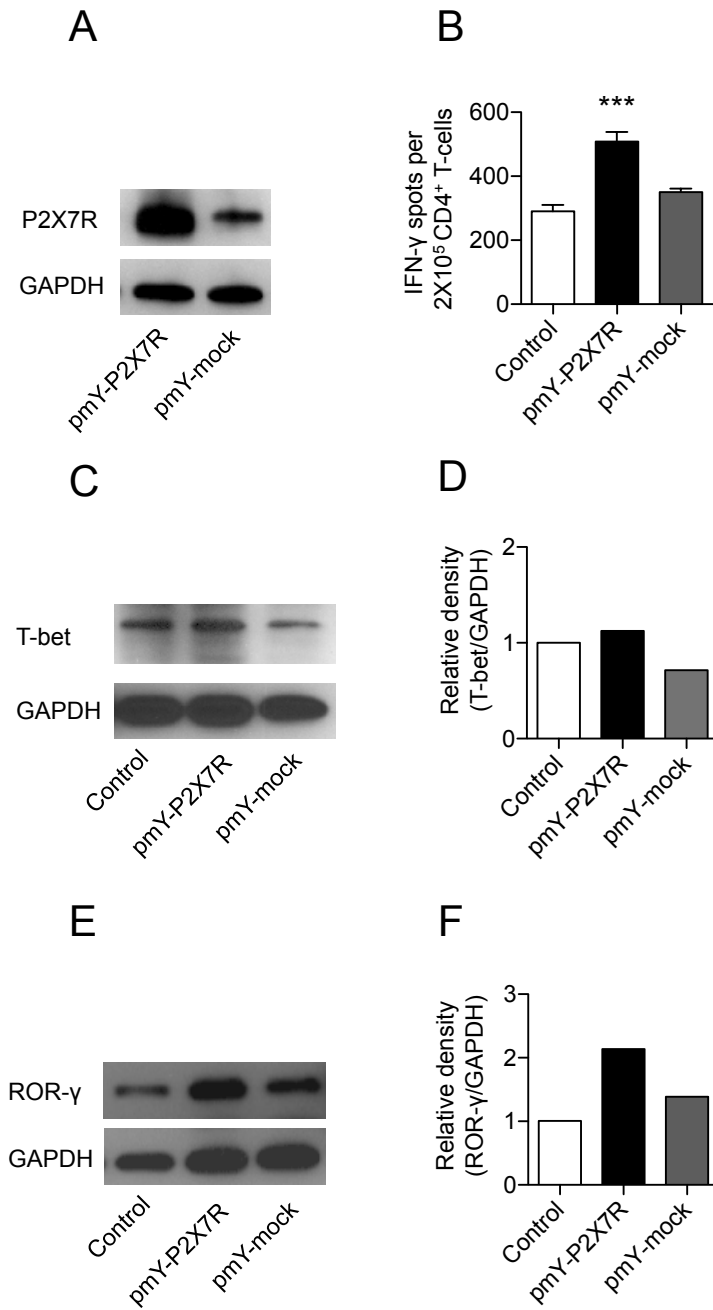
Figure S1

Figure S2