Effect of the Purinergic Inhibitor Oxidized ATP in a Model of Islet Allograft Rejection

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The lymphocytic ionotropic purinergic P2X receptors (P2X1R–P2X7R, or P2XRs) sense ATP released during cell damage–activation, thus regulating T-cell activation. We aim to define the role of P2XRs during islet allograft rejection and to establish a novel anti-P2XRs strategy to achieve long-term islet allograft function. Our data demonstrate that P2X1R and P2X7R are induced in islet allograft-infiltrating cells, that only P2X7R is increasingly expressed during allogeneic immune response, and that P2X1R is augmented in both allogeneic and syngeneic transplants. In vivo short-term P2X7R targeting (using periodate–oxidized ATP [oATP]) delays islet allograft rejection, reduces the frequency of Th1/Th17 cells, and induces hyporesponsiveness toward donor antigens. oATP-treated mice displayed preserved islet grafts with reduced Th1 transcripts. P2X7R targeting and rapamycin synergized in inducing long-term islet function in 80% of transplanted mice and resulted in reshaping of the recipient immune system. In vitro P2X7R targeting using oATP reduced T-cell activation and diminished Th1/Th17 cytokine production. Peripheral blood mononuclear cells obtained from long-term islet-transplanted patients showed an increased percentage of P2X7R+CD4+ T cells compared with controls. The beneficial effects of oATP treatment revealed a role for the purinergic system in islet allograft rejection, and the targeting of P2X7R is a novel strategy to induce long-term islet allograft function. Diabetes 62:1665–1675, 2013

The current mainstay of treatment for type 1 diabetes (T1D) is insulin therapy, which has proven to be a lifesaving breakthrough. However, insulin treatment cannot fully prevent the severe complications related to the disease, including kidney failure and coronary heart disease (1,2). Successful islet transplantation cures T1D, improves glycometabolic control, reduces hypoglycemic episodes, and halts diabetes complications (3–5). Unfortunately, the rate of functioning islet allografts at 5 years is well below 20% (6), primarily although not exclusively, due to alloreactive and autoreactive immune responses (7–9). The anti-islet immune response involves a complex interplay between pathogenic and inflammatory immune pathways, which promote rejection, and regulatory or anti-inflammatory immune pathways, which facilitate tolerance toward transplants (10–12); one such pathway is the purinergic system (13).

The purine ATP is a small molecule (14) present at high concentrations within cells and released after cell damage or death (15) and immune cell activation (16,17); it acts as a danger signal and potent chemotactic mediator (15,18). ATP is abundant at inflammation sites and is sensed by ionotropic purinergic P2X receptors (seven receptors named P2X1–P2X7, or P2XRs) (19–21). In leukocytes, P2XRs regulate cytokine production, activation, and apoptosis (thus constituting an “autocrine alerting system”) (13,22–24). In particular, P2X7R (16,21,25,26) has been linked to T-cell activation and function, serving as a signal amplification mechanism for antigen recognition (13). Recent studies have shown that ATP may be a factor that determines the fate of T cells by promoting Th17 differentiation (24), and Th17 cells have been demonstrated to be relevant in islet allograft rejection (27–29). Inhibiting P2X7R halts the delivery of ATP signals and may redirect the immune system from a Th1/Th17 profile to a more tolerogenic state (30). P2X7R inhibitors are available for human use, including periodate–oxidized ATP (oATP) and CE-224535, thereby rendering P2X7R targeting a potential path to be tested in transplantation. Of particular interest is oATP, a small Schiff-base molecule that irreversibly antagonizes P2X7R through the selective modification of lysine residues in the vicinity of the ATP-binding site (31,32).

We explored the role of P2X7R in islet allograft rejection as a means of establishing an anti–P2X7R-based strategy to redirect the immune system toward a more tolerogenic profile and thus achieving stable tolerance to islet allografts.

RESEARCH DESIGN AND METHODS

Patients. Peripheral blood mononuclear cell (PBMC) fractions were isolated by Ficoll (GE Healthcare, Piscataway, NJ) from patients with T1D (n = 8) and patients who had received allogeneic islet transplantation <3 (n = 8) or ≥3 (n = 8) years earlier, as well as healthy control patients (n = 8). Patient characteristics are presented in Table 1.

Human islet transplantation and immunosuppression. Islet-transplanted patients received the standard triple immunosuppressive regimen: anti-Thymoglobulin (Fresenius, Waltham, MA), followed by FK506 (target blood levels, 6–8 ng/mL; Astellas, Deerfield, IL) and/or cyclosporine (100 ng/mL; Novartis, Basel, Switzerland) and/or rapamycin (8–15 ng/mL; Pfizer, New York, NY) and/or mycophenolate (2 g/day; Roche, Basel, Switzerland) and prednisone (5–10 mg/day; Bruno Farmaceutici, Rome, Italy) (33).

Mice. C57BL/6, BALB/c, and C57BL/6 P2X7R−/− mice were obtained from the Jackson Laboratory, Bar Harbor, Maine. All mice were cared for and used in accordance with institutional guidelines approved by the Harvard Medical School.

See accompanying commentary, p. 1394.
TABLE 1
Characteristics of islet-transplanted patients and healthy volunteers

<table>
<thead>
<tr>
<th></th>
<th>Healthy volunteers (n = 8)</th>
<th>T1D patients (n = 8)</th>
<th>Islet-transplanted patients</th>
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<tbody>
<tr>
<td></td>
<td>T1D</td>
<td>Long-term</td>
<td>Short-term</td>
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<td></td>
<td>(n = 8)</td>
<td>(n = 8)</td>
<td>(n = 8)</td>
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<tr>
<td>Sex (n)</td>
<td>Male 4</td>
<td>4</td>
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<tr>
<td></td>
<td>Female 4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Age (years)</td>
<td>42.3 ± 3.3</td>
<td>39 ± 5</td>
<td>43.7 ± 6.0</td>
</tr>
<tr>
<td>T1D (years)</td>
<td>25 ± 10</td>
<td>29.0 ± 9.1</td>
<td>31.3 ± 3.5</td>
</tr>
<tr>
<td>Time since islet infusion (years)</td>
<td>—</td>
<td>1.2 ± 0.5</td>
<td>7.0 ± 1.2</td>
</tr>
<tr>
<td>C-peptide (ng/mL)</td>
<td>0.01 ± 0.3</td>
<td>1.9 ± 1</td>
<td>1.7 ± 0.8</td>
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<tr>
<td>Exogenous insulin requirement (units/day)</td>
<td>—</td>
<td>32 ± 3.2</td>
<td>7.2 ± 1</td>
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<tr>
<td>HbA1c (%)</td>
<td>—</td>
<td>8.8 ± 1.5</td>
<td>6.8 ± 1.1</td>
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<td>Rapamycin + FK506 (n)</td>
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<tr>
<td>FK506 + mycophenolate (n)</td>
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<td>Cyclosporine + mycophenolate (n)</td>
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</tr>
<tr>
<td>Rapamycin + azathioprine (n)</td>
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Continuous data are expressed as mean ± SD. HbA1c, glycated hemoglobin.

RESULTS
P2X7R is specifically expressed in islet allograft-infiltrating lymphocytes during the alloimmune response in vivo. We first evaluated the expression of P2X7R mRNA during the alloimmune response in vivo in an islet transplantation model. BALB/c (H-2b) islets were transplanted under the kidney capsule of C57BL/6 (H-2b) mice, rendered diabetic through streptozotocin, and islet allografts and spleens were harvested at day 14, according to the kinetics of islet allograft rejection (39). P2X7R expression levels were compared with baseline values using untransplanted BALB/c islets or naive C57BL/6 splenocytes. A significantly higher expression of P2X1R (10-fold increase vs. baseline, n = 3; P = 0.0002) and P2X7R (10-fold increase vs. baseline, n = 3; P = 0.01) was evident in the graft (Fig. 1A). No changes were observed in the expression of the remaining P2XR in the graft (Fig. 1A) or in any P2XR in the spleen (data not shown).

To explore the possible effects of ischemia/reperfusion injury and peritransplant inflammation on P2XR expression levels, we evaluated P2XR expression in the setting of syngeneic islet transplantation—C57BL/6 islets into hyperglycemic C57BL/6 mice—in which the effects of the alloimmune response are absent. At day 14 after transplantation, P2X1R expression was increased in the syngeneic graft compared with baseline (14-fold increase, n = 3; P = 0.008), similarly to what we observed in allogeneic
Targeting P2X7R in vivo reduces the severity of the alloimmune response. We performed immune profiling of islet-transplanted oATP-treated and untreated mice at day 14 after transplantation. When splenocytes harvested from oATP-treated recipients were challenged with donor-derived splenocytes, a reduction was observed at day 14 in the number of cells producing IFN-γ (oATP-treated = 117 ± 9 vs. untreated = 418 ± 26, n = 5; P < 0.0001; Fig. 2B), with no effect on the number of cells producing IL-4 (Fig. 2C). No significant changes in the percentages of peripheral CD4+ T effector cells were observed between groups (CD44highCD62LlowCD4+ T cells; Fig. 2D). Slight reductions were evident in the percentages of CD8+ T effector cells (Fig. 2E) and of Th17 cells (Fig. 2F). No differences were observed in the percentages of regulatory T-cells (CD25+FoxP3+CD4+ T-cells, or Tregs; Fig. 2G). The cytokine profile of sera showed that IL-6, a Th17 cytokine, was reduced in oATP-treated recipients (Fig. 2K), whereas only minor changes were observed for all other cytokines examined (Fig. 2H–J, L and M).

Targeting P2X7R in vivo reduces infiltration and Th1 transcripts in islet allografts. We next analyzed the pathology of islet allografts 14 days after transplantation. Islet structure was substantially compromised, and insulin or glucagon staining were undetectable in untreated mice (Fig. 3A, E and F, respectively), with diffuse infiltration primarily constituted by CD3+ T cells and MAC2+ macrophages, with very few FoxP3+ cells (Fig. 3A–D). Conversely, islet structure and insulin and glucagon staining were maintained in oATP-treated mice (Fig. 3G, K and L, respectively), with infiltrate mainly confined to the islet borders with several FoxP3+ cells (Fig. 3G–J). Semiquantitative analysis confirmed preserved insulin staining at day 14 in oATP-treated mice but not in the untreated mice (Fig. 3M), with no major differences in the overall infiltrate (Fig. 3N). A reduction in the mRNA expression of
targeting of P2X7R on islet graft survival. P2X7R targeting robustly inhibited IFN-γ production (cells producing IFN-γ; oATP 100 μmol/L = 30 ± 2 vs. control = 190 ± 4, n = 5; \( P < 0.0001 \); Fig. 4A), but a dose-dependent increase in IL-4 production was observed (Fig. 4B). To confirm in vitro suppression of the alloantigen response, we performed a mixed leukocyte reaction experiment in which alloantigen-mediated cell proliferation appeared to be suppressed (number of proliferating cells as measured by \(^{3}H\)-thymidine incorporation: oATP 100 μmol/L = 136 ± 5 vs. control = 6,199 ± 498, n = 5; \( P < 0.0001 \); Fig. 4C). C57BL6 splenocytes were also challenged with allogeneic cells in the presence of oATP (100 μmol/L) for 24 h, and apoptosis was assessed by fluorescence-activated cell sorter as the percentage of annexin V^+7AAD^− cells; no increase in the percentage of apoptotic cells was detected (Fig. 4D).

We then tested the effect of increasing doses of oATP on Th1/Th17 cytokine levels. A dose-dependent response was evident, in which oATP reduced the number of cells producing Th1 cytokines (IFN-γ and IL-2) and Th17 cytokines (IL-6 and IL-17; Fig. 4E–H). Because it has been reported that oATP may affect other P2XRs, we made use of the availability of the selective P2X1R inhibitor NF 449 and the selective P2X3R inhibitor NF 110. C57BL6 splenocytes were stimulated with irradiated BALB/c splenocytes, and IFN-γ production was evaluated in the presence of these inhibitors. P2X1R and P2X3R inhibition failed to modulate alloantigen-mediated IFN-γ production (Fig. 4I and J); these data confirmed the marginal role of P2X1R and P2X3R in the alloimmune response, at least in vitro.

**Genetic targeting of P2X7R slightly prolongs islet graft survival.** We also determined the effect of genetic targeting of P2X7R on islet graft survival. P2X7R^−/− C57BL6 mice were transplanted with allogeneic BALB/c islets, and partial prolongation of graft survival was observed compared with wild-type recipients (MST: P2X7R^−/− = 19 days, n = 10; \( P < 0.0001 \) vs. wild-type; Fig. 5A). oATP treatment on P2X7R^−/− recipients was able to further...
prolong graft survival (MST: oATP-treated P2X7R−/− = 26 days, n = 5; P = 0.04 vs. untreated P2X7R−/−; Fig. 5A).

To investigate the purinergic system function in P2X7R−/− mice, we analyzed the effect of benzoyl-ATP (Bz-ATP), a synthetic agonist of the purinergic receptors, in CD4+ and CD8+ T cells. When 1 μmol/L Bz-ATP was added to the cultured cells, IFN-γ production increased in CD4+ (Fig. 5B) and CD8+ T cells (Fig. 5D) isolated from wild-type mice. Bz-ATP similarly stimulated IFN-γ production from CD4+ (Fig. 5C) and CD8+ T cells (Fig. 5E) isolated from P2X7R−/− mice. We hypothesized that compensatory mechanisms may have arisen in P2X7R−/− mice, and in particular, we investigated a possible upregulation of P2X1R, P2X3R, and P2X4R, which also have been associated with the immune response (25). CD4+ and CD8+ T cells were extracted from wild-type and P2X7R−/− mice, and the relative expression of P2XR is was assessed by real-time PCR. Higher levels of P2X1R and P2X4R were found...
P2X7R targeting with oATP was tested in vitro. In an ELISPOT assay in which C57BL/6 splenocytes were challenged in vitro with irradiated BALB/c splenocytes (Control), oATP treatment greatly reduced the number of cells producing IFN-γ (n = 5, *P < 0.05, ***P < 0.0001 vs. Control) (A), as well as increased the number of cells producing IL-4 (n = 5, *P < 0.05 vs. Control) (B). C: oATP also inhibited the proliferation of C57BL/6 splenocytes measured as incorporation of 3H-thymidine (n = 5, ***P < 0.0001 vs. Control). D: No significant differences in the percentages of apoptotic cells (Annexin V−7-ADD− cells) were evident in controls compared with oATP−cultured splenocytes (representative of three different experiments). Luminex analysis of the supernatant showed reduced IFN-γ (E), IL-2 (F), IL-6 (G), and IL-17 (H) production in C57BL/6 splenocytes challenged with irradiated BALB/c splenocytes (n = 5, *P < 0.05 vs. Control). Conversely, targeting of P2X1R using the specific inhibitor NF 449 (I) or targeting of P2X3R using the specific inhibitor NF 110 (J) was unable to modulate IFN-γ production by C57BL/6 splenocytes challenged in an ELISPOT assay with irradiated BALB/c splenocytes. (A high-quality color representation of this figure is available in the online issue.)
Control). CD4+ T cells and CD8+ T cells were extracted from wild-type and P2X7R untreated P2X7R2 mice and were assessed for P2XRs expression. Higher levels of P2X1R and P2X4R were observed in P2X7R−/− mice (Fig. 6J and F) compared with wild-type recipients (Fig. 6I and F). Among CD4+ T cells, an expansion of P2X7R+CD45RO+ cells (Fig. 7A) and a slight decrease in the number of Tregs were observed in oATP-treated mice (Fig. 6L). A better protection in lym- phocytes obtained from islet-transplanted patients to de- termine the relevance of the P2X7R/ATP system in human T cells and, potentially, in islet rejection in humans. PBMCs from islet-transplanted patients, T1D patients, and healthy control subjects were harvested, and CD4+ T cells, a key population in islet rejection, were analyzed by flow cytometry (Table 1). In healthy controls, the P2X7R/CD4+ T-cell population represented 17% of all PBMCs (Figs. 7A and F), and P2X7R was expressed in almost 50% of peripheral CD4+ T cells (data not shown). The percentage of P2X7R/CD4+ T cells observed in recently well-functioning transplanted patients (<3 years from last islet infusion) were comparable to those of healthy control subjects (Fig. 7A, C, and F). Conversely, the frequency of the P2X7R/CD4+ T-cell population significantly increased in long-term islet-transplanted patients (>3 years; Fig. 7A, D, and F) and in T1D patients (Figs. 7A, B, and F). Among CD4+ T cells, an expansion of P2X7R+/CD45RO+ cells (T cells with a mature phenotype or memory T cells) was observed in long-term islet-transplanted patients (Fig. 7G, J, and L) and in T1D patients (Fig. 7G, H, and L) compared with healthy control subjects. Conversely, a reduction in naive P2X7R−/CD45RA− cells (T cells with a mature phenotype or memory T cells) was observed in long-term islet-transplanted patients (Fig. 7G, J, and L) and in T1D patients (Fig. 7G, H, and L) compared with healthy control subjects. A different pattern was expressed on recently islet-transplanted patients and healthy control subjects.

FIG. 5. A: A significant prolongation of islet allograft survival was shown in P2X7R−/− C57BL/6 recipients of allogeneic BALB/c islets compared with wild-type recipients (n = 10, ***P < 0.0001 vs. wild-type untreated), and oATP treatment prolonged islet graft survival in P2X7R−/− recipients (n = 5, ³P < 0.05 vs. P2X7R−/− untreated; ***P < 0.0001 vs. wild-type untreated). CD4+ and CD8+ T cells purified from splenocytes of wild-type (B and D, respectively) or P2X7R−/− (C and E, respectively) C57BL/6 mice were challenged with anti-CD3 Ig and anti-CD28 Ig in the presence or absence of different concentrations of Bz-ATP and were tested for IFN-γ production in an ELISPOT assay. A preserved and efficient Bz-ATP response, despite the absence of P2X7R, was evident in P2X7R−/− mice (C and E) and in wild-type mice (B and D) (n = 5, *P < 0.05, **P < 0.01 vs. Control). CD4+ T cells and CD8+ T cells were extracted from wild-type and P2X7R−/− mice and were assessed for P2XRs expression. Higher levels of P2X1R and P2X4R were observed in P2X7R−/− CD4+ (F) and CD8+ (G) T cells (n = 3, *P < 0.05, **P < 0.01 vs. wild-type) compared with wild-type.
The specific human P2X7R antagonist CE-224535 inhibits the alloimmune response in vitro. To confirm the immunomodulatory effect of P2X7R inhibition, we challenged PBMCs obtained from healthy control subjects with allogeneic irradiated PBMCs obtained from different donors in an ELISPOT assay in the presence of different concentrations of the human-specific P2X7R antagonist CE-224535, which has been investigated in clinical trials for rheumatoid arthritis. A reduction in the number cells producing IFN-γ was observed with CE-224535 treatment at 100 μmol/L (29 ± 1 vs. control 51 ± 3, n = 5, P = 0.003; Fig. 7S).

DISCUSSION
Successful islet transplantation can improve metabolic control and can reestablish normoglycemia in subjects with T1D (3). Unfortunately, transplanted islets are subjected to the damaging effects of the alloimmune response and the recurrence of autoimmunity (20% of the patients insulin-free at 5 years) (3,7,8,40). The better outcomes of autologous islet transplantation from patients with chronic pancreatitis (47% insulin-free at 5 years) (41), in whom the allo- and autoimmune responses are absent, confirms the primary role of immune response in islet graft failure,
although multiple factors are ultimately responsible for islet graft loss (3). Considering the potential advantages of islet transplantation in diabetes complications and the relatively low invasiveness of the procedure, additional research is required to develop novel strategies to lessen the need for lifelong immunosuppression (42).

We studied the role of the ionotropic purinergic P2X receptors in islet allograft rejection, particularly in light of recent studies that have demonstrated roles for the ATP/P2XR system in T-cell activation and in Th1/Th17-cell generation (24–26). P2X7R may represent a crossroads for naïve T cells to become activated during the alloimmune response. Our data show that the expression of P2X1R and P2X7R is higher in the islet allograft during rejection, whereas in the syngeneic setting, only P2X1R has higher expression. These data seem to link P2X7R expression specifically to the alloimmune response, whereas P2X1R expression appears to be more related to ischemia/reperfusion and peritransplant inflammation.

In our model of islet allotransplantation, P2X7R targeting with oATP was able to promote long-term graft function in 30% of recipients, and this effect was associated with an extensive reshaping of the immune system, specifically in a reduction of the anti-islet Th1 response, a reduction in CD4+ T effector and Th17 cells, which are well known to promote the alloimmune response (29), and a preservation of Tregs, which, on the contrary, promote graft acceptance (43). Of particular interest in the clinical setting is the synergistic effect observed between rapamycin, which is a pivotal drug in clinical islet transplantation (44), and oATP. This combination treatment resulted in a preponderance of islet-transplanted mice accepting a fully major histocompatibility complex–mismatched allograft long-term, with hyporesponsiveness toward alloantigen and preservation of islet graft morphology and insulin staining.

Although oATP is described as one of the most effective P2X7R inhibitors in vivo (45,46), the targeting of P2X7R using the specific human inhibitor CE-224535 in an ELISPOT assay in which human PBMCs were challenged with allogeneic human PBMC reduced the number of cells producing IFN-γ (n = 5, **P < 0.01 vs. control).

**FIG. 7.** PBMCs from healthy control subjects, T1D patients, and islet-transplanted patients were harvested and analyzed by flow cytometry for P2X7 expression. A higher percentage of P2X7+CD4+ T cells was evident in T1D patients and in long-term islet-transplanted patients (>3 years, n = 8) compared with recently islet-transplanted (<3 years, n = 8) or healthy control subjects (n = 8; **P < 0.01, ***P < 0.0001 vs. healthy controls and recently islet-transplanted patients) (A, B, C, D, and F). Within the CD4+ T-cell population, an increase in the percentage of memory P2X7R+CD45RO+ cells was observed in T1D patients and in long-term islet-transplanted patients (**P < 0.01, ***P < 0.0001 vs. healthy controls and recently islet-transplanted; §§P < 0.01 vs. healthy controls) (G, H, I, J, and L). Conversely, a reduction in the percentage of naïve P2X7R+CD45RA+ cells was observed in T1D patients and in long-term islet-transplanted patients (***P < 0.0001 vs. healthy controls and recently islet-transplanted; ‡P < 0.05 vs. healthy volunteers) (M, N, O, P, R). S: The targeting of P2X7R using the specific human inhibitor CE-224535 in an ELISPOT assay in which human PBMCs were challenged with allogeneic human PBMC reduced the number of cells producing IFN-γ (n = 5, **P < 0.01 vs. control).
the CD4+ and CD8+ T cells obtained from P2X7R−/− mice. However, some caution should be used during analysis of data using P2X7R−/− recipients; in these mice, the immunological function of the other P2XRs may in fact become more relevant, as suggested by the preserved response to Bz-ATP in P2X7R−/−-derived lymphocytes, accompanied by the upregulation of other P2XRs. Further development of specific antagonists for the different P2XRs suitable for in vivo use (22,46) will help us to better dissect the relative contribution of the targeting various P2XRs in islet graft rejection.

In vitro, the targeting of P2X7R with oATP during allostimulation inhibited the production of Th1/Th17 cytokines and T-cell activation, confirming that P2X7R signaling is required to trigger the Th1/Th17 immune response (21,25); on the contrary, the expansion of Tregs is not evident in our model, although this has been reported in other settings (30).

By studying PBMCs obtained from different populations of human subjects, we were able to evaluate the expression of P2X7R in different lymphocyte subsets. The P2X7R+CD4+ T-cell population appeared to be upregulated in T1D patients and islet-transplanted patients in the long-term, suggesting a link between activation of the immune system, the anti-islet response, and expression of P2X7R. Interestingly, an increase in memory and decrease of naive CD4+ T-cells expressing P2X7R was observed in long-term islet-transplanted recipients. Considering that progressive loss of islet graft function may be associated with a mounting of memory allo- and autoimmune cell responses (40,47), identifying potential new targets for the memory-cell subpopulation is highly desirable. In this sense, the targeting of P2X7R may represent a viable option, particularly in light of the availability of clinical-grade therapeutics, such as the human-specific inhibitor CE-224535.

In conclusion, P2X7R-mediated alloimmunity is an important pathway in the alloimmune response that contributes to priming of Th1/Th17 effector cells. Targeting P2X7R may thus be beneficial in preventing islet allograft rejection and inducing tolerance, thereby reducing the need for immunosuppressive agents. Indeed, complete tolerance induction may require a specific P2X7R-targeting approach to abrogate Th1/Th17 alloimmunity and to facilitate a Treg sparing/expansion strategy. In view of the novel anti-P2X7R inhibitors available for clinical use (e.g., CE-224535, AZD9056, and GSK1482160), our data may have significant clinical relevance for the field of islet transplantation.

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A.V. conducted this study as partial fulfillment of his PhD in Molecular Medicine, San Raffaele University, Milan, Italy.

A.V. designed and performed research, analyzed data, and wrote the paper. C.F. performed research, analyzed data, and wrote the paper. F.D., S.T., M.P., F.Ga., M.C., R.B., R.D.M., D.C., R.G., M.E.F., A.S., and P.M. performed research. F.Gr. edited the paper. C.R. designed research and edited the paper. M.H.S. designed research. A.P. and P.F. designed research and wrote and edited the paper. P.F. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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