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A novel *in vivo* regulatory role of P-glycoprotein in alloimmunity

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Abstract

P-glycoprotein (P-gp) is required for adaptive immunity through defined functions in T cell activation and antigen presenting cell (APC) maturation. The potential role of P-gp as an *in vivo* regulator of alloimmunity is currently unknown. Here we show that P-gp blockade prolongs graft survival in a murine heterotopic cardiac allotransplantation model through *in vivo* inhibition of the T helper 1 (Th1) cytokine IFN- γ and the Th2 product IL-4, and via downregulation of the APC-expressed positive costimulatory molecule CD80. *In vitro*, the P-gp antagonist PSC833, a non-calcineurin-inhibitory cyclosporine A analogue, specifically inhibited cellular efflux of the P-gp substrate rhodamine-123 in wild-type CD3⁺ T cells and MHC class II⁺ APCs but not their P-gp knockout counterparts that lacked rhodamine-123 efflux capacity. Additionally, P-gp blockade significantly inhibited murine alloimmune T cell activation in a dose-dependent fashion. *In vivo*, P-gp blockade significantly prolonged graft survival in Balb/c recipients of C57BL/6 cardiac allografts from 8.5 $\pm$ 0.5 to 11.7 $\pm$ 0.5 days ($P<0.01$), similar in magnitude to the effects of monotherapy with cyclosporine A. Moreover, P-gp blockade, compared to controls, attenuated intragraft expression of CD3 and CD80, but not CD86, and inhibited IFN- γ and IL-4 production ($P<0.05$). In the setting of systemic CD86 inhibition, P-gp blockade suppressed IFN- γ and IL-4 production significantly further (to 98% and 89% inhibition, respectively) compared to either P-gp or anti-CD86 blockade alone, and markedly prolonged allograft survival compared to anti-CD86 blockade alone (40.5 $\pm$ 4.6 vs. 22.5 $\pm$ 2.6 days, respectively, $P<0.01$). Our findings define a novel *in vivo* regulatory role of P-gp in alloimmunity and identify P-gp as a potential therapeutic target in allotransplantation.

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Keywords

P-glycoprotein; immunity; transplantation; T cell; antigen presenting cell; CD86

Introduction

The human multidrug resistance 1 (MDR1) gene product P-gp (ABCB1), and its murine gene homologue *mdr1a/1b* P-gp (*Abcb1a/1b*) [1], are members of the ATP-binding cassette (ABC) superfamily of active transporters that also includes the two human P-gp paralogues, ABCB4 (MDR3) and ABCB5. Overexpression of P-gp has been associated with cancer MDR. Moreover, MDR1 P-gp (ABCB1) is also expressed in normal tissues including peripheral immune cells [2;3;4], where it serves distinct roles in T cell [4;5;6;7], natural killer cell [8;9] and APC functions [4;10;11;12]. In human T cells, P-gp function has been implicated in cytokine secretion, T cell survival and differentiation, and cytotoxic T cell effector function [13]. Our laboratory previously assessed for the first time the effects of pharmacological human P-gp blockade on cytokine production in the setting of alloimmune stimulation *in vitro* [4]. Specifically, P-gp blockade inhibited alloantigen-dependent IL-2, IFN- γ and TNF- α production along with T cell proliferation in the human mixed lymphocyte reaction (MLR). Addition of exogenous IL-2 restored MLR-induced T cell proliferation, demonstrating that P-gp was critical to alloimmune T cell activation, occurring prior to IL-2 secretion. P-gp can also function in T cell activation via APC-dependent mechanisms [4]. Selective blockade of APC-expressed P-gp prior to MLR co-culture results in significant inhibition of IL-12 secretion and inhibition of allogeneic CD4⁺ T cell proliferation. Additionally, we showed that P-gp functions in human dendritic cell (DC) maturation as a result of its role in IL-12 secretion: P-gp blockade inhibits APC CD1a and costimulatory CD80 expression, but not expression of CD86, during *in vitro* IL-4/GM-CSF-induced CD14⁺ monocyte differentiation, inducing the generation of APCs that are incapable of stimulating IFN- γ production by co-cultured allogeneic T cells [11]. Most recently, this role of P-gp as a key regulator of DC function was confirmed *in vivo*, in a murine autoimmune disease model of multiple sclerosis [12]. In this study, DC function and disease severity were found to be severely impaired in P-gp knockout mice (*mdr1a/1b*^{-/-}), with significantly attenuated DC maturation and T cell stimulatory capacities, and inhibited secretion of the pro-inflammatory cytokine IFN- γ .

Thus, P-gp represents an immunomodulatory molecule and potential target for immunotherapy [4;11;12;13]. However, the potential role of P-gp as a regulator of *in vivo* alloimmunity, strongly suggested by previous *in vitro* findings, has not been investigated to date. Here, we used a vascularized murine heterotopic cardiac allotransplantation model and the non-calcineurin inhibitory cyclosporine A analogue and designer P-gp antagonist PSC833 [11] to examine the effects of P-gp blockade on alloimmune responses and allograft survival *in vivo*.

Materials and Methods

Animals

C57BL/6 (H-2^b), Balb/c (H-2^d), *mdr1a/1b* targeted mutation (*mdr1a/1b*^{-/-}) [14], and wild-type FVB (H-2^q) mice were obtained from Taconic (Germantown, NY). Mice were maintained in accordance with the institutional guidelines of Children's Hospital Boston and experiments were performed according to approved experimental protocols.

Reagents and Antibodies

PSC833 was provided by Novartis (East Hanover, NJ). PSC833 stock (50 mg/ml in 2:1 cremophor EL:ethanol (v/v)) was diluted to 1 mg/ml in physiological saline solution. Anti-CD80 and anti-CD86 mAbs were obtained from BioExpress (West Lebanon, NH), rat IgG control from Sigma-Aldrich (St. Louis, MO), PE-conjugated anti-mouse CD3, CD4, CD8, MHC class II, and isotype control Abs, and unconjugated anti-mouse CD86 and isotype control Abs from BD Pharmingen (San Diego, CA). Unconjugated anti-mouse CD3 and isotype control Abs were purchased from Serotec (Raleigh, NC), unconjugated anti-mouse CD80 Ab from Chemicon (Temecula, CA), and HRP- and AP-conjugated secondary Abs from Dako (Carpinteria, CA). ³H-thymidine was purchased from Perkin Elmer, Boston, MA.

RT-PCR

The murine *mdr1a* gene-specific oligonucleotide primer pair 5'-CCTCGTCAGACAGCCTCACATTT-3' (forward) and 5'-ATCAAGTCCGCGTTCTGGATGG-3' (reverse), and the *mdr1b* gene-specific primers 5'-TGTCCACAGAAAGCAAGACCAAG-3' (forward) and 5'-CACTGGTTGTATGCACCCATTTAT-3' (reverse) were used for PCR amplification of reverse transcribed total mRNA derived from Balb/c murine splenocytes. Standard cDNA synthesis reactions and PCR analysis were performed as described [15; 16]. β -actin primers were used as controls to ensure RNA integrity.

Rhodamine-123 Efflux Studies

Rhodamine-123 efflux studies were performed as described [16]. Isolated wild-type or *mdr1a/1b*^{-/-} knockout murine splenocytes were stained with PE-conjugated CD3, CD4, CD8, MHC class II, or isotype control mAb (all 20 μ g/ml) and subsequently loaded with the P-gp transport substrate rhodamine-123 (1 μ g/ml) (Molecular Probes, Eugene, OR) for 30 min at 37° C and 5% CO₂. Subsequently, cells were washed and then incubated in the presence or absence of the P-gp antagonist PSC833 (5 μ m) for 15 minutes at 4° C. Dual color flow cytometry [15] was subsequently performed for acquisition of cellular rhodamine-123 content, immediately (T=0min) and upon further incubation at 37° C (T=120 min) at the F11 emission spectrum with specific F12 gating on CD3⁺, CD4⁺, CD8⁺ or MHC class II⁺ cell subpopulations. As a control, samples were incubated for 120 min on ice. Under those conditions, ATP hydrolysis and thus ATP-dependent P-gp-mediated rhodamine-123 efflux transport are inhibited.

Mixed Lymphocyte Reaction (MLR)

Splenocytes isolated from naïve C57BL/6 mice were used as stimulators (irradiated at 1,750 rad) or responders in 5-day cocultures with allogeneic wildtype or *mdr1a/1b*^{-/-} FVB mouse splenocytes (used as responders or irradiated stimulators, respectively) at 1:1 stimulator to responder ratios (2.5 $\times$ 10⁵ cells/well) in standard one-way MLRs as described [4;11]. In additional experiments, splenocytes isolated from naïve Balb/c mice were seeded at 2.5 $\times$ 10⁵/well in round-bottom 96-well cell culture plates for use in a standard one-way MLR. Cells were pre-incubated with PSC833 (1-5 μ m), anti-CD86 mAb, anti-CD80 mAb, isotype control Ig (all 20 μ g/ml) or combinations thereof before addition of irradiated (1,750 rad) allogeneic stimulator splenocytes (2.5 $\times$ 10⁵/well) isolated from naïve C57BL/6 mice. Cells were cocultured in the presence of inhibitors and cell proliferation was assessed after a culture period of 5 days by ³H-thymidine incorporation as described [4;11].

Quantification of Cell Death by Annexin V-PE/7-Amino-actinomycin D Staining

To assess the effects of P-gp blockade by PSC833 on cell viability in the MLR, freshly isolated splenocytes derived from naïve Balb/c mice were cocultured with irradiated, allogeneic stimulator splenocytes isolated from naïve C57BL/6 mice at cellular densities as above, in the presence or absence of PSC833 (1, 2, 5 μ m). As a positive control, the effects of toxic concentrations of PSC833 (50 μ m) were examined. On days 1 and 5 of coculture, cell death was quantified by annexin V-PE/7-amino-actinomycin D (7-AAD) (BD Pharmingen) staining and subsequent flow cytometric analysis, as described [17].

Heart Transplantation and Treatment Protocols

Abdominal heterotopic cardiac transplantation was performed by microsurgery [18]. To assess the role of P-gp blockade on acute cardiac allograft rejection, donor hearts were harvested from wild-type C57BL/6 mice and transplanted into wild-type Balb/c recipient mice. Allograft recipients received i.p. injections either with PSC833 (50mg/kg on day -1, 10mg/kg from day 0 to 14), cyclosporine (10mg/kg from day 0 to 14) or with vehicle control. In some experiments, PSC833 treatment was combined with either anti-CD86 mAb or control Ig (250 μ g, i.p. on days 0, 2, 4, and 6, respectively). The survival of cardiac allografts was assessed daily by palpation, with rejection defined as the complete cessation of detectable beating.

Histology and Immunohistochemistry

Cardiac allografts were harvested 10 days following transplantation. For evaluation of lymphocytic infiltration and preservation of myocardium, paraffin-embedded axial ventricular tissue sections were stained with haematoxylin and eosin (H& E) and images obtained as described [19]. In order to characterize intragraft inflammatory infiltrates, HRP immunohistochemical analysis for CD3, CD80, and CD86 marker expression was performed as described [15], in frozen axial ventricular tissue sections. Numbers of graft-associated cells staining for each surface marker were counted in three separate microscopy fields for each experimental condition and mean percentages from replicate animals were calculated and statistically analyzed using the unpaired student *t* test.

Enzyme-Linked Immunosorbent Spot Assay (ELISPOT)

For determination of cytokine production by cocultures of Balb/c responder splenocytes (1×10^5 /well) isolated 10 days post transplantation from spleens of cardiac allograft recipients with freshly isolated, irradiated (3000 rad) naïve C57BL/6 donor-strain stimulator splenocytes (2.5×10^5 /well), ELISPOT analyses were performed as described [11;17], using ELISPOT sets for murine IFN- γ and IL-4 (BD Pharmingen). IFN- γ and IL-4 production was assessed at 24 and 48 hours, respectively, by counting resulting spots on an ELISPOT analyzer, as described [11;17].

Statistical Methods

Statistical differences between Kaplan-Meier graphs of allograft survival were assessed using the log-rank test. Results of cell proliferation, cell death, and ELISPOT assays were compared statistically using the unpaired student *t* or Mann-Whitney tests. A two-sided value of $P < 0.05$ was considered statistically significant.

Results

Functional Expression of P-gp in Murine Immune Cells

Using gene-specific oligonucleotide primer pairs for murine mdr1a and mdr1b, the mouse homologues of human MDR1, and PCR amplification of reverse transcribed total mRNA,

we detected *mdr1a* cDNA as a 162 bp band, but not 169 bp *mdr1b* cDNA in murine splenocytes (Fig.1A), demonstrating that murine immune cells preferentially express *mdr1a* P-gp. Flow cytometric analysis of P-gp-mediated fluorescent rhodamine-123 efflux by wild-type FVB strain murine splenocyte subpopulations revealed expression of functional P-gp in 39% of CD3⁺, 12% of CD4⁺ and 49% of CD8⁺ lymphocytes and in 11% of MHC class II⁺ murine APCs (Fig.1B), as evidenced by the de novo accumulation of the respective cell percentages in the rhodamine-123^{low}-measuring R1 gate upon a 120 min rhodamine-123 efflux period. Both P-gp blockade by the specific antagonist PSC833 (5 μ m) and by temperature-dependent inhibition of ATP hydrolysis (incubation of cell samples on ice) blocked rhodamine-123 efflux by >90% ($P<0.05$) in CD3⁺, CD4⁺, CD8⁺ and MHC class II⁺ splenocyte subpopulations (Fig.1B). In contrast, neither CD3⁺ lymphocytes nor MHC class II⁺ APCs derived from spleens of FVB *mdr1a/1b* KO mice exhibited significant rhodamine-123 efflux capacities, and no significant effects on cellular rhodamine-123 content were observed in these cell populations as a result of either specific P-gp blockade or temperature-dependent inhibition of ATP hydrolysis (Fig.1C), demonstrating specificity of the rhodamine efflux assay to measure *mdr1a/1b* expression, in the absence of available murine *mdr1a/1b*-specific monoclonal antibodies. These results establish the functional and predominant expression of *mdr1a* P-gp in murine immune cells and confirmed functional and specific inhibition of murine P-gp by the P-gp antagonist PSC833.

P-gp Blockade Inhibits Murine Alloimmune T Cell Activation

To determine the effect of *mdr1a/1b* gene knockout on alloimmune responses, we cocultured splenocytes isolated from wildtype or *mdr1a/1b*^{-/-} FVB mice (responders) with irradiated naïve allogeneic C57BL/6 splenocytes (stimulators) in a standard one-way MLR [4;11]. We found that *mdr1a/1b*^{-/-} lymphocytes proliferated significantly less (>90% inhibition, $P<0.0001$) compared to wildtype FVB splenocytes in response to alloimmune stimulation (Fig.2A). These results are consistent with a role of T cell-expressed P-gp in alloimmune proliferation [4] and/or APC-expressed human P-gp in *indirect* allorecognition [4;11]. In contrast, alloimmune proliferation of wildtype allogeneic C57BL/6 responders was not significantly different upon coculture with wildtype or *mdr1a/1b*^{-/-} FVB stimulators (Fig. 2A), excluding a significant role for APC-expressed P-gp in *direct* allorecognition. We next investigated the effects of pharmacologic P-gp blockade on murine alloimmune T cell proliferation *in vitro*. P-gp blockade by PSC833 (1-5 μ m) inhibited a murine MLR using C57BL/6 and Balb/c splenocytes as stimulators and responders, respectively, in a dose-dependent fashion by up to >95% ($P<0.05$) (Fig.2B). Combining non-inhibitory PSC833 concentrations (1 μ m) with CD86-directed, but not CD80-directed costimulatory blockade significantly inhibited the MLR by 58% ($P<0.05$), whereas anti-CD86 or anti-CD80 mAbs alone exerted no significant inhibition compared to isotype control Ab treatment (Fig.2B). Quantification of cell death by annexin V-PE/7-AAD staining and flow cytometry revealed that P-gp blockade by concentrations of PSC833 up to 5 μ m did not significantly affect cell viability in the MLR (Fig.2C), in contrast to *toxic* concentrations of PSC833 (50 μ m), not otherwise used in this study, which induced significant cell death above control after both 1-day and 5-day incubation periods (20.3 $\pm$ 0.1 % and 42.9 $\pm$ 2.2%, respectively, $P<0.05$) (Fig. 2C). Thus, murine P-gp blockade inhibits alloimmune T cell proliferation *in vitro*.

P-gp Blockade Prolongs Murine Cardiac Allograft Survival

We next studied the effects of P-gp blockade *in vivo* on murine cardiac allograft survival, using a murine C57BL/6 to Balb/c vascularized heterotopic cardiac allotransplantation model. P-gp blockade in Balb/c recipients of C57BL/6 allografts significantly prolonged allograft survival compared to that observed in drug vehicle only-treated controls (mean survival time $\pm$ SEM: 11.7 $\pm$ 0.5 days, $n=7$ versus 8.5 $\pm$ 0.5 days, $n=6$; $P<0.01$) (Fig.3A). The survival prolongation via P-gp blockade was not significantly different from the likewise

significant effect of cyclosporine A treatment alone (10.8 ± 0.5 days, $n=4$) (Fig.3A). We next examined the effects of P-gp blockade in combination with CD86-directed costimulatory blockade on allograft survival, based on our previous demonstration that P-gp blockade does not inhibit CD86 induction during APC maturation [11] and our current result of synergistic *in vitro* inhibition of T cell proliferation by this combination regimen. In the setting of systemic mAb-mediated CD86 inhibition, P-gp blockade resulted in marked prolongation of allograft survival (40.5 ± 4.6 days, $n=6$) compared to control treatment (8.8 ± 0.7 days, $n=5$, $P<0.01$) (Fig.3B). This prolongation of allograft survival was also significantly greater compared to the significant prolongation achieved by either P-gp blockade (+control Ig) (12.2 ± 1.1 days, $n=5$, $P<0.01$) or CD86 inhibition alone (22.5 ± 2.6 days, $n=6$, $P<0.01$) (Fig. 3B). Severe acute allograft rejection including myocardial necrosis was observed in control-treated recipients (Fig.4A), whereas allografts dissected from P-gp-blocked recipient mice showed only moderate, scattered infiltration of inflammatory cells within the myocardium, with relatively preserved myocytes (Fig.4A). P-gp blockade also significantly inhibited intragraft CD3 and CD80 expression by 43% and 55%, respectively ($P<0.05$) but did not affect CD86 expression compared to controls (Figs.4A and 4B).

P-gp Regulates Alloimmune IFN- γ and IL-4 Production

In order to further dissect the mechanism by which P-gp blockade alone or in the setting of concurrent CD86 inhibition prolonged murine cardiac allograft survival, we examined the phenotype of donor-specific T cell responses. Splenocytes isolated from treated or control Balb/c recipients of C57BL/6 cardiac allografts were cocultured with naïve C57BL/6 donor strain stimulators, and Th1 IFN- γ and Th2 IL-4 responses were measured by ELISPOT analysis. We found that *in vivo* P-gp blockade alone significantly inhibited alloreactive IFN- γ production by 69% ($P<0.05$) and IL-4 production by 58% ($P<0.05$) compared to controls (Fig.4C). CD86 blockade alone, like P-gp blockade, also suppressed IFN- γ production (94%, $P<0.05$), while exerting no significant effects on IL-4 production (Fig.4C). P-gp blockade in the setting of CD86 inhibition further decreased the significantly inhibited alloreactive IFN- γ production resulting from either P-gp or CD86 blockade alone ($P<0.05$, respectively), to 98% inhibition (Fig.4C). P-gp blockade in the setting of CD86 inhibition also inhibited further the significantly decreased alloreactive IL-4 production resulting from P-gp blockade alone ($P<0.05$), to 89% inhibition (Fig.4C).

Discussion

Our results demonstrate for the first time a function of P-gp in allograft rejection, thereby defining a novel *in vivo* regulatory role of P-gp in alloimmunity. Consistent with earlier reports [20], we found that murine immune cells predominantly express the mdrla but not the mdrlb P-gp isoform. Our results show that Pgp is functionally expressed by subpopulations of murine T cells and APCs at frequencies consistent with respective expression ranges previously reported for human immune cell subsets [2;3;4;5;11] and that Pgp function is effectively blocked by the specific P-gp inhibitor PSC833. Earlier *in vitro* results had demonstrated a role of P-gp in human alloimmune T cell activation [4] and in APC maturation [4;10;11]. Our current study now critically extends these results, demonstrating that P-gp function is also required for alloimmunity *in vivo*. In elucidating the mechanism underlying the *in vivo* immunosuppressive effect of P-gp blockade, we found significantly inhibited intragraft CD80 but not CD86 expression. Moreover, P-gp blockade resulted in attenuated CD3⁺ and CD8⁺ graft inflammatory infiltration and inhibited Th1 IFN- γ and Th2 IL-4 production by graft recipient-derived *ex vivo* allostimulated T cells. These findings parallel our demonstration in a previous human *in vitro* study that P-gp blockade in the course of cytokine-induced APC maturation inhibits CD80 but not CD86 expression, and results in the generation of APCs deficient in inducing IFN- γ responses by

allostimulated T cells [11]. Our current findings in alloimmunity are also consistent with the recent demonstration of P-gp function in autoimmunity, i.e. in APC maturation and pro-inflammatory IFN- γ production in an *in vivo* disease model of multiple sclerosis [12]. In aggregate, our results suggest that the predominant mechanism for the prolonging effects of P-gp blockade on allograft survival was impaired P-gp-dependent CD80 expression by APCs and, as a result, inhibited alloimmune IFN- γ production, because Th1 responses are critically involved in allograft rejection [21]. Importantly, our results further suggest that ultimate graft rejection in P-gp-blocked animals occurred predominantly via CD86-dependent costimulation, since P-gp blockade under conditions of concurrent CD86 blockade resulted in further prolongation of allograft survival. This observed prolongation likely resulted from further inhibition of both Th1 IFN- γ and Th2 IL-4 production, because Th2 responses, in addition to Th1 alloimmunity, are also required for murine cardiac allograft rejection [22]. The dual role of *in vivo* P-gp blockade in inhibiting both alloimmune IFN- γ and IL-4 production is consistent with previously reported *in vitro* effects of P-gp blockade [6].

In conclusion, our results define a novel *in vivo* regulatory role of P-gp in alloimmunity and identify P-gp as a potential therapeutic target for immune modulation in transplantation medicine.

Acknowledgments

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Figure 1. P-gp gene expression and function in murine splenocyte subsets

(A) RT-PCR amplification of murine splenocyte P-gp cDNA. (B) Flow cytometry analysis of P-gp-mediated rhodamine-123 efflux transport (FL1 fluorescence) in untreated, P-gp-inhibited (PSC833) or ATP hydrolysis-inhibited (ice) wild-type FVB strain splenocyte subsets (gated for PE-labeled CD3⁺, CD4⁺, CD8⁺ or MHC class II⁺ subsets at the FL2 emission spectrum) as a time course (T=0 min and T=120 min upon rhodamine-123 loading). The percentages of rhodamine-123-effluxing cells are shown in the R1 gates. (C) Flow cytometry analysis of mdr1a/1b KO FVB strain splenocyte subsets studied and analyzed as in (B).

Figure 2. Effects of *in vitro* P-gp blockade on murine alloimmune T cell proliferation

(A) Top panel: MLR proliferation (^3H -thymidine uptake) of wildtype (wt) versus *mdr1a/1b* knockout (KO) FVB splenocytes stimulated with allogeneic wt C57BL/6 splenocytes. Bottom panel: MLR proliferation (^3H -thymidine uptake) of wt C57BL/6 splenocytes stimulated with allogeneic wt versus *mdr1a/1b* KO FVB splenocytes. Illustrated are mean values of $n=16-32$ wells $\pm$ SEM representative of $n=3-4$ independent experiments, respectively. (B) Top panel: ^3H -thymidine uptake of C57BL/6 donor strain (MLR)-stimulated Balb/c splenocyte cultures compared to untreated controls, plotted against PSC833 concentration (μM). Illustrated are mean values of quadruplicate wells $\pm$ SEM. Bottom Panel: ^3H -thymidine uptake of C57BL/6 donor strain (MLR)-stimulated Balb/c splenocyte cultures treated with PSC833, anti-CD86 mAb, anti-CD80, control Ig, or combinations thereof. Illustrated are means of quadruplicate wells $\pm$ SEM. (C) Assessment of cell death by Annexin V-PE/7-AAD staining of untreated and PSC833-treated C57BL/6 donor strain (MLR)-stimulated Balb/c splenocyte cultures at 1 day (top panel) and 5 days (bottom panel) of incubation. Illustrated are mean values $\pm$ SD of percentage changes in cell death ($n=3$ independent experiments).

Figure 3. Effects of *in vivo* P-gp blockade on murine cardiac allograft survival

(A) Effects of *in vivo* P-gp blockade on C57BL/6 cardiac allograft survival in Balb/c recipients. Kaplan-Meier analyses of the effects of PSC833, vehicle, or cyclosporine A monotherapy on cardiac allograft survival are illustrated. (B) Effects of concurrent P-gp and CD86 blockade on C57BL/6 cardiac allograft survival in Balb/c recipients. Kaplan-Meier analyses of the effects of PSC833+anti-CD86 compared to vehicle+anti-CD86, PSC833+control Ig, or vehicle+control Ig are illustrated.

Figure 4. Effects of *in vivo* P-gp blockade on graft inflammatory infiltration and T helper responses

(A) Depicted are H&E- or HRP-stained (isotype control, CD3, CD80, or CD86) representative axial ventricular tissue sections of cardiac allografts dissected 10 days post transplantation from vehicle- (top row) or PSC833-treated (bottom row) allograft recipients. Size bars: 50 μ m. (B) Quantitative analysis of intragraft CD3, CD80, and CD86 expression from vehicle- versus PSC833-treated allograft recipients. Mean percentages of positive cells $\pm$ SEM are illustrated (*: $P < 0.05$, NS: not significant). (C) ELISPOT analysis of INF- γ (left) and IL-4 (right panel) production by Balb/c recipient splenocytes isolated from vehicle- or PSC833-treated allograft recipients 10 days post transplantation. Naïve, irradiated C57BL/6 donor-strain splenocytes were used as stimulators. Spots per well (mean $\pm$ SEM, $n=6$) are shown for each treatment group.