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Pirfenidone Inhibits T Cell Activation, Proliferation, Cytokine and Chemokine Production, and Host Alloresponses

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Abstract

Background—We previously showed that pirfenidone, an anti-fibrotic agent, reduces lung allograft injury/rejection. In this study, we tested the hypothesis that pirfenidone has immune modulating activities and evaluated its effects on the function of T cell subsets, which play important roles in allograft rejection.

Method—We first evaluated whether pirfenidone alters T cell proliferation and cytokine release in response to T cell receptor (TCR) activation, and whether pirfenidone alters regulatory T cells (CD4+CD25+) suppressive effects using an *in vitro* assay. Additionally, pirfenidone effects on alloantigen-induced T cell proliferation *in vivo* were assessed by adoptive transfer of CFSE-labeled T cells across a parent->F1 MHC mismatch, as well as using a murine heterotopic cardiac allograft model (BALB/c->C57BL/6).

Results—Pirfenidone was found to inhibit the responder frequency of TCR-stimulated CD4+ cell total proliferation *in vitro* and *in vivo*, whereas both CD4 and CD8 proliferation index were reduced by pirfenidone. Additionally, pirfenidone inhibited TCR-induced production of multiple pro-inflammatory cytokines and chemokines. Interestingly, there was no change on TGF- β production by purified T cells, and pirfenidone had no effect on the suppressive properties of naturally occurring regulatory T cells. Pirfenidone alone showed a small but significant ($p < 0.05$) effect on the *in vivo*

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allogeneic response while the combination of pirfenidone and low dose rapamycin had more remarkable effect in reducing the alloantigen response with prolonged graft survival.

Conclusion—Pirfenidone may be an important new agent in transplantation, with particular relevance to combating chronic rejection by inhibiting both fibroproliferative and alloimmune responses.

Keywords

rodent; T cells; transplantation

Introduction

The small molecule, pirfenidone (5-methyl-1-phenyl-2-(1H)-pyridone), has protective effects in experimental models of fibrosis and/or inflammation (1-4). Pirfenidone (185 Da) is formulated as a water-soluble crystalline powder that readily penetrates into cells and is well tolerated with relatively minor adverse effects (5,6). The underlying mechanism of the beneficial effect of pirfenidone in fibrotic disease is not fully understood, though pirfenidone inhibits expression of fibrogenic cytokines, such as transforming growth factor (TGF)- β , interleukin (IL)-4, IL-13 (7-9), and inhibits expression of tumor necrosis factor (TNF)- α (10, 11). Pirfenidone also inhibits expression of intracellular adhesion molecule (ICAM)-1 (12) and enhances expression of the anti-inflammatory cytokine, IL-10 (13,14).

Our laboratory and others have shown that pirfenidone has graft-protective effects (7,15-18). As with its anti-fibrotic and anti-inflammatory effects, the beneficial actions of pirfenidone in improving graft survival may result from the inhibition of profibrotic/inflammatory cytokines (19). Although inhibiting profibrotic cytokines such as TGF- β may have benefits towards the fibroproliferative aspect of chronic rejection (20,21), the inhibitory effects of pirfenidone on TGF- β expression could potentially be detrimental to early graft survival with increased acute rejection (22,23) and potentially long term graft survival due to reducing the development of inducible regulatory T cells (24). We previously found that pirfenidone prolonged survival and inhibited fibrosis in mouse tracheal and rat lung allograft models (7,15,17). The protection afforded by pirfenidone required treatment prior to the onset of fibrosis to be effective in attenuating the obstructive airway disease and associated fibrosis. These data suggest pirfenidone has important effects beyond control of fibrosis, including the possibility of having direct effects on lymphocyte activation and/or function. In this study, we examined whether pirfenidone has immune modulating properties and if it can directly inhibit T cell activation/function. Since CD4+ and CD8+ T cell subsets play a role in transplant rejection (25-27), we evaluated the effects of pirfenidone on these subsets of lymphocytes and also its effects on regulatory T cell (Treg) function, and its potential to promote long-term graft survival using a mouse cardiac allograft model.

Materials And Methods

Reagents

We purchased carboxyfluorescein diacetate succinimidyl ester (CFSE) (Molecular Probes, Carlsbad CA), pirfenidone (CAS 53179-13-8, Department of Chemistry, University of Florida, Gainesville FL), rapamycin (LC Laboratories, Woburn MA), TGF- β ELISA assay kit (MB100B R&D systems Inc, Minneapolis MN), and Luminex Multiplex Panel (Invitrogen Inc, Carlsbad CA).

Mice

Six to eight weeks old BALB/c (H-2^d), C57BL/6 (H-2^b) (B6), and C57BL/6/DBA F1 (H-2^{b/d}) mice (The Jackson Laboratory, Bar Harbor ME) were housed in specific pathogen-free conditions. All protocols were approved by the Institutional Animal Care and Use Committees of the Children's Hospital of Philadelphia and Children's Hospital Boston.

T cells phenotypic analysis

Spleen and lymph nodes (para-aortic, mesenteric, and ilioinguinal) were isolated and single cell suspensions prepared in phosphate buffered saline with 0.1% bovine serum albumin (Sigma-Aldrich, St Louis Mo). Cells were first incubated with anti-CD16/32 (FcγIII/II receptor antibody) to block non-specific binding, and then stained with anti-CD4, anti-TCR and anti-CD8 (BD Biosciences, San Diego CA) fluorochrome-conjugated monoclonal antibodies (mAb). For the identification and phenotypic analysis of Treg cells (TCR+CD4+FOXP3+), we first performed extracellular staining of the cells with anti-TCRβ and anti-CD4 (Invitrogen Inc, Carlsbad CA) mAb. This was followed by intracellular staining with anti-mouse/rat FOXP3 (FJK-16a) antibody using the mouse/rat FOXP3 Staining kit (eBioscience Inc, San Diego CA) per the manufacturer's instructions. Viable cells were selected for flow cytometric analysis (LSR II BD, San Jose CA) based on forward and side scatter light properties. The analysis was performed with FlowJo software (Tree Star software Inc, Portland OR).

T cell proliferation

Lymphocytes were isolated from spleen and lymph node samples by magnetic cell separation. Briefly, single cell suspensions were prepared and red blood cells removed by hypotonic lysis. Magnetic beads conjugated mAbs (Miltenyi Biotec Inc, Auburn CA) were used to separate CD3, CD8, and CD4 subsets. Lymphocytes (1×10^5) labeled with the tracking fluorochrome CFSE and stimulated with mouse anti-CD3 mAb (1 μg/ml) and anti-CD28 (1 μg/ml), and proliferation assessed 72 h later by flow cytometry of CFSE dilution.

In vivo mixed lymphocyte response (MLR)

Alloreactive T cell proliferation was assessed using an in vivo allogeneic MLR, involving parent to F1 hybrid activation of CFSE-labeled T cells, as previously described (28). Donor cells were isolated from the lymph nodes and spleens of wild-type C57BL/6 mice (H-2^b). Pooled cells were labeled with CFSE and 4×10^7 cells were injected intravenously into C57BL/6/DBA F1 (H-2^{b/d}) recipients. Recipients were divided into groups receiving no additional therapy (control); pirfenidone by oral gavages or subcutaneous injection (400 mg/kg/day); rapamycin i.p (0.1 mg/kg/day); or both pirfenidone and rapamycin treatment. Spleens were harvested from F1 mice after 3 days and splenocytes incubated with fluorochrome-conjugated mAb anti-CD4 and anti-CD8 and biotin-conjugated anti-H-2K^d and anti-H-2D^d mAb, followed by fluorochrome-conjugated secondary antibody. Donor alloreactive T cells were distinguished from recipient T cells by gating on H-2K^d and H-2D^d cells. T-cell proliferation was assessed using CFSE division profiles.

Cytokines and chemokines assay

CD3+ and CD4+ T cells were stimulated by TCR activation and cell culture supernatant collected. The concentrations of IFN-γ, IL-1β, TNF-α, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p, IL-13, IL-17, IP-10, MIP-1, Mig, FGF-β, GM-CSF and VEGF were measured by Luminex-200 System using the Cytokine Mouse 20-Plex Panel (Invitrogen Inc, Carlsbad CA) per the manufacturer's instructions. TGF-β levels were determined from stimulated CD3+ and CD4+ T cells or isolated protein from cardiac allografts using ELISA kit (R&D system Minneapolis, MN) per manufacturer's directions and values were within the linear range based on the generation of a standard curve for each individual experiment. There were four individual

experiments for each group and each sample was run in duplicate (TGF- β ELISA) or triplicate (mouse cytokine panel). Pirfenidone and vehicle treated heart allografts were homogenized in phosphate-buffered saline containing protease inhibitor cocktail (Sigma-Aldrich, St Louis Mo) and centrifuged at 20,000g for 20 min at 4 °C and 200 μ g of the protein concentration from supernatant was used for analysis.

Suppression assay

CD4+CD25+ (Treg), CD4+CD25- (Teff), and APCs were isolated from total splenocytes by using magnetic beads conjugated mAbs (Miltenyi Biotec Inc). APCs were irradiated (3300 rad) and plated onto 96-well plates. 1×10^5 Teff cells labeled with CFSE were co-cultured with or without Treg cells at the indicated ratios, and a combination of 1 μ g/ml of soluble anti-CD3 provided the polyclonal stimulus. To analyze the effect of pirfenidone on the suppression activity of Treg cells we used optimum concentration of 1mM. Proliferation of Teff cells was assessed by CFSE dilution 72 h later.

Cardiac transplantation

Heterotopic abdominal cardiac allograft with end-to-side anastomosis of aorta to aorta and pulmonary artery to vena cava (29) were undertaken using BALB/c donors and B6 recipients (5-8 allograft/group). Graft function was monitored daily by palpation, and rejection was confirmed by histology. Allograft recipients were sacrificed and grafts harvested for analysis at the time of rejection (lack of heart beat), or in additional recipients at one week post-transplant, so as to allow direct comparison of intragraft events. Grafts were removed, fixed in formalin, paraffin embedded and 5 μ sections were cut and stained with hematoxylin and eosin (H&E).

Statistics

Data were expressed as mean $\pm$ SEM, and statistical analyses were performed using Prism statistical program (Graph Pad, San Diego, CA). We used one-way ANOVA with Tukey's Multiple Comparison Test to evaluate differences between groups, student t-test to compare two groups, and Kaplan-Meier survival curve for heart allograft survival; a P value less than 0.05 was considered significant.

Results

Pirfenidone doesn't change percentage of T cells subset at the steady state level

Our previous studies using both mouse tracheal and rat lung transplant models showed that pirfenidone treatment had graft protective properties with a reduction in the extent of rejection (7,15,17). To determine if the beneficial effects of pirfenidone on graft survival include modulation of allo responses, we first examined whether pirfenidone has any direct effects on T cells in steady state level. Our initial studies examined whether pirfenidone (400 mg/kg for 7 days) has negative effect on T cell subsets and Treg percentage in spleen and lymph nodes in C57BL/6 mice. There are no significant differences in TCR+CD4+, TCR+CD8+, and TCR + CD4+ Foxp3+ Treg cells in spleen and lymph nodes (Figure 1, Tables 1 and 2) ($p > 0.05$), which indicate pirfenidone does not have any negative effect on the steady state level of T cells subsets in mice.

Pirfenidone inhibit T cells proliferation in vitro and in vivo

We then tested whether pirfenidone has direct effects on T cell proliferation. Pirfenidone was found to significantly inhibit T cell receptor (TCR)-stimulated CD4+ T cells proliferation in vitro ($p < 0.01$), whereas CD8+ T total cell proliferation was not significantly affected (Figure 2a). Although we observed no significant inhibition of CD8+ T cells based on responder

frequency (percent of original cells that divided), we observed attenuation for both CD4+ and CD8+ T cells proliferation index (number of divisions of proliferating cells) in response to pirfenidone (Figure 2b) in a dose dependent manner.

To examine the effects of pirfenidone on T cell allogeneic activation and proliferation *in vivo*, we used a parent into F1 *in vivo* one-way MLR assay (28). We found that pirfenidone treatment resulted in attenuation of both CD4+ and CD8+ T cell proliferation. There was a small but consistent reduction in CD4+ and CD8+ T cells proliferation based on responder frequency; however, this was most evident based on proliferation index with a reduction of proliferation at the higher division populations. We also examined whether there was an additive effect of pirfenidone and rapamycin on alloreactive T cell proliferation. Using low dose rapamycin plus pirfenidone, we observed a greater inhibition of both CD4+ and CD8+ T cells proliferation than when either agent was used alone (Figure 3).

Pirfenidone significantly reduced some cytokine and chemokine expression

Many of pirfenidone actions are thought to occur through its inhibitory actions on cytokine expression. Additionally, cytokine production is a component of T cell activation (30,31). We therefore examined whether pirfenidone inhibited cytokine/chemokine release in response to TCR activation *in vitro*. We first observed a significant ($p < 0.05$) decrease in multiple cytokines in CD3+ T cells, including those linked with Th1 (IFN- γ , IL-1 β , TNF- α), Th2 (IL-4) and Th17 (IL-17) differentiation, plus at least one profibrotic cytokine fibroblast growth factor (FGF- β), and pro-inflammatory chemokines (IP-10, MIP-1, Mig). No significant change in response to pirfenidone was observed in production of IL-2, IL-6, IL-12p, IL-5, IL-10, IL-13, GM-CSF or VEGF. Since we observed a greater effect of pirfenidone on CD4+ T cells proliferation *in vitro* than CD8+ T cells, we also evaluated the effects of pirfenidone on TCR-stimulated cytokine expression by purified CD4+ T cells. We found a significant inhibition of CD4+ T cells production of IFN- γ , IL-1 β , IL-4, IL-5, IL-17, IP-10, MIP-1, and Mig in response to pirfenidone (Figure 4a). Also of note was a lack of inhibition of TGF- β in either purified CD3+ or CD4+ T cells with pirfenidone (Figure 4b), in contrast to our previous data from both rat lung and mouse tracheal transplant models in which pirfenidone resulted in decreased TGF- β in peripheral blood and grafts (7,17). However, similar to our previous studies, there was an inhibition of TGF- β expression of isolated protein from cardiac allografts treated with pirfenidone (Figure 4c) indicating cell specific responses of TGF- β to pirfenidone.

Pirfenidone doesn't interfere with regulatory T cell function

Since pirfenidone had an inhibitory effect on T cell activation and production of multiple cytokines (both Th1- and Th2-associated), we examined whether pirfenidone had effects on Treg cell activity. A standard *in vitro* Treg assay was performed using varying ratios of CD4+CD25+ co-cultured with CFSE-labeled CD4+CD25- T cells plus CD3 mAb and APC. As expected, there was less overall TCR stimulated proliferation in the pirfenidone-treated group as compared to the control population; however, the suppressive effects of CD4+CD25+ population on effector cells was maintained in the presence of pirfenidone (Figure 5). Hence, pirfenidone does not appear to affect Treg function.

Pirfenidone promotes long term heart allograft survival in low dose rapamycin treated recipient

To examine the effect of pirfenidone on immune responses in a transplant model, we undertook heterotopic vascularized cardiac allografting across a full MHC mismatch, BALB/c (H-2^d) donors and B6 (H-2^b) recipients. Whereas low dose pirfenidone (40 mg/kg) had no effect on allograft survival, a higher dose of pirfenidone (400 mg/kg) significantly ($p < 0.05$) prolonged graft survival (Figure 6a) and there was a remarkable reduction in the histological evidence of acute rejection after 7 days. Untreated cardiac allografts showed extensive mononuclear cell

infiltration and multifocal myocyte necrosis characteristic of acute cellular rejection, grade 3R, while pirfenidone and/or rapamycin treated grafts showed only mild or low grade acute cellular rejection with diffuse interstitial pattern of lymphocytes and little perivascular infiltrate without myocyte damage, grade 1R (Figure 6b). Similar to the in vivo MLR, the combination of pirfenidone plus a subtherapeutic regimen of rapamycin significantly prolonged allograft survival ($p<0.05$) and delayed the onset of rejection and the development of key features of chronic rejection, including arteriosclerosis and myocardial fibrosis.

Discussion

Pirfenidone's anti-fibrotic effects have elicited an interest in its use as a potential therapeutic agent to limit the fibroproliferative response of chronic rejection (19,32). Our laboratory and others have previously shown that pirfenidone has graft protective effects and reduced the development of fibrosis in lung and tracheal transplant models (7,15-18). This was associated with an inhibition of the profibrotic cytokine TGF- β and a reduction in collagen formation. However, in addition to anti-fibrotic effects, pirfenidone also limited the alloimmune/inflammatory response, as reflected by reduction of intra-graft neutrophil and lymphocyte recruitment (15). The current study was performed to assess whether pirfenidone had a direct effect on T cell activation and proliferation, thereby having an immunosuppressive role in addition to its known anti-fibrotic effect. We found that pirfenidone exposure attenuated T cell proliferation, activation and production of multiple cytokines and chemokines, thereby indicating that the graft protective effects of pirfenidone may be two-fold, encompassing both the alloimmune and non-alloimmune phases of rejection.

Our in vitro studies demonstrated that pirfenidone reduced TCR-stimulated proliferation of CD4⁺ T cells and a more limited effect on CD8⁺ T cells proliferation. However, using an in vivo allogeneic model of stimulated T cell proliferation (parent to F1 model), we observed an inhibitory effect on both CD4⁺ and CD8⁺ T cells proliferation. Further evidence of pirfenidone's inhibition of the allogeneic response was observed by its significant delay of allograft loss in a fully MHC-mismatched murine cardiac allograft model. Interestingly, we observed that pirfenidone with low dose rapamycin also potentiated the inhibition of allogeneic-stimulated T cell proliferation using the parent to F1 in vivo MLR, and more importantly, further prolonged mouse cardiac allograft survival.

Hirano et. al. showed that pirfenidone inhibited the antigen-specific proliferation of lymph node mononuclear cells in OVA sensitized mice, and inhibited production of the profibrotic Th2 cytokines, IL-4 and IL-13 (8). In addition, pirfenidone was shown to inhibit the expression of the Th1 cytokines TNF- α and IFN- γ (11,14). We also found that pirfenidone inhibits a number of proinflammatory cytokines/chemokines from activated lymphocytes including IFN- γ , IL-1 β , TNF- α , IL-4, IL-17, FGF- β , IP-10, MIP-1, Mig. Pirfenidone's protective effects on graft survival may still be related to its inhibition of a number of these cytokines and chemokines, thereby reducing the inflammatory response, T cell activity, and possibly trafficking with its effects on chemokines. The down regulation of these cytokines and chemokines does not appear to be from a general inactivation of lymphocytes and therefore inhibiting the release of all products since we observed no change in IL-2, IL-6, IL-12p, IL-5, IL-10, IL-13, GM-CSF, and VEGF. In addition, we did not see a decrease in CD25 or CD69 expression upon TCR activation of T cells in vitro (data not shown).

As noted, pirfenidone's anti-fibrotic effects are commonly attributed to its suppression of TGF- β production. TGF- β is a pleiotropic cytokine with complex regulatory functions including the differentiation and proliferation of a wide variety of cell types, and a central mediator in the development of fibrosis (33-35). TGF- β has an early pro inflammatory role in tissue, which can recruit monocytes and granulocytes to injured areas by acting as a chemoattractant (34,

36), but is subsequently anti-inflammatory with effects on a variety of inflammatory cells which is most apparent upon T cells (37,38). The later inhibitory effects are believed to help resolve inflammation (35). TGF- β also has anti-proliferative actions on T cells, largely attributed to inhibition of IL-2 production (39), and plays a role in the development of both natural and inducible Tregs (40). Similar to our previous observations for mouse tracheal and rat lung allografts, pirfenidone inhibited TGF- β expression in heart allografts. Pirfenidone's inhibition of the early TGF- β and/or other acute inflammatory mediators could reduce the early transplant response while its effects on the later TGF- β response and/or profibrotic cytokines reduce the fibroproliferative injury. In contrast to what we observed in lung or heart tissue, we found that pirfenidone had no suppressive effects on TGF- β release from TCR-activated T cells showing a differential cellular response to pirfenidone. More importantly, since TGF- β can mediate the generation of Tregs, and these cells may play a role in long term tolerance of allografts (40), it is reassuring that pirfenidone did not interfere with the suppressive effects of CD4⁺ CD25⁺ cells/Tregs on T cell proliferation.

This study supports the potential of pirfenidone as an additive therapeutic agent in prolonging allograft survival. As previously shown, it may reduce the fibroproliferative response that is part of the pathophysiology of chronic rejection. However, it also has inhibitory actions against the alloimmune phase with a reduction in lymphocyte activation and proliferation, and prolongs graft survival especially in combination with low dose rapamycin. It is encouraging that despite having the ability to reduce TGF- β expression, we did not see an inhibition of TGF- β in activated T cells and more importantly it did not reduce the number of Tregs in the transplant model or interfere with Treg suppressive activities.

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Abbreviations

APC	antigen presenting cells
CFSE	carboxyfluorescein diacetate succinimidyl ester
H&E	hematoxylin and eosin

Figure 1.

The ratio of TCR+CD4+, TCR+CD8+ and TCR+CD4+foxp3+ T cell. Mice were treated with pirfenidone or vehicle for 7 days, and isolated cells from spleen and lymph nodes were stained for anti-TCR β , CD4, CD8 and Foxp3. The ratio of T cells subpopulation from isolated spleen and lymph nodes was assessed by flow cytometry, and shown is a representative Dot Plot of CD4+foxp3+ cells ratio among TCR+CD4+ cells isolated from spleen and lymph nodes.

Figure 2a

Figure 2b

Figure 2.

a) Suppressive effect of pirfenidone (PFD, 1 mM) on TCR-stimulated (APC + anti-CD3) CD4 + T cell proliferation (mean $\pm$ SEM from 6 independent experiments, * p <0.01, control to PFD) as assessed by flow cytometry, including all dividing populations (R3). A representative FACS experiment of CFSE dilution is shown. **b)** CD4+ and CD8+ T cell proliferation upon stimulation with CD3 and CD28 mAbs upon addition to increasing Pirfenidone dose (0, 0.01, 0.1, and 1 mM) (* p <0.05 as compared to untreated group, n =4). A representative FACS experiment of CD4+ and CD8+ T cell proliferation based on CFSE dilution and illustrating a reduction in cell division proliferation upon addition of PFD (note overall shift to the right with PFD use).

Figure 3.

Results of a parent-F1 hybrid in vivo MLR illustrating both CD4+ and CD8+ T cells proliferation (all divisions and last three divisions) in untreated, PFD treated (400 mg/kg/day), Rapamycin (Rap, 0.1 mg/kg/day), or a combination of PFD plus Rap using CFSE dilution (* $p < 0.05$ as compared to untreated group, $n=4$), and a representative FACS experiment for both CD4+ and CD8+ T cells proliferation is shown for the experimental groups.

Figure 4a

Figure 4b

TGF- β from isolated lymphocytes

Figure 4c

TGF- β from heart allografts

Figure 4.

a) Graphs of cytokines and chemokines released from TCR stimulated CD3 and CD4+ T cells under control (untreated) and PFD treated conditions. The agents listed are those that showed significant inhibition in response to PFD ($p < 0.05$ with $n=4$). **b)** In addition the results of TGF- β are shown in which there were no significant difference observed between untreated and treated CD3+ and CD4+ T cells. **c)** Graph illustrating TGF- β expression from pirfenidone ($n=6$) and vehicle ($n=6$) in isolated protein from PFD and untreated heart allografts 7 days after transplantation. Pirfenidone treated heart allografts significantly ($p < 0.05$) express lower TGF- β compared to vehicle treated grafts.

Figure 5.

This is a representative experiment evaluating the effects of PFD on Treg function. The first set of panels illustrates TCR stimulated proliferation of CD4+ CD25-, T effector cells (Teff), with increasing concentration of CD4+CD25+, regulatory T cell population (Treg). The second set illustrates the results with the addition of PFD. The graph shows the mean of three independent experiments.

a.

b.

Figure 6.

a) Survival curve of heterotopic heart transplants from BALB/c donors into B6 recipients for untreated (control) and those treated Rap, PFD, or the combination of PFD plus Rap (n=5-8 transplants/group). $P < 0.05$ for PFD and PFD/Rap treated groups as compared to untreated allografts. **b)** Representative microphotograph (H&E stain, $\times 200$) of a normal mouse heart and heart allografts from untreated (vehicle), Rap, and PFD treated mice at day 7 post-transplantation.

Table 1

Spleen	Vehicle	Pirfenidone
%TCR+CD4+	10.24 ± 0.62 N=6	9.69 ± 0.72 N=6
%TCR+CD8+	7.82 ± 0.77 N=5	6.25 ± 0.29 N=5
%TCR+CD4+FOXP3+	15.30 ± 0.38 N=6	15.57 ± 0.42 N=6

Table 2

Abdominal Lymph	Vehicle	Pirfenidone
%TCR+CD4+	29.82 ± 1.99 N=6	30.30 ± 1.15 N=5
%TCR+CD8+	26.94 ± 3.50 N=5	28.80 ± 3.86 N=4
%TCR+CD4+FOXP3+	15.55 ± 0.81 N=6	15.22 ± 0.88 N=6

There is no significant difference in TCR+CD4+, TCR+CD8+ and TCR+CD4+foxp3+ T cell ratio from spleen (Table1) and lymph nodes (Table2) in pirfenidone and vehicle treated mice by unpaired student t test ($p>0.05$).