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Pirfenidone restricts Th2 differentiation in vitro and limits Th2 response in experimental liver fibrosis

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ABSTRACT

Polarized T helper type 2 (Th2) response is linked with fibrosis. Here, we evaluated the effect of the anti-fibrotic agent pirfenidone on Th type 1 (Th1) and Th2 responses. For in vivo testing; Wistar rats were made cirrhotic by intraperitoneal administration of thioacetamide. Once hepatic damage was established, pirfenidone was administered intragastrically on a daily basis during three weeks. Gene expression of Th marks was evaluated by RT-PCR and Western blot assays from liver homogenates. Pirfenidone therapy induced down-regulation of Th2 transcripts and proteins (GATA3 and IL-4), without affecting significantly Th1 genes expression (T-bet and IFN-γ). We found that the activated form of p38 MAPK (identified by Western blot) was reduced by pirfenidone treatment, which is consistent with the anti-Th2 activity observed. Pirfenidone reduced GATA3 nuclear localization without modifying its DNA binding activity (evaluated by electrophoretic mobility shift assay). For in vitro testing; human naive CD4+ T cells were cultured in either Th1 or Th2 polarizing conditions in the presence of pirfenidone and flow cytometric analysis of intracellular synthesis of IFN-γ and IL-4 was conducted. Pirfenidone impaired development of Th2 subpopulation. In conclusion, pirfenidone is capable of impairing Th2 differentiation and limits Th2 profibrogenic response. The mechanism involves p38 inhibition and regulation of GATA3 expression and translocation.

1. Introduction

Liver fibrosis is an aberrant wound healing process characterized by the accumulation of extracellular matrix proteins in response to chronic liver injury. Recruitment of leukocytes takes place following hepatic injury and persists during the process. This chronic inflammatory reaction is characterized by a large infiltrate of mononuclear cells including T lymphocytes (Wynn, 2008). CD4+ T-helper (Th) lymphocytes are divided into four major subsets based on their expression profile of transcription factors and secreted cytokines: 1) Th1 interferon-γ (IFN-γ) producing cells, 2) Th2 expressing interleukins 4, 5 and 13 (IL-4, IL-5 and IL-13), 3) regulatory T cells that produce IL-10 and transforming growth factor beta (TGF-β), and 4) Th17 that synthesize IL-17.

Th lymphocytes have an important role in the progression of fibrotic disease in several tissues including the liver (Wynn, 2004). Fibrogenesis is strongly linked with the development of a dominant (polarized) type 2 CD4+ T-cell response (T helper 2 or Th2 response), whereas anti-fibrotic activity has been described when type 1 CD4+ T-cell response (Th1 response) dominates (Wynn, 2008).

The Th differentiation process is complex. The T-box transcription factor T-bet is the central regulator of Th1 differentiation (Szabo et al., 2000) and directly activates transcription of IFN-γ gene (Lee et al., 2004; Shnyreva et al., 2004; Szabo et al., 2000), and also blocks Th2 differentiation and production of the Th2 cytokines (Hwang et al., 2005; Usui et al., 2006). On the other hand, the zinc-finger transcription factor GATA-binding protein 3 (GATA3) determines Th2 cell differentiation and selectively activates promoters of IL-4, IL-5 and IL-13 genes through chromatin remodeling (Lavenu-Bombled et al., 2002; Yamashita et al., 2004).

The anti-fibrotic agent pirfenidone (5 methyl-1-phenyl-2-(1H)-pyridone) has proven effectiveness for preventing and resolving the accumulation of fibrous tissue in pulmonary and liver fibrosis (Armendariz-Borunda et al., 2006; Garcia et al., 2002; Iyer et al.,

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[4] Author contributions: Study concept and design, drafting of the manuscript, analysis and interpretation of data, critical revision of the manuscript for important intellectual content, statistical analysis, study supervision.

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1995; Oku et al., 2008; Raghu et al., 1999; Simone et al., 2007). In addition to pirfenidone anti-fibrotic properties, anti-oxidant (Giri et al., 1999; Salazar-Montes et al., 2008) and anti-inflammatory activities have been documented (Oku et al., 2002).

In the context of Th response, pirfenidone was found to inhibit the responder frequency of TCR-stimulated CD4+ cell total proliferation in vitro and in vivo and to diminish significantly Th cytokines in the bronchoalveolar lavage fluid from animals in airway chronic allergen challenge with ovalbumin (Hirano et al., 2006; Visner et al., 2009).

In this study, we have demonstrated an immunoregulatory effect of pirfenidone on Th differentiation in vitro and in Th2 response in vivo. Essentially, our data showed that pirfenidone impaired Th2 development, limited Th2 cytokine production, reduced expression of GATA3 and partially blocked its translocation to the nucleus. A decline in Th2 response due to pirfenidone treatment resulted in a significant improvement on fibrosis in vivo.

2. Materials and methods

2.1. Animals and pirfenidone dosing

Male Wistar rats (150–180 g) were randomized into three experimental groups (each n = 10), as follows: 1) Non-TAA or control, 2) TAA or fibrotic and 3) TAA-pirfenidone or pirfenidone. Thioacetamide (TAA) induced liver fibrosis was achieved using a dose of 200 mg/kg of this hepatotoxic agent, administrated intraperitoneally at 3 times per week for 7 weeks, as previously described (Li et al., 2002; Neef et al., 2006). At the end of TAA intoxication regimen, the group on TAA-pirfenidone was given the anti-fibrotic agent (200 mg/kg) orally by gavage, daily during three weeks (Garcia et al., 2002). Liver extraction from all groups was performed after pirfenidone treatment was concluded. With the purpose of monitoring the expression of Th and fibrosis markers in TAA-induced liver fibrosis model, additional rats were intoxicated with TAA as described above and were sacrificed at weeks 1, 4, 7 and 10 (each n = 5) after TAA administration started. Immediately after sacrifice, representative liver sections were excised and either fixed with 4% buffered paraformaldehyde for histological examination or frozen at −70 °C for molecular analysis.

All animals were fed *ad libitum* and received humane care according to criteria outlined in the "Guide for the Care and Use of Laboratory Animals" published by the National Institutes of Health (NIH publication 86-23 revised 1985).

2.2. Determination of gene expression for IL4, IFN-γ, T-bet, GATA3, α1(I) collagen and TGF-β1 by quantitative real-time RT-PCR

RNA was isolated from liver homogenates with Trizol reagent (Invitrogen, Carlsbad CA, USA). Reverse transcription was performed with 2μg of total RNA, using M-MLV reverse transcriptase (Invitrogen, Carlsbad CA, USA). Then, 2μl of cDNAs was subjected to real-time PCR using Rotor Gene Termocycler under the following conditions: 2 min/50 °C, 10 min/95 °C, and 45 cycles of 15 s/95 °C and 1 min/60 °C. Specific probes designed to align with IL4, IFN-γ, T-bet, GATA3, α1(I) collagen and TGF-β1 rat mRNAs were acquired from Applied Biosystems (Carlsbad CA, USA). Gene amplification was normalized against 18S rRNA expression. Relative quantification by $2(-\Delta\Delta CT)$ method was carried out comparing to control group as internal calibrator (Livak and Schmittgen, 2001; Yuan et al., 2006).

2.3. Analysis of activation of p38 MAPK and Th1 and Th2 proteins in liver homogenates

Western blot assays were conducted to analyze Th1 and Th2 protein expressions, as well as to identify the active form of P38 MAPK (phosphorylated p38 MAPK) from liver homogenates. Proteins were extracted from 400 mg of liver using lysis buffer (50 mM Tris–HCl pH 8.0, 150 nM NaCl, 0.02% NaN_3). After centrifugation at 13,000 rpm/5 min/4 °C supernatant was collected and quantified by Bradford assay. Briefly, 20–25 μg of total proteins was separated by 10% SDS-PAGE under reducing conditions and transferred to PVDF membranes (Bio-Rad Laboratories, Hercules CA, USA). Blocking was carried out using 3% nonfat dry milk for 2 h; primary antibody dilution was 1:500 for GAPDH (loading control), IFN-γ, IL-4 and phosphorylated p38 antibodies and 1:800 for GATA3 and T-bet antibodies (Santa Cruz Biotechnology, Santa Cruz CA, USA). Antibody binding was revealed with a secondary anti-antibody diluted 1:5000–1:6000 using BM Chemiluminescence kit (Roche Diagnostics, Indianapolis IN, USA). Densitometric analysis was realized with a Kodak 1D 3.5 Image analyzer (Eastman Kodak Co., Rochester NY, USA).

2.4. Assessment of T-bet and GATA3 translocation

Western blot assays of cellular fractions (nucleus and cytoplasm) were performed to evaluate translocation of T-bet and GATA3. Cytoplasmic and nuclear extracts were isolated from liver biopsies according to Andrews and Faller (1991). Briefly, 20 μg of cytoplasmic or nuclear extract proteins was used on each western blot assay performed as previously described in Section 2.3. GAPDH was used as cell fractionation control.

2.5. Evaluation of T-bet and GATA3 DNA binding activity

A LightShift Chemiluminescent Electrophoretic Mobility Shift Assay (EMSA) Kit (Pierce, Rockford IL, USA) was used to detect DNA–protein interactions. Briefly, double-stranded oligonucleotides corresponding to the specific DNA binding consensus sequences for GATA3 and T-bet transcription factors were biotin-end labeled with a Biotin 3′ End DNA Labeling Kit (Pierce, Rockford IL, USA) and then incubated with 1 μg of nuclear protein obtained as described in a previous section. For identification of DNA-bound protein, a binding reaction included the specific antibody, Anti-T-bet or Anti-GATA3 (Santa Cruz Biotechnology, Santa Cruz CA, USA). These reactions were then subjected to gel electrophoresis on a native 5% polyacrylamide gel and transferred to a nylon membrane. Biotin end-labeled DNA was detected using the streptavidin horseradish peroxidase conjugate and the chemiluminescent substrate. Densitometric analysis was realized with a Kodak 1D 3.5 Image analyzer (Eastman Kodak Co., Rochester NY, USA).

2.6. Histological examination

Hepatic sections were randomly taken from the right, median and left lobes of rat livers and immediately fixed by immersion in 4% paraformaldehyde diluted in phosphate saline buffer (PBS), dehydrated in graded ethylic alcohol and embedded in paraffin. Sections (5 mm thick) were stained with Masson's trichrome technique. Then, 20 random fields from each liver section were analyzed with light-microscopy (20X) using a computer-assisted morphometric analyzer (Image Proplus, Bethesda MD, USA). The percentage of liver tissue affected by fibrosis was determined calculating the ratio of connective tissue to the whole area of the liver (Garcia et al., 2002).

2.7. Cells

Peripheral blood mononuclear cells (PBMCs) were isolated by a Ficoll Paque Plus (Amersham, Piscataway NJ, USA) density gradient from healthy donors following informed consent. Naive CD4+ T cells were positively purified from PBMCs. Briefly, PBMCs were incubated at 4 °C for 30 min with a mixture of anti-CD4 PE-Cy7 and anti-CD45RA-APC (eBioscience, San Diego CA, USA). After washing twice in buffer [phosphate-buffered saline (PBS)], cells were resuspended

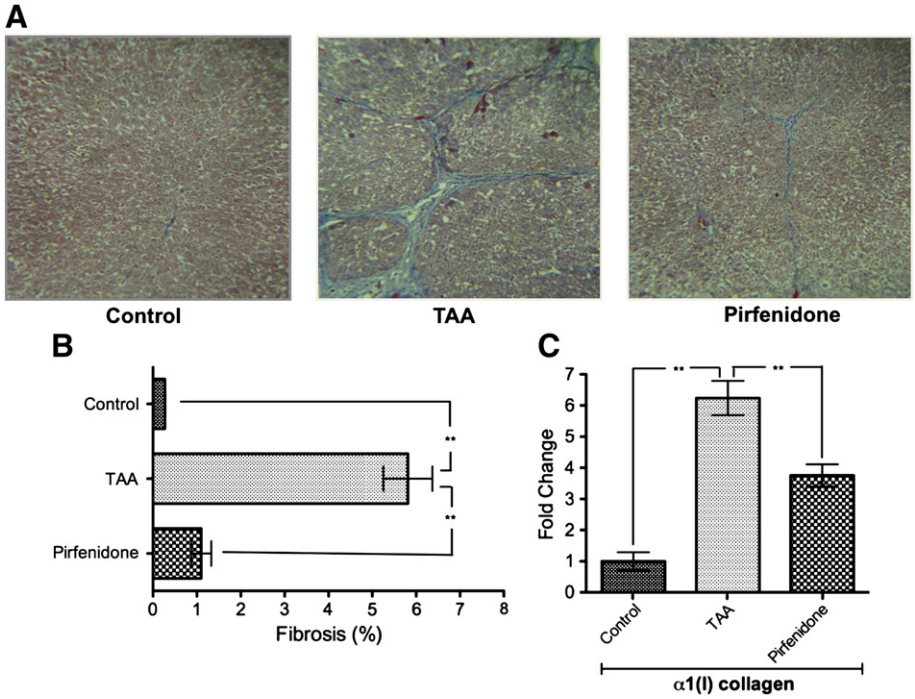

Fig. 1. Anti-fibrotic effect of pirfenidone in TAA-injured rats. (A) Anti-fibrotic effect of pirfenidone was confirmed in TAA-induced liver fibrosis. Microphotographies (20×) of liver sections stained with Masson's trichrome technique revealed that pirfenidone treatment reduced extracellular matrix proteins accumulation. (B) Computer-based image analysis of microphotographies showed that pirfenidone therapy diminished in 68% extracellular matrix proteins accumulation. (C) Pirfenidone reduced significantly α1(I) collagen transcript (evaluated by real-time RT-PCR) after established TAA-induced liver fibrosis. Bars and plots represent the mean ± standard deviation of the mean of 5–6 rats per group. Asterisks indicate values significantly different. ** $P<0.01$.

in the buffer and sorted on a MoFlo cell sorter (Dako Cytomation, Fort Collins CO, USA). Purity of cell population was assessed by flow cytometry. We obtained >98% purity for naive CD4+ T cells.

2.8. Culture conditions

Naive CD4+ T cells were plated at a density of 3×10^5 in 24-well tissue culture plate. Plates were precoated with a mixture (2 μg/ml) of anti-CD3 and anti-CD28 antibodies (eBioscience, San Diego CA, USA). Cells were cultured for 1 h in the presence of 100 μM of pirfenidone in RPMI supplemented with 10% of fetal bovine serum (FBS), 2 mM-glutamine, penicillin (50 units/ml) and streptomycin (50 μg/ml). Cells were washed with 1× phosphate-buffered saline (PBS 1×) and cultured for 6 days with 10 ng/ml of rIL-2, 5 ng/ml of rIL-12 and 30 μg/ml of anti-IL-4 for Th1polarization, or cultured with 10 ng/ml of rIl-2, 40 ng/ml of rIL-4, 30 μg/ml of anti-IL-12 and 30 μg/ml of anti-IFN-γ for Th2 polarization (all recombinant cytokines and antibodies against cytokine were purchased from PeproTech Inc, Rocky Hill NJ, USA). Three days after culture, fresh pirfenidone was added. At this period, cells were washed, plated at density of 3×10^5 and cultured in either Th1 or Th2 polarizing conditions as described above.

2.9. Flow cytometric analysis of intracellular cytokine synthesis

Cultured CD4+ T cells were harvested, washed twice with PBS and activated during 6 h with anti-CD3 and anti-C28 in the presence of 100 μM pirfenidone and 10 μg/ml of Brefeldin A. Then cells were harvested, washed twice with PBS, fixed and permeabilized with Perm Buffer II (B&D, San Diego CA, USA) for 15 min at room temperature. Afterwards cells were incubated with anti-IFNg-PE/Cy7 and anti-IL-4-PE or respective isotype control antibody (eBioscience, San Diego CA, USA) at 4 °C for 30 min. Finally, cells were washed with PBS and 2×10^5 cells were acquired by CyAn flow cytometer (Beckman Coulter, Fullerton CA, USA) and analyzed using FlowJo software (TreeStar, Ashland OR, USA).

2.10. Statistical analysis

For non-normally distributed data the non-parametric tests Kruskal–Wallis/Dunn's and Mann–Whitney U were used. Non-normally distributed data are shown as the median. Normally distributed data were analyzed using one-way ANOVA. Then,

Table 1
Expression of Th and fibrosis markers in TAA-induced liver fibrosis model.

	T-bet	GATA3	T-bet/ GATA3	IFN-γ	IL-4	TGF-β1	α1(I) collagen
Basal	1.00 ± 0.13	1.00 ± 0.35	1	1.00 ± 0.76	1.00 ± 1.46	1.00 ± 0.19	1.00 ± 0.14
1 week	0.81 ± 0.38	1.30 ± 0.28	0.62	1.8 ± 0.23	0.79 ± 0.31	1.53 ± 0.21	2.15 ± 0.34
4 weeks	1.40[b] ± 0.20	2.53[b] ± 0.42	0.55	3.2[b] ± 0.42	0.75 ± 0.26	2.96[a] ± 0.64	10.15[b] ± 0.75
7 weeks	1.96[b] ± 0.36	2.36[b] ± 0.46	0.83	17.6[b] ± 0.10	13.39[b] ± 0.08	2.55[a] ± 0.15	9.96[b] ± 0.91
10 weeks	23.68[b] ± 0.36	63.78[b] ± 0.58	0.37	512[b] ± 0.23	799.8[b] ± 0.57	1.55 ± 0.86	7.62[b] ± 0.59

During TAA intoxication, expression of both Th1 and Th2 markers as well as expression of TGF-β1 and α1(I) collagen was augmented across the time. At 10 weeks of follow-up, a clear Th2 polarization occurred evidenced by the drop in T-bet/GATA3 ratio. The expression of Th2 markers (GATA3 and IL-4) clearly exceeds the expression of Th1 markers (T-bet and IFN-γ). Values are fold change ± standard deviation of 5 rats per group. Letters indicate values significantly different from the basal levels. [a] $P<0.05$, [b] $P<0.01$.

Holm–Sidak or Dunnett's *post tests* were used for determination of statistical significance. Normally distributed data are shown as the mean ± standard deviation. Significance was defined as a P value less than 0.05.

3. Results

3.1. Pirfenidone reduced liver fibrosis in TAA-injured rats

TAA is a known hepatotoxic agent that produces stable experimental liver fibrosis (Neef et al., 2006). With the aim to generate this model of hepatic injury, we intoxicated male Wistar rats with TAA for seven weeks. Three weeks later, fibrotic animals were sacrificed. As expected at this point, liver fibrosis and over expression of α1(I) collagen gene were achieved ($P<0.01$) (Fig. 1A and C). Pirfenidone treatment reduced in 68% extracellular matrix proteins accumulation ($P<0.01$) (Fig. 1B) and diminishes α1(I) collagen transcript ($P<0.01$) (Fig. 1C) in this experimental model of liver fibrosis.

3.2. Pirfenidone limited Th2 response in TAA-injured rats

There is no current information about Th1/Th2 balance in hepatotoxic induced liver fibrosis models. Thus, we first analyzed Th response in TAA-induced liver fibrosis. During 7 weeks of TAA intoxication, expression of both Th1 and Th2 markers as well as expression of TGF-β1 and α1(I) collagen was augmented across time (Table 1). At 10 weeks of follow-up, T-bet/GATA3 ratio fell dramatically suggesting that Th2 polarization occurred (Table 1). At this moment, the

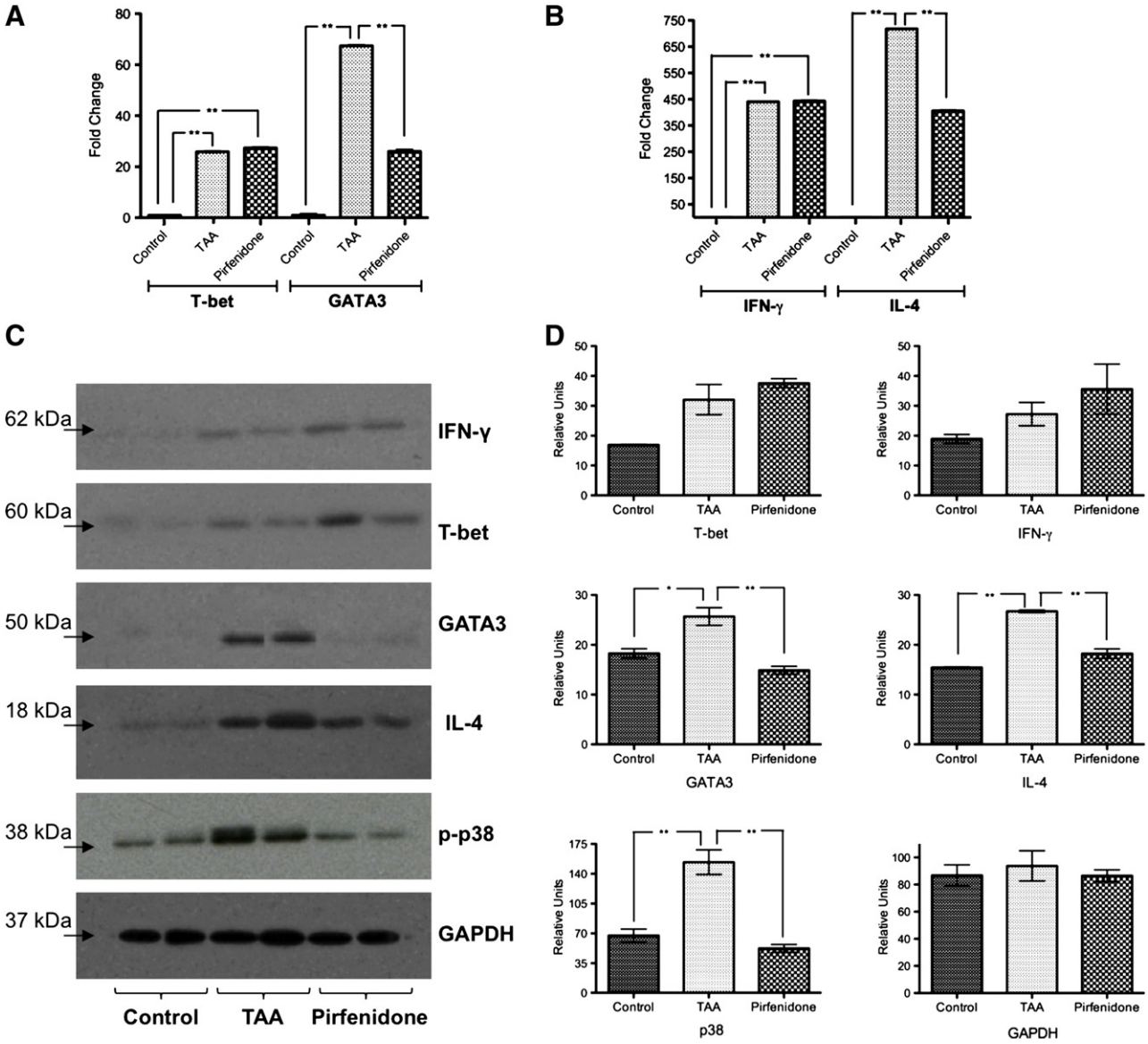

Fig. 2. Pirfenidone diminishes Th2 mRNAs and proteins. (A and B) Gene expression analysis by real-time RT-PCR showed that Th1 transcripts T-bet and IFN-γ were unaffected or slightly augmented in response to TAA (TAA group), whereas mRNAs for Th2 transcription factor GATA3 and Th2 cytokine IL-4 were strongly increased in liver homogenates. Dominant expression of Th2 mRNAs over Th1 transcripts in TAA group indicates a polarized Th2 response. (A) GATA3 mRNA was strongly diminished with pirfenidone therapy (pirfenidone group). (B) As expected, repression of GATA3 by pirfenidone was consistent with significant reduction in mRNA expression for Th2 cytokine IL-4. (C and D) Th1 proteins; IFN-γ and T-bet, as well as Th2 proteins; IL-4 and GATA3 analyzed by Western blot assay, were found increased in TAA injured rats (TAA group). Pirfenidone treatment resulted in reduced Th2 protein expression and coincides with reduction in p38 activation (as measured by the phosphorylated form of p38). (C) Western blots bands and (D) densitometric analyses are presented. Bars and plots represent the mean ± standard deviation of the mean of 5–6 rats per group. Asterisks indicate values significantly different. * $P<0.05$, ** $P<0.01$.

expression of Th2 markers (GATA3 and IL-4) exceeded expression of Th1 markers (T-bet and IFN-γ). As expected, TGF-β1 and α1(I) collagen gene transcription rose after the first week of TAA administration and remained high during TAA intoxication. TGF-β1 expression dropped by the end of the 10th week (Table 1). Taken altogether, these data suggest that Th2 polarized response was developed in rat liver after TAA intoxication.

A clear anti-Th2 effect was observed with pirfenidone administration in TAA-induced liver fibrosis. The Th2 transcription factor GATA3 was repressed at transcriptional and translational levels ($P<0.01$) (Fig. 2A and C). It is important to emphasize, that pirfenidone achieved to reduce both GATA3 mRNA and protein in a highly Th2 polarized environment. As expected, repression of GATA3 by pirfenidone was consistent with the reduction in both IL-4 mRNA and protein expression ($P<0.01$) (Fig. 2B and C).

Messenger RNA and protein expression of Th1 genes (T-bet and IFN-γ) were not significantly modified as a result of pirfenidone administration (Fig. 2).

3.3. Pirfenidone effects on Th2 response were p38 mediated

On the basis that p38 is a key regulator in Th response, we explored p38 activation using an antibody directed against residues Thr^{180} and Tyr^{182} to recognize the activated form of phosphorylated p38 MAPK. We showed that pirfenidone at doses of 200 mg/kg reduced p38 activation in the liver, which coincides with the anti-Th2 effect previously observed with pirfenidone administration (Fig. 2C and D).

3.4. Pirfenidone limited GATA3 translocation without modifying its DNA binding activity

GATA3 nuclear localization and increased GATA3 DNA binding activity were characteristic of TAA group (Fig. 3) ($P<0.05$). Although we found increased T-bet translocation (Fig. 3A) ($P<0.05$), the DNA binding activity of this factor was reduced in TAA group (Fig. 3B). No differences were observed in GATA3 localization among cellular fractions in pirfenidone group (Fig. 3A), suggesting that pirfenidone therapy limited GATA3 nuclear translocation. On the other hand, T-bet nuclear localization was promoted by pirfenidone treatment (Fig. 3A) ($P<0.05$). DNA binding activity of GATA3 was unmodified by pirfenidone administration (Fig. 3B), whereas T-bet activity was increased in response to this anti-fibrotic drug ($P<0.05$) (Fig. 3B). In brief, pirfenidone therapy reduced GATA3 translocation without influencing its DNA binding activity, whereas pirfenidone induced an amplified translocation and activity of T-bet.

3.5. Pirfenidone impairs Th2 cells development

In an attempt to correlate our findings in vivo with a direct effect of pirfenidone, the effect of pirfenidone during activation and polarization of naive cultured CD4+ T cells was assessed. Pirfenidone impairs development of both types of CD4+ T cell subpopulations (Fig. 4). However, pirfenidone affects more severely Th2 generation than Th1. It can be clearly seen a 50 times reduction of Th2 cells cultured in the presence of pirfenidone as compared with only 4 times reduction for Th1 cells (Fig. 4).

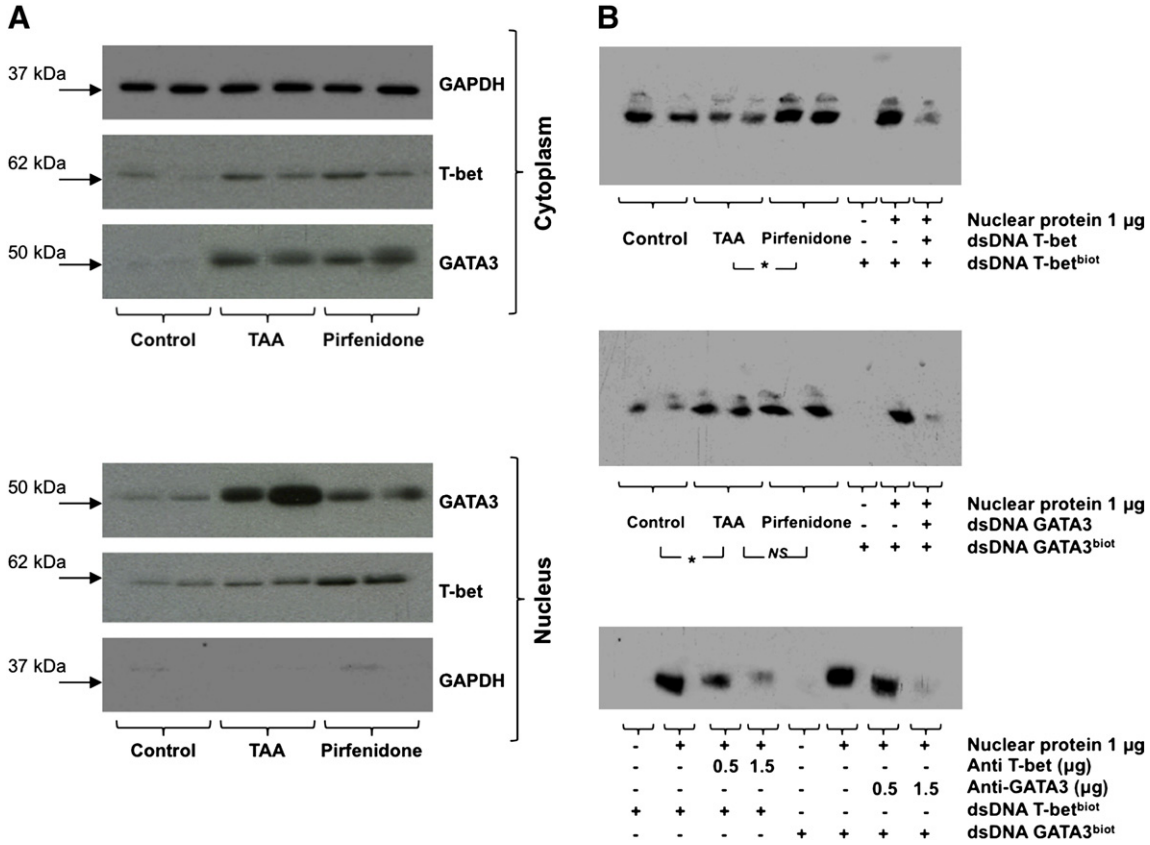

Fig. 3. Pirfenidone limits GATA3 translocation and increases T-bet DNA binding. (A) Translocation of Th transcription factors was evaluated trough Western blot assays for T-bet and GATA3 from cytoplasm and nuclear fractions (top and inferior images respectively). No differences were observed in T-bet localization between cell fractions in TAA group. Pirfenidone therapy increased T-bet nuclear localization ($P<0.05$). In TAA group GATA3 was located primarily in the nucleus; whereas in the pirfenidone group no significant difference was found in the location of that factor among the cellular compartments, suggesting that pirfenidone therapy limits GATA3 translocation. (B) DNA binding activity for T-bet and GATA3 (top and middle images respectively) was analyzed by EMSA assays. Increment in GATA3 DNA binding activity was characteristic of TAA group. Pirfenidone therapy amplified T-bet DNA binding activity without affecting GATA3. Incubation of DNA binding reactions with specific antibodies (inferior image) decreased DNA binding activity for T-bet and GATA3 in a concentration-dependent way. * $P<0.05$, ^{NS} Non-significative, ^{dsDNA} double strand DNA, ^{biot} biotinylated.

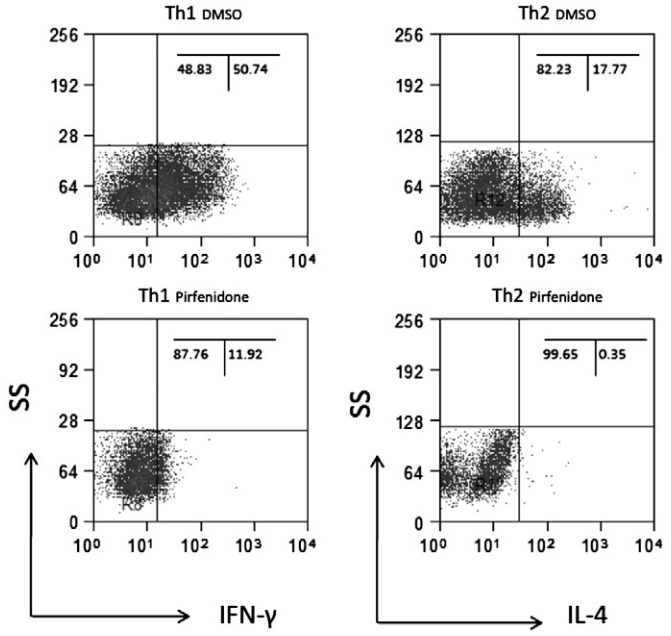

Fig. 4. Effect of pirfenidone on human Th1 and Th2 subpopulation. Naive CD 4 T cells (CD4 + CD45RA +) were cultured in Th1 (left) or Th2 (right) polarization conditions in the presence of pirfenidone or DMSO (control) for 12 days. Afterwards, the cells were analyzed for IFNγ and IL-4 production. Pirfenidone strongly impairs development of CD4+ IL-4-producing cells (Th2 subpopulation). A representative FACS dotplot shows cytokine staining and side scatter and represent at least three different experiments.

4. Discussion

The activated hepatic stellate cell (HSC) is primarily responsible for the increased synthesis and deposition of type I collagen during hepatic fibrosis (Friedman, 2000). TGF-β1 constitutes the most potent profibrogenic cytokine for HSC, capable of inducing its activation and proliferation, and stimulates α1(I) collagen expression via Smad proteins (Friedman, 2000). Despite TGF-β1 stimulus has been recognized primordial to collagen synthesis in liver fibrosis, alternative signaling pathways have been described to regulate collagen expression as well as TGF-β (Aoudjehane et al., 2008; Sugimoto et al., 2005). Profibrogenic effect has been documented in several studies for Th2 cytokines (IL-4 and IL-13). In vitro IL-4 has proved to induce type I collagen expression in skin and conjunctival fibroblast, in the hepatic stellate cell line LI90 and in cultured human intrahepatic myofibroblast (Aoudjehane et al., 2008; Doucet et al., 1998; Liu et al., 2003; Sempowski et al., 1994; Sugimoto et al., 2005). In fact, studies in skin fibroblast suggest that IL-4 is twice more efficient on collagen synthesis than TGF-β (Fertin et al., 1991). IL-4 regulates α1(I) expression by binding and stimulating its transmembrane receptor (IL-4R), involving the Janus kinases (JAK) 1 and JAK-3 and the signal transducer and activator of transcription (STAT)-6 pathway (Aoudjehane et al., 2008).

Here, we found a substantial increase in IL-4 mRNA and protein synthesis that partially explains α1(I) collagen transcript over-expression in TAA group. Besides, it is important to emphasize that in conjunction with IL-4 increment other Th2 phenotype markers such as GATA3 expression, GATA3 translocation and GATA3 DNA binding activity were found magnified in response to TAA intoxication. In conjunction these findings associated suggest Th2 polarization in this liver fibrosis model.

Similar to what has been reported elsewhere in other clinical and experimental liver fibrosis studies (Di Sario et al., 2002; Garcia et al., 2002; Tada et al., 2001), pirfenidone therapy proved to possess a potent anti-fibrotic activity in the experimental model shown here. It is well known that pirfenidone limits type I collagen synthesis because it is a potent anti-TGF-β1 agent (Iyer et al., 1999; Shihab et al., 2002). We observed that pirfenidone is also capable of reducing IL-4, the main Th2 cytokine implicated in fibrogenesis in vivo, and even capable of limiting expression and translocation of GATA3, the principal transcriptional factor implicated in Th2 differentiation. Additionally, pirfenidone was found to impair preferably Th2 cells development in vitro, evidenced by the cytokines produced in polarizing conditions. As showed previously, pirfenidone impairs CD4+ cell proliferation in vitro (Visner et al., 2009), however we demonstrated that pirfenidone affects more severely Th2 generation than Th1.

The mechanisms through pirfenidone limited Th2 response should be clarified in brief, but our observations suggest that it is mediated by p38. Some researches have proposed that pirfenidone declines p38 activation and reduces p38 activity acting like p38 MAPK inhibitor binding competitively to ATP-binding site of p38 MAPK. We showed that the active form of p38 (phosphorylated p38) was reduced with pirfenidone administration. Active p38 is decisive in Th2 differentiation and in Th2 phenotype maintenance (Lu et al., 2001; Owaki et al., 2006; Schafer et al., 1999). Different studies have proven that p38 activity is crucial for Th cytokine expression through regulating mRNA stability of IL-4 and IFN-γ (Dodeller et al., 2005; Guo et al., 2008) and even controlling the T-bet gene transcription and GATA3 nuclear translocation (Jones et al., 2003; Maneechotesuwan et al., 2007). To direct Th2 phenotype, the zinc-finger transcription factor GATA3 has to leave the cytoplasm and translocate into the nucleus to reach Th2 cluster gene. GATA3 nuclear translocation is critical to Th2 cytokine expression. This process is dependent on GATA3 phosphorylation on serine residues by p38 MAPK, which facilitates GATA3 interaction with the nuclear transporter protein Importin-α (Maneechotesuwan et al., 2007). Pirfenidone as inhibitor of p38 activation prevents p38 MAPK activity and in consequence avoids GATA3 phosphorylation, impairs GATA3 nuclear translocation to finally block Th2 cytokine production. This explanation results logic, and agree with our results, but additional experiments should be conducted in order to ratify this hypothesis.

The anti-Th2 effect of pirfenidone is clear, however when we explored the effect of the drug on Th1 response, we found that T-bet translocation and activity were amplified with pirfenidone, though IFN-γ mRNA and protein expression were slightly amplified in response to pirfenidone treatment (statistically non-significant). It is possible that failure to induce IFN-γ by pirfenidone is due to p38 inhibition. Experiments conducted with multiple p38 isoform inhibitors have revealed that IFN-γ gene expression and its mRNA stability are dependent on p38 activity (Mavropoulos et al., 2005). Contrasting effects that we have observed on Th1 and Th2 responses with pirfenidone therapy in experimental liver fibrosis could be the result of inhibition to specific isoform by pirfenidone.

In short, fibrosis and an exacerbated Th2 response coexist in experimental TAA-induced liver fibrosis. These pathologic characteristics are effectively limited by pirfenidone. The broad-spectrum therapeutic activity that pirfenidone has, potentially gives opportunity to use this agent in diseases where inflammation, fibrosis and exacerbated Th2 response are characteristic.

In conclusion, in this study, we demonstrated that pirfenidone impairs Th2 differentiation in vitro, and limits Th2 pro-fibrotic response without affecting the Th1 anti-fibrotic in a Th2 polarized environment in vivo. Our results suggest that anti-Th2 activity of pirfenidone could be p38 mediated and involves regulation of GATA3 expression and translocation.

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