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IL-10 production in non–small cell lung carcinoma patients is regulated by ERK, P38 and COX-2

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Abstract

Immune dysfunction is hallmark of patients with non–small cell lung carcinoma (NSCLC). The molecular mechanism involved in COX-2– and PGE2-mediated production of immunosuppressive cytokine IL-10 is not well-understood. Our study addresses the involvement of T cell downstream signalling intermediates, cytokines (IL-10 and IFN-γ) and their transcription factors (T-bet and GATA-3) in COX-2–mediated regulation of lymphocyte functions in NSCLC patients. In comparison to healthy individual, a marked decrease in lymphocyte proliferation to anti-CD3 MAb was observed in NSCLC patients by thymidine incorporation assay. Using flow cytometry, decrease in intracellular calcium release with increase in reactive oxygen species was observed in lymphocytes of NSCLC patients. These patients showed increased IL-10 and PGE2 with reduced IFN-γ production by ELISA. Results demonstrated defect in regulation of transcription factors T-bet and GATA-3 as analysed by Western blotting (WB), immunoprecipitation and EMSA. Overexpression of p-p38, p-ERK and COX-2 were observed with diminished p-JNK by WB. IL-10/IFN-γ levels were found to be differentially regulated *via* p38 and ERK mitogen-activated protein kinase (MAPK) pathways in cooperation with COX-2. Inhibition of these pathways using selective inhibitors lead to increased lymphocyte proliferative response to anti-CD3 MAb and IFN-γ production with decrease in IL-10 production. Studies showed involvement of ERK, p38 and COX-2 pathways in high IL-10 production, driven by lung tumour derived PGE2. The selective COX-2 inhibitor rofecoxib showed ability to alter the cytokine balance by affecting regulation of T-bet and GATA-3 transcription factors.

Keywords: non–small cell lung carcinoma • cyclooxygenase-2 • prostaglandin E2 • rofecoxib • lymphocytes • IFN-γ • interleukin-10 • T-bet • GATA-3 • MAPK pathways

Introduction

Tumour-induced immune suppression has been well-documented in several malignancies including non–small cell lung carcinoma (NSCLC) [1–2]. In patients with NSCLC, lung cancer cells are known to elaborate immunosuppressive mediators including type 2 cytokines (IL-10, IL-4 and IL-13), PGE2 and TGF-β that may act as inhibitors of anti-tumour directed cell-mediated immune responses [3–5]. PGE2 can inhibit apoptosis and increase proliferation; mobility and metastatic potential of tumour cells and can contribute to angiogenesis and invasion of tumour cells [6, 7]. PGE2 appears to play an important role in pro-inflammation [8]. PGE2 has also been found to have an effect on cellular functions of monocytes, dendritic cells and lymphocytes leading to suppression of anti-tumour activities in these patients [1, 9]. In addition, PGE2 have the ability to modulate immune responses and acts as immune suppressor [10, 11]. Type 2 cytokines (IL-4, IL-10 and IL-13) negatively regulate COX-2 and PGE2 production in normal and tumour cells [12].

In NSCLC, IL-10 overproduction at the tumour site has been implicated in tumour-mediated immunosuppression [13, 14], enhanced angiogenesis [15] and serum IL-10 appears to be an indicator of poor prognosis [4, 5]. Also the lung tumour derived PGE2 in these patients is known to trigger the synthesis of IL-10 from host immune cells [3].

COX-2 and PGE2 have been reported to play a pivotal role in IL-10 production in NSCLC patients. However, the molecular

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mechanism involved in COX-2–mediated regulation of IL-10 production and lymphocyte function is not well-understood. Lymphocyte activation and cytokine production involves tight regulation of different mitogen-activated protein kinases (MAPKs), which get activated on T cell receptor (TCR) activation. COX-1 and -2 are identified as critical regulators of these signalling cascades [16] but the molecular mechanism behind regulation of cytokine production and lymphocyte functions by COX-1 and -2 is not well-understood.

Our study focuses on understanding the mechanisms of PGE2-mediated immune suppression and regulation of IL-10 production in lymphocytes of NSCLC patients. We demonstrate that COX-2 inhibitor; rofecoxib can restore the lymphocyte functions and cytokine balance in these patients by regulating the transcription factors GATA-3 and T-bet. Further, we have demonstrated the involvement of reactive oxygen species (ROS) and MAPK pathways in PGE2-mediated IL-10 production in NSCLC patients. Understanding these molecular events will help in designing specific targeted therapies in these patients.

Materials and methods

Study group

Peripheral blood samples from lung cancer patients, clinically and histologically diagnosed for NSCLC ($n = 75$) were collected before any treatment from out patients department (OPD) of Tata Memorial Hospital after obtaining ethical consent as per the norms laid down by ethical committee of Tata Memorial Centre. All patients were staged according to the TNM system of classification and patients in all the four stages (stages I–IV) were included in the study. As controls, blood samples were collected from age and sex-matched healthy individuals (HI, $n = 58$).

Inhibitors

COX-2 selective inhibitor rofecoxib (Sigma-Aldrich, St. Louis, MO, USA), ERK Inhibitor (PD-098059; Sigma-Aldrich), p38 Inhibitor (SB-203580; Sigma-Aldrich) and JNK Inhibitor (SP-600125; Sigma-Aldrich) were used. An appropriate stock solution was prepared in dimethyl sulfoxide (Sigma-Aldrich) and filter sterilized before use. The concentration of rofecoxib was titrated and 100 μM was selected as optimum concentration for all experiments. One microgram of MAPK inhibitors was used for proliferation and cytokine experiments.

Antibodies

Antibodies used in the studies were purified human anti-CD3 monoclonal antibody (Sigma-Aldrich), purified monoclonal antibodies against human T-bet and GATA-3 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), antimouse monoclonal antibody conjugated to horseradish peroxidase (HRPO) (Sigma-Aldrich), purified monoclonal antibodies against human actin, p-p38, p-ERK, COX-2 (Santa Cruz), p-JNK, HSP-90 (BD Biosciences, San Diego, CA, USA).

Separation of lymphocytes from peripheral blood

Lymphocytes were separated from peripheral blood of NSCLC patients and HI by Ficoll Hypaque (FH; Sigma-Aldrich) density gradient centrifugation. The viability obtained was >98% as tested by erythrosine B dye.

Lymphocyte proliferation assay

Proliferative responses in peripheral blood lymphocytes (PBL) of NSCLC patients and HI were analysed using 72-hr tritiated thymidine incorporation assay. PBL (1.5×10^5) were stimulated with 1 μg plate-coated anti-CD3 MAb for 72 hrs at 37°C in 96-well plates (Nunc, Denmark). As controls, lymphocytes in medium alone (RPMI-1640 + 10%FCS) were incubated at 37°C for 72 hrs. 0.5 μCi/10 μl/well tritiated thymidine (specific activity 240 GBq/mmol; Board of Radiation and Isotype Technology, India) was added during the last 18 hrs of the assay. The cells were harvested onto glass-fibre filter paper (Titertek, Norway) using a cell harvester (Titertek) and tritiated thymidine incorporation was measured in a liquid β scintillation counter (Model 1900; Packard, USA) as counts per minute (cpm). The proliferation assay was performed in triplicate sets, and data were expressed as mean cpm. Stimulation index (S.I.) was calculated as the ratio of mean cpm of cultures stimulated with anti-CD3 MAb and unstimulated cultures.

For treatment with COX-2 inhibitor (rofecoxib) and MAPKs inhibitors, PBL (1.5×10^5/200 μl/well) of NSCLC patients were stimulated with 1 μg plate-coated anti-CD3 MAb in presence of inhibitors in the above assay. As controls, unstimulated and PBL stimulated with anti-CD3 MAb without any inhibitors were used. The concentration of inhibitors used were COX-2–specific inhibitor (rofecoxib; 100 μM/well), ERK Inhibitor (PD-098059; 1 μg/well), p38 Inhibitor (SB-203580; 1 μg/well) and JNK Inhibitor (SP-600125; 1 μg/well).

Flow cytometry

PBL from HI and NSCLC were stimulated with COX-2 inhibitor (rofecoxib) for 48 hrs. After incubation, unstimulated and stimulated PBL (1×10^6 cells/ml) were suspended in PBS containing 2% FCS (Invitrogen, La Jolla, CA, USA) and sodium azide (Sigma-Aldrich). Cells were further incubated with fluorochrome labelled mouse anti-human MAb CD3PE, CD4PE, CD8PE, CD56PE, CD14PE and CD19PE (BD Biosciences) for surface staining.

Detection of T regulatory cells was performed by using four colour flow cytometric analysis. Cells were incubated with CD4 Alexa Fluor700, CD25PE and CD127APC MAb (BD Biosciences) at 4°C for 45 min. Cells were washed and fixed with 1% paraformaldehyde followed by permeabilization with 1% saponin (Sigma-Aldrich) for 20 min. Cells were washed and incubated with anti-FoxP3 FITC MAb (e Biosciences, USA) for 30 min in dark. Appropriate isotype controls were used in all experiments. A minimum of 10,000 events were acquired on FACS Aria flow cytometer (BD Biosciences). The data were analysed with FACS Diva software.

Estimation of intracellular calcium release

PBL (1×10^6/1 ml 0.01M PBS) of NSCLC patients and HI were loaded with 5 μM Fluo-3-AM (Sigma-Aldrich) for 30 min. at 37°C. PBL were washed with 0.01M PBS to remove excess dye and acquired on the FACS Calibur flow cytometer (Becton Dickinson, Mountain View, USA) for the determination of basal level of fluorescence in unstimulated cells. One micrograms of anti-CD3 MAb was added as stimulant and fluorescence intensity was

measured immediately at 0 sec. The cells were acquired at pre-determined time (0–330 sec.) from the time of the addition of the stimulant. The increase in mean fluorescence intensity (MFI) was measured in seconds. Calibration of fluo-3 fluorescence into absolute intracellular calcium was made by a method using calcium ionophore A 23187 (10 μM, 10 μl; Sigma-Aldrich) and manganese chloride (2 mM, 5 μl; Qualigens, India) [17, 18].

To analyse the effect of rofecoxib on intracellular calcium release PBL (1×10^6/1 ml 0.01M PBS) of NSCLC patients were loaded with Fluo-3-AM (5 μM) dye. PBL were washed with 0.01M PBS and incubated with rofecoxib (100 μM, 200 μM, 400 μM) for 15 min. at 37°C. After incubation PBL were immediately acquired on the flow cytometer for the determination of calcium released as described earlier.

Measurement of intracellular reactive oxygen species in lymphocytes

Reactive oxygen species (ROS) level in PBL was measured using 2′, 7′ dichloro-fluorescein diacetate (DCFH-DA) dye (Sigma-Aldrich) [17]. PBL (1×10^6/250 μl 0.01M PBS) were loaded with 4 μM dye and incubated for 30 min. at 37°C. PBL were washed to remove all extracellular dye. PBL were then acquired on the FACS Calibur flow cytometer (Becton Dickinson) for the determination of basal level of fluorescence in unstimulated cells. One micrograms anti-CD3 MAb was added as stimulant and increase in MFI was measured from 0 to 45 min. Analysis was performed using Cell Quest software and results were expressed as MFI as described [17].

To analyse the effect of COX inhibitors on intracellular ROS generation PBL of NSCLC patients were loaded with DCFH-DA dye as described earlier. PBL were washed with 0.01M PBS and incubated with rofecoxib for 15 min. at 37°C. After incubation, PBL were immediately acquired on the FACS Calibur flow-cytometer (Becton Dickinson) and generation of ROS on addition of 1 μg anti-CD3 MAb was determined at different time points (0–45 min).

Cytokine and PGE2 ELISA

PBL (1.5×10^5/200 μl cRPMI/well) were stimulated with 1 μg anti-CD3 MAb for 48 hrs at 37°C. After incubation, supernatants were collected and analysed for secreted IL-10 and IFN-γ using Opt-EIA sandwich ELISA kit (BD Biosciences) as per the manufacturer's instructions. Serum samples from HI and NSCLC patients were analysed for IL-10 and IFN-γ using same kits. PGE2 production was measured using the PGE2 screening assay kit (Cayman Chemicals, Ann Arbor, MI, USA) as per the manufacturer's instructions.

PBL were incubated with COX-2, ERK, p38 and JNK inhibitors and stimulated with 1 μg anti-CD3 MAb for 48 hrs at 37°C. After stimulation, supernatants were collected and cytokines (IL-10, IFN-γ) and PGE2 were quantitated.

Reverse transcription polymerase chain reaction

Total RNA was extracted from PBL of NSCLC patients using TRIZOL Reagent (Invitrogen) according to manufacturer's protocol. cDNA was synthesized in 20 μl reaction volume using reverse transcription by random primer method. cDNA was further amplified by using primers specific for human COX-2 and GAPDH. The product was visualized by agarose gel electrophoresis and ethidium bromide staining.

Preparation of nuclear extracts

For detection of T-bet and GATA-3, nuclear extracts were prepared from PBL of HI and NSCLC patients stimulated with anti-CD3 MAb for 48 hrs. Briefly, PBL (15×10^6) were washed with 0.01M PBS twice and incubated with 1.2 ml of buffer-A (10 mM HEPES, 10 mM KCl, 2 mM $MgCl_2$, 1 mM DTT, 0.1 mM PMSF, 2 μg/ml aprotinine; Sigma-Aldrich) on ice for 15 min. After 15 min. 75 μl of 10%NP-40 was added and vortexed for 10 sec. PBL were centrifuged at 13,000 rpm for 1 min. at 4°C. The pellet was collected and incubated with 200 μl of buffer-C (50 mM HEPES, 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 0.1 mM PMSF, 10% glycerol, 2 μg/ml aprotinine, 2 μg/ml leupeptine, 1 μg/ml pepstatin; Sigma-Aldrich) for 15 min. on ice. Supernatant containing nuclear protein was collected by centrifuging at 13,000 rpm for 5 min. at 4°C. Protein estimation was performed using Bradford reagent (Sigma-Aldrich).

Immunoprecipitation (IP) and Western blotting (WB) for T-bet and GATA-3

Anti-human T-bet and GATA-3 monoclonal antibodies conjugated sepharose A beads were prepared and used for immunoprecipitation of T-bet and GATA-3 from nuclear extract. Separated antibody–antigen complexes were loaded on 12.5% denaturing polyacrylamide gel and WB was performed using primary purified monoclonal antibodies against human T-bet and GATA-3. Secondary antibody conjugated to HRPO was used to probe primary antibodies. Specific proteins were detected by enhanced chemiluminescence plus detection kit (ECL-plus; Amersham, Buckinghamshire, UK) as per manufacturer's instructions.

Electrophoretic mobility shift assay (EMSA)

Synthetic single-stranded oligonucleotides DNA sequences containing consensus binding sites for T-bet and GATA-3 were obtained from (IDT Inc., USA). The sequences for T-bet and GATA-3 oligonucleotides were 5′-AAT TTC ACA CCT AGG TGT GAA ATT-3′ and 5′-CTC TTA ACT GAT AAA GAA AAT-3′, respectively. Oligonucleotides were annealed and end labelled with γ-[^{32}P]-ATP 1 μl (from stock of 10 mCi/ml; BRIT, India) using bacteriophage T4 polynucleotide kinase (20 μl/reaction; Sigma-Aldrich), 5× kinase buffer (0.25M Tris pH-7.4, 0.05M $MgCl_2$, 0.25M DTT; Sigma-Aldrich) and water to make final reaction volume to 20 μl. Labelled sequences were purified using probequant^{TM} G-50 microcolumns (Amersham). For binding reaction (20 μl), end labelled DNA probe (0.4 pm), 2 μg nuclear extract and 5× binding buffer (10 mM HEPES pH 7.9, 50 mM KCl, 1 mM DTT, 10% glycerol, BSA 330 μg/ml) were mixed and incubated for 30 min. at room temperature and then loaded on 4% native polyacrylamide gel. After electrophoresis gel was dried and exposed to Kodak intensifying hyper films at −80°C overnight.

Preparation of total cell lysate

PBL were separated from HI and NSCLC patients and cell lysates were prepared using lysis buffer (50 mM Tris, 150 mM NaCl, 1 mM EGTA pH 7.4, 1.5 mM $MgCl_2$, 10% glycerol, 1% Triton X100, 10 μg/ml aprotinine, 10 μg/ml leupeptine, 1 mM PMSF; Sigma-Aldrich). Briefly PBL (10×10^6) were collected and washed with ice cold 0.01M PBS. PBL were incubated with lysis buffer for 45 min. on ice. After incubation PBL were sonicated. Supernatant containing proteins were collected after centrifuging at 14,000 rpm for 10 min. Protein estimation was performed by using Bradford reagent and then subjected to SDS-PAGE.

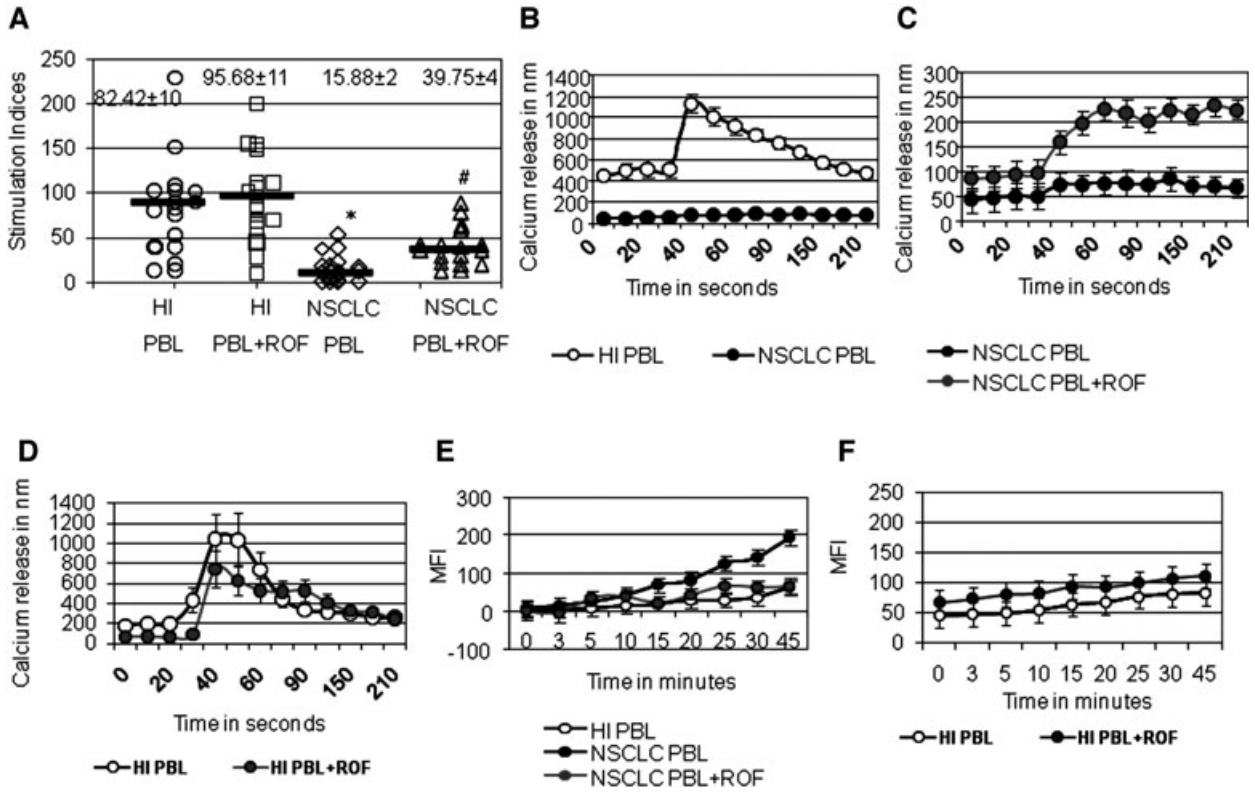

Fig. 1 (**A**) Proliferative response on anti-CD3 MAb stimulation in NSCLC patients ($n = 17$) compared to HI ($n = 17$) before and after treatment with rofecoxib. This figure represents stimulation indices (SI) from each individual with black bar indicating mean±SE value. $^{*}P \leq 0.05$ when results of HI PBL were compared with NSCLC PBL. $^{\#}P \leq 0.05$ when results of NSCLC PBL were compared with NSCLC PBL+ROF. (**B**) Intracellular calcium release in PBL of NSCLC patients ($n = 5$) compared to HI ($n = 5$) on anti-CD3 MAb stimulation. This figure represents intracellular calcium content (nm) at different time intervals after stimulation. (**C**) Intracellular calcium levels in PBL of NSCLC patients ($n = 5$) on anti-CD3 MAb stimulation before and after treatment with rofecoxib (ROF). This graph represents mean values at each time point for each group. (**D**) Intracellular calcium levels in PBL of HI ($n = 3$) on anti-CD3 MAb stimulation before and after treatment with rofecoxib. This graph represents mean values at each time interval for each group. (**E**) ROS generation from untreated PBL of HI ($n = 6$) and untreated and rofecoxib-treated PBL of NSCLC patients ($n = 6$) on stimulation with anti-CD3 MAb at different time intervals. This figure represents MFI values. (**F**) ROS generation from untreated and rofecoxib-treated PBL of HI ($n = 6$) on stimulation with anti-CD3 MAb at different time intervals. This figure represents MFI values.

Western blotting

PBL were separated from blood of HI and NSCLC patients and cell lysates and nuclear extracts were prepared as described before. Cell lysates were separated on 12.5% denaturing polyacrylamide gel, electro blotted to nitrocellulose membrane (Amersham). Proteins were probed using primary antibodies against human actin, p-p38, p-ERK1/2, p-JNK1/2, COX-2, HSP-90. Secondary antibody conjugated to HRPO was used to probe primary antibodies. Specific proteins were detected by enhanced chemiluminescence plus detection kit (ECL-plus; Amersham) as per manufacturer's instructions.

Statistical analysis

Statistical significant difference between HI and NSCLC patients and between untreated and inhibitors treated PBL of NSCLC patients were calculated using Wilcoxin signed rank test and Student's *t*-test.

Results

Impaired functional ability of PBL from NSCLC patients was restored by rofecoxib

To assess the functional status of lymphocytes of NSCLC patients, proliferative responses of PBL to anti-CD3 MAb were analysed. PBL of NSCLC patients and HI were stimulated with anti-CD3 MAb in presence of rofecoxib a COX-2 inhibitor to analyse the involvement of lymphocyte expressed COX-2 in immune dysfunction of NSCLC patients. A significant reduction in proliferative response of PBL to anti-CD3 MAb was observed in NSCLC patients compared to HI (Fig. 1A). The reduced proliferative responses of NSCLC patients were found to be restored significantly on treatment of PBL with rofecoxib (Fig. 1A). Proliferative responses of

Table 1 Subset analysis in PBL of HI and NSCLC patients before and after treatment with rofecoxib

	Mean% positive cells ± SE			
	HI PBL	**HI PBL+ROF**	**NSCLC PBL**	**NSCLC PBL+ROF**
$CD3^+$	73.50 ± 6.06	75.30 ± 6.12	54.00 ± 8.47*	60.77 ± 0.57
$CD4^+$	36.77 ± 2.11	36.70 ± 1.47	27.00 ± 3.47*	26.65 ± 4.85
$CD8^+$	47.47 ± 4.55	50.57 ± 4.49	25.49 ± 0.79*	27.91 ± 7.91
$CD56^+$	32.90 ± 5.49	35.65 ± 1.48	27.50 ± 8.93	26.21 ± 9.68
$CD14^+$	9.43 ± 3.61	11.16 ± 3.78	2.95 ± 0.81*	2.90 ± 0.75
$CD19^+$	10.80 ± 2.82	12.83 ± 2.76	14.41 ± 10.13	12.29 ± 8.57

*$P \leq 0.05$ compared to HI PBL; HI ($n = 5$) and NSCLC patients ($n = 5$).

PBL to anti-CD3 MAb were compared in paired samples of NSCLC patients and HI (Fig. S1). The results demonstrate a marked increase in lymphocyte proliferation in response to anti-CD3 stimulation after treatment of PBL with rofecoxib. The increase in proliferative response was clearly evident in majority of NSCLC patients after rofecoxib treatment compared to untreated PBL of NSCLC patients. Immunophenotyping of PBL of NSCLC patients and HI showed a significant decrease in CD3, CD4, CD8 and CD14 markers in NSCLC compared to HI (Table 1).

Interestingly, the level of regulatory T cells ($CD4^+CD25^+CD127^{low/-}$) were significantly increased in NSCLC patients (13.16 ± 2.32) compared to HI (6.33 ± 0.76) (Table 2). FoxP3 expression was high in regulatory T cells of HI and NSCLC patients before and after treatment with rofecoxib (ranged from 86.9% to 90.1%). On treatment with rofecoxib, significant decrease in regulatory T cells was observed in NSCLC patients ($P \leq 0.05$). In contrast, the levels of regulatory T cells remained unchanged in HI after rofecoxib treatment (Table 2).

To analyse involvement of COX-2 in signalling events associated with TCR engagement, the intracellular calcium flux was monitored in PBL of NSCLC patients on anti-CD3 MAb stimulation in presence and absence of rofecoxib (100 μM). A rapid and sustained increase in intracellular calcium flux was observed in lymphocytes of HI whereas a decreased mobilization of intracellular calcium was observed in PBL of NSCLC patients (Fig. 1B). Treatment of PBL of NSCLC patients with rofecoxib caused a marked increase in intracellular calcium flux (Fig. 1C). Increasing the rofecoxib concentration (200 and 400 μM) and the time up to 330 sec. did not further increase the intracellular calcium flux in PBL of NSCLC patients (Fig. S2). Although maximum increase in calcium flux was seen with 100 μM of rofecoxib in NSCLC patients, the level remained low compared to that observed with HI PBL after rofecoxib treatment (Fig. 1B and C). In contrast, the intracellular calcium flux was slightly decreased in HI PBL after treatment with rofecoxib (Fig. 1D) indicating that rofecoxib has a selective effect (increase) on intracellular calcium flux of NSCLC PBL compared to HI.

Table 2 Circulatory $CD4^+$ regulatory T cells in HI and NSCLC patients before and after treatment with rofecoxib

Groups	**Mean% positive cells ± SE**	
	Gated on $CD4^+$	**Gated on $CD4^+CD25^+CD127^{low/-}$**
	$CD4^+CD25^+CD127^{low/-}$	**Foxp3+**
HI PBL	6.33 ± 0.76	88.10 ± 4.46
HI PBL+ROF	5.80 ± 0.40	88.90 ± 3.47
NSCLC PBL	13.16 ± 2.32*	90.10 ± 7.26
NSCLC PBL+ROF	9.27 ± 3.27†	86.97 ± 9.12

*$P \leq 0.05$ compared to HI PBL; HI ($n = 5$). †$P \leq 0.05$ compared to NSCLC PBL; NSCLC patients ($n = 5$).

On anti-CD3 MAb stimulation, about twofold increase in ROS generation was also observed in PBL of NSCLC patients compared to HI (Fig. 1E). However, rofecoxib treatment of PBL caused a significant reduction in ROS generation (Fig. 1E). In HI PBL, rofecoxib did not caused a major change in ROS generation as was observed with NSCLC PBL on treatment with rofecoxib (Fig. 1F). Our data suggest that lymphocyte expressed COX-2 contributes to immune hyporesponsiveness in patients with NSCLC. Rofecoxib, selective COX-2 inhibitor is able to reverse the observed hyporesponsiveness in these patients.

NSCLC patients showed high IL-10 production and GATA-3 expression in PBL

The production of IL-10 and IFN-γ is regulated by predominant transcription factors GATA-3 and T-bet, respectively [19, 20]. To

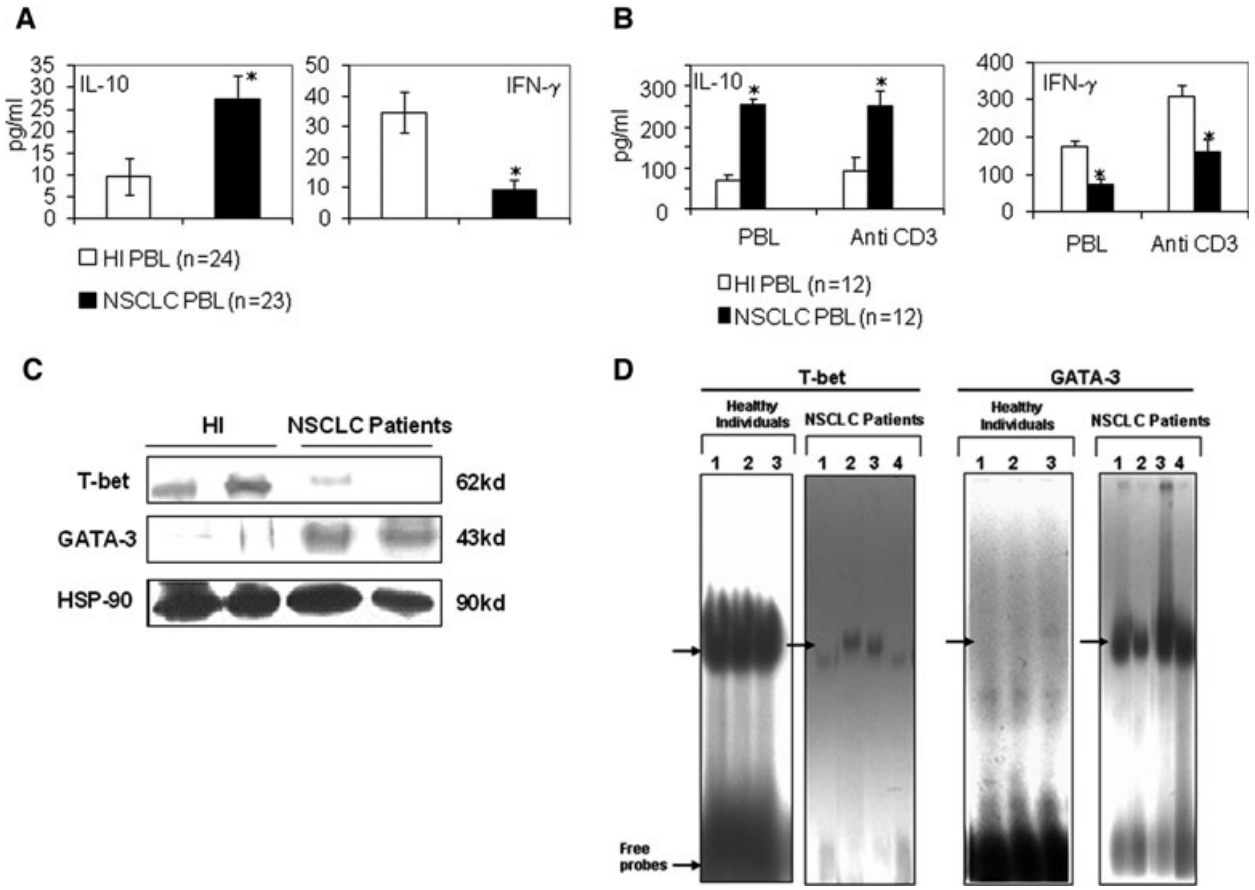

Fig. 2 (**A**) Comparison of IL-10 and IFN-γ (pg/ml) from sera of NSCLC patients ($n = 23$) and HI ($n = 24$). (**B**) IL-10 and IFN-γ (pg/ml) from 48 hrs cultured supernatants of PBL of NSCLC patients ($n = 12$) and HI ($n = 12$) before and after stimulation with anti-CD3 MAb. (**C**) Representative figures for IP and WB for T-bet and GATA-3 from nuclear extracts of anti-CD3 MAb-stimulated PBL of NSCLC patients ($n = 2$) and HI ($n = 2$). HSP-90 was used as internal control for nuclear extract. (**D**) EMSA for T-bet and GATA-3 from nuclear extracts of anti-CD3 MAb-stimulated PBL of NSCLC patients ($n = 4$) and HI ($n = 3$). Arrow in middle indicates binding of transcription factors to DNA sequences. $^*P \leq 0.05$ when results of HI PBL were compared with NSCLC PBL.

study the defects in cytokine regulation, we analysed cytokines IL-10 and IFN-γ in serum and in supernatant of anti-CD3 MAb-stimulated lymphocytes of HI and NSCLC patients. Serum analysis for IL-10 and IFN-γ showed significant high levels of IL-10 and reduced IFN-γ in NSCLC patients (Fig. 2A). Mean values of IL-10 in culture supernatants of unstimulated PBL of HI and NSCLC patients was 69 ± 15 pg/ml and 250 ± 12 pg/ml, respectively (Fig. 2B). Stimulation of PBL of HI and NSCLC patients with anti-CD3 MAb changed IL-10 levels in culture supernatants to 93 ± 30 pg/ml and 231 ± 35 pg/ml, respectively (Fig. 2B). On the other hand, culture supernatants of unstimulated PBL of HI and NSCLC patients showed mean IFN-γ levels of 176 ± 16 pg/ml and 74 ± 16 pg/ml, respectively (Fig. 2B). After stimulation with anti-CD3 MAb, IFN-γ levels in culture supernatants of PBL of HI and NSCLC patients increased to 309 ± 38 pg/ml and 160 ± 25 pg/ml, respectively (Fig. 2B). Thus, supernatants from unstimulated and anti-CD3 MAb stimulated PBL of NSCLC patients showed increased levels of IL-10 with significantly reduced IFN-γ compared to HI (Fig. 2B).

IP and WB of nuclear extracts from PBL of NSCLC patients showed a marked increase in GATA-3 with reduced T-bet expression compared to HI (Fig. 2C). On the contrary T-bet expression was high compared to GATA-3 in PBL of HI (Fig. 2C). Expressions of T-bet and GATA-3 were further confirmed by evaluating DNA binding ability of these transcription factors using specific consensus sequences. High DNA binding activity of GATA-3 with low binding of T-bet was observed in nuclear extract from PBL of NSCLC patients (Fig. 2D). In HI, high DNA binding of T-bet was observed with low binding of GATA-3 to the target sequence. The results of EMSA corroborated our data of T-bet and GATA-3 expression in PBL of NSCLC patients obtained with IP and WB (Fig. 2C and D). The specificity of T-bet and GATA-3 binding to DNA sequences was confirmed by competition EMSA. The binding

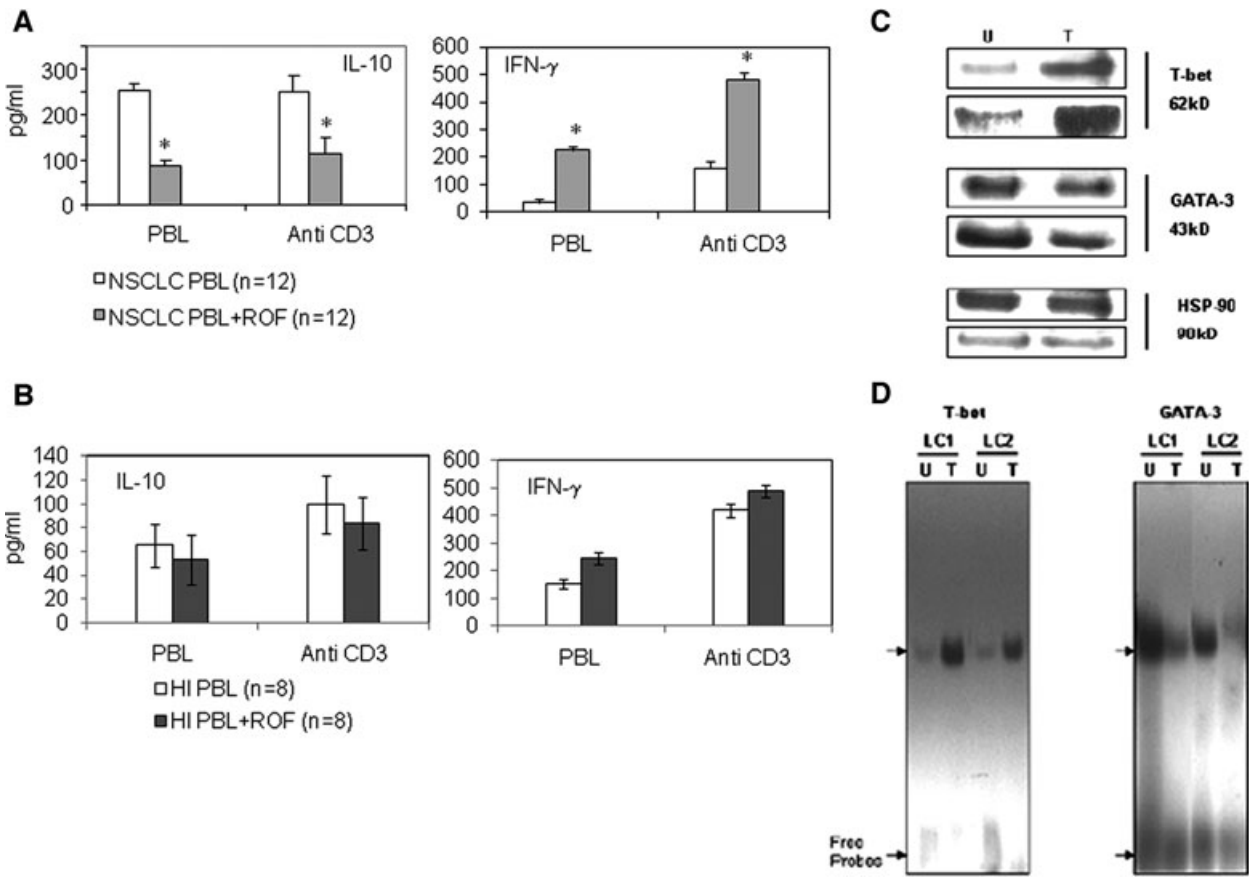

Fig. 3 (**A**) IL-10 and IFN-γ (pg/ml) from 48 hrs cultured supernatants of anti-CD3 MAb-stimulated and -unstimulated PBL of NSCLC patients ($n = 12$), before and after treatment with rofecoxib $^*P \leq 0.05$ when results of NSCLC PBL were compared with NSCLC PBL+ROF. (**B**) IL-10 and IFN-γ (pg/ml) from 48 hrs supernatants of anti-CD3 MAb-stimulated and -unstimulated PBL of HI ($n = 8$), before and after treatment with rofecoxib. (**C**) IP and WB for T-bet and GATA-3 from nuclear extracts of anti-CD3 MAb-stimulated PBL of NSCLC patients ($n = 5$) before (U) and after (T) treatment with rofecoxib (ROF). (**D**) Analysis of DNA binding activity of T-bet and GATA-3 to their specific consensus DNA sequences by EMSA from rofecoxib-treated and -untreated PBL of NSCLC patients. Representative figures with arrow in middle indicate binding of transcription factors to DNA sequences.

of labelled DNA-protein complexes was competed by specific consensus unlabelled sequences of T-bet or GATA-3, respectively (data not shown).

The results indicate that lymphocytes of NSCLC patients showed high GATA-3 activity with diminished T-bet which explains the high IL-10 secretion in these patients.

Rofecoxib induces T-bet expression and IFN-γ production in NSCLC patients

Our earlier data demonstrated restoration of functional ability of PBL of NSCLC patients by rofecoxib (Fig. 1). We therefore analysed effect of rofecoxib on IL-10 and IFN-γ production by these lymphocytes. PBL of NSCLC patients were stimulated with anti-CD3 MAb in presence of rofecoxib and culture supernatants were analysed for IL-10 and IFN-γ production. Treatment of PBL of NSCLC patients with rofecoxib resulted in a significant decrease in IL-10 with concomitant increase in IFN-γ production (Fig. 3A). In HI, significant increase was observed in IFN-γ production in supernatant of anti-CD3–stimulated PBL in presence and absence of rofecoxib (Fig. 3B). It was observed that irrespective of the activation status of PBL, IFN-γ levels in HI increased marginally after treatment with rofecoxib. Similarly, no significant decrease in IL-10 was noted in HI PBL (unstimulated or stimulated) after treatment with rofecoxib (Fig. 3B). These results are in marked contrast to the effect of rofecoxib observed in NSCLC patients (Fig. 3A).

We further analysed the expression and DNA binding activity of T-bet and GATA-3 in PBL of NSCLC patients after treatment with rofecoxib. A marked increase in protein expression of T-bet with decreased GATA-3 was observed (by IP and WB) in nuclear proteins of rofecoxib-treated PBL stimulated with anti-CD3 MAb compared to untreated PBL (Fig. 3C). Nuclear extracts from anti-CD3 MAb-stimulated and rofecoxib-treated PBL of NSCLC patients also

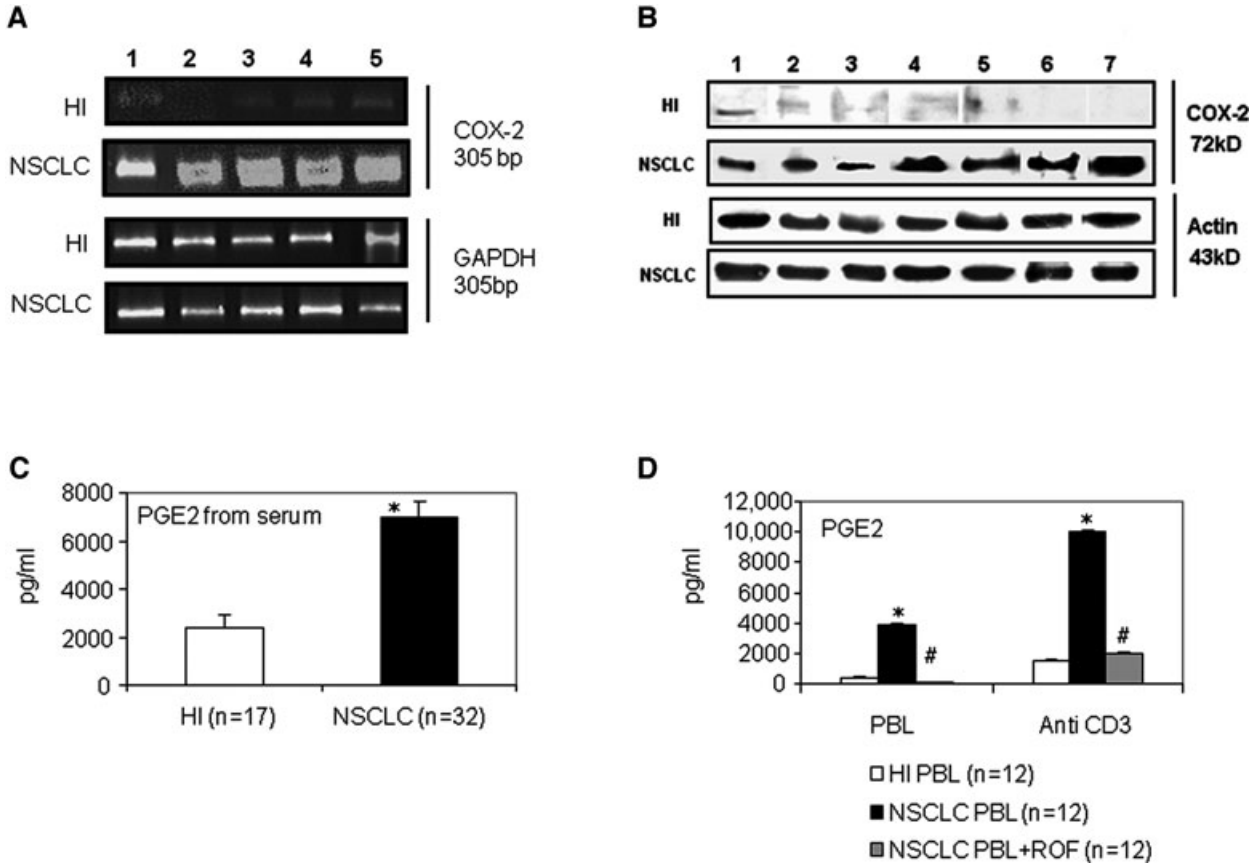

Fig. 4 (**A**) Reverse transcriptase-polymerase chain reaction (RT-PCR) for mRNA expression of COX-2 from unstimulated PBL of NSCLC patients ($n = 5$) and HI ($n = 5$). Expression of GAPDH was used as internal PCR control. (**B**) WB analysis of cell lysate from unstimulated PBL of NSCLC patients ($n = 7$) and HI ($n = 7$). Actin was used as internal control. (**C**) Levels of PGE2 (pg/ml) from sera of NSCLC patients ($n = 32$) and HI ($n = 17$). This figure represents mean value of PGE2 for each group. (**D**) PGE2 levels (pg/ml) from cultured supernatants of unstimulated and anti-CD3 MAb-stimulated PBL of HI ($n = 12$) and rofecoxib (ROF) treated and untreated NSCLC patients ($n = 12$). $^{*}P \leq 0.01$ when results of NSCLC PBL were compared with HI PBL. $^{\#}P \leq 0.01$ when results of NSCLC PBL were compared with NSCLC PBL+ROF.

showed increased binding of T-bet but decrease in GATA-3 binding to their target sequences (Fig. 3D). Our data reveals that rofecoxib treatment leads to significant induction of T-bet in NSCLC patients which helps in IFN-γ production.

PBL of NSCLC patients showed increased expression of COX-2 and PGE2

COX-1 and COX-2 play an important role in lymphocyte activation and function [16]. The increase in lymphocyte activation with restoration of cytokine balance (high IFN-γ and decrease IL-10) observed on rofecoxib treatment in NSCLC patients indicates involvement of COX-2 (Figs 1 and 3). Overexpression of COX-2 mRNA and protein was observed in PBL of NSCLC patients compared to HI (Fig. 4A and B). We further analysed level of PGE2 in serum and culture supernatants of PBL as a read out for functional measurement of COX-2 activity in HI and NSCLC patients. A marked increase in serum PGE2 levels was observed in NSCLC patients compared to HI (Fig. 4C). Unstimulated and anti-CD3 MAb-stimulated PBL of NSCLC patients showed high levels of PGE2 production which were significantly reduced on treatment with rofecoxib (Fig. 4D). Our data suggest that increased IL-10 production in NSCLC patients may be the result of increased PGE2 production due to overexpression of COX-2 in PBL of NSCLC patients.

Altered expression of signalling intermediates in PBL of NSCLC patients

Although PGE2 is known to induce IL-10, the exact mechanism is not well-understood. To investigate the involvement of specific molecular pathways underlying high IL-10 production by NSCLC

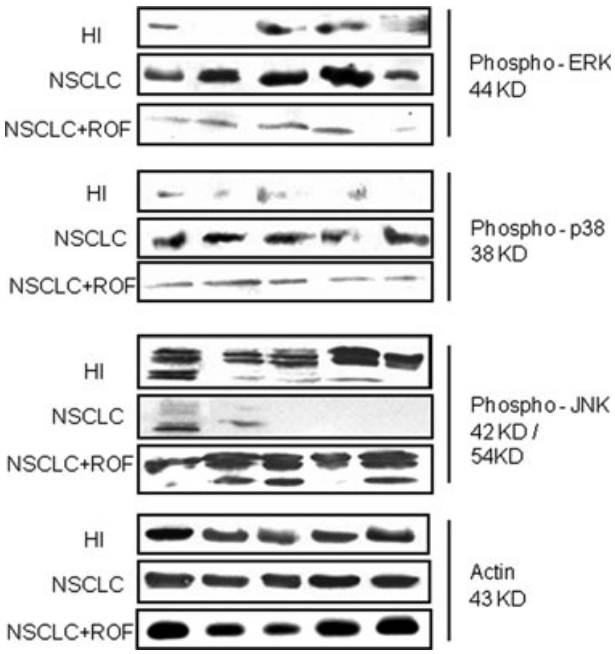

Fig. 5 WB analysis for detection of p-ERK, p-p38, p-JNK and actin from unstimulated PBL of HI ($n = 5$) and NSCLC patients ($n = 5$) before and after treatment with rofecoxib.

patients, we analysed the expression of active forms of three crucial signalling molecules, phosphorylated-ERK (p-ERK), p38 (p-p38) and JNK (p-JNK) of MAPK pathways in PBL of NSCLC patients as compared to HI. WB of lysates of PBL from NSCLC patients and HI with specific MAb demonstrated that the expression of p-ERK and p-p38 was high whereas that of p-JNK was low in PBL of NSCLC patients (Fig. 5). On treatment with rofecoxib PBL of NSCLC patients showed marked decrease in p-ERK and p-p38 with increase in p-JNK expression compared to untreated PBL of NSCLC patients (Fig. 5). Thus, the data indicate that p-ERK and p-p38 pathways may be involved in high IL-10 production by PBL of NSCLC patients.

PGE2 *via* p38 and ERK MAPK pathway regulates the high IL-10 production in NSCLC patients

To substantiate the involvement of ERK and p38 pathways in increased IL-10 production *via* PGE2 observed in NSCLC patients, we used specific inhibitors for p38 (SB-203580), ERK (PD-98059), JNK (SP-600125) and COX-2 (rofecoxib). PBL of NSCLC patients were stimulated with anti-CD3 MAb in presence and absence of these inhibitors and the supernatants of cultured PBL were analysed for IL-10, IFN-γ and PGE2 production. The proliferation of PBL in the presence of these inhibitors was simultaneously monitored. We also used the combinations of MAPK inhibitors with rofecoxib to understand the cooperation of MAPK pathways with COX-2.

A significant increase in proliferative response to anti-CD3 MAb was observed when PBL were treated with PD-98059 (ERK inhibitor), SB-203580 (p38 inhibitor) and rofecoxib compared to untreated PBL and anti-CD3 MAb-stimulated PBL of NSCLC patients (Fig. 6A). Further, when PBL of NSCLC patients were treated with combinations of PD-98059 with rofecoxib and SB-203580 with rofecoxib, a significant increase in proliferative response to anti-CD3 MAb was observed in PBL of NSCLC patients compared to PBL treated with rofecoxib alone (Fig. 6A). However, the increase in proliferation of PBL was significantly high when combination of SB-203580 was used along with rofecoxib compared to rofecoxib or SB-203580 alone. In presence of JNK inhibitor, PBL of NSCLC patients showed reduced proliferative response to anti-CD3 MAb compared to untreated but stimulated PBL (Fig. 6A). Similar results were obtained when PBL of NSCLC patients were treated with combinations of SP-600125 (JNK inhibitor) with rofecoxib (Fig. 6A).

Analysis of cytokines in culture supernatants of anti-CD3 MAb-stimulated PBL treated with MAPKs and COX-2 inhibitors revealed significantly decreased IL-10 (Fig. 6B) with concomitantly increased IFN-γ (Fig. 6C) production in presence of PD-98059, SB-203580 and rofecoxib when each inhibitor was used alone. Combination of SB-203580 with rofecoxib further reduced IL-10 production in comparison to the treatment with SB-203580 or rofecoxib alone (Fig. 6B). Combined treatment of PD-98059 with rofecoxib although reduced the IL-10 level (compared to PD-98059), the effect was comparable to that observed with rofecoxib alone (Fig. 6B). Stimulation of PBL with anti-CD3 MAb in presence of rofecoxib and MAPK inhibitors (PD-98059 or SB-203580) was unable to further increase level of IFN-γ above that observed with MAPK inhibitors alone (Fig. 6C) but was significantly increased above rofecoxib alone. SP-600125 (JNK inhibitor) did not show any increase in proliferative response or IFN-γ production (Fig. 6A and C).

We also investigated the effect of these inhibitors on PGE2 production from anti-CD3–stimulated and unstimulated PBL of NSCLC patients. In the presence of PD-98059, SB-203580 and SP-600125 alone or in combination with rofecoxib, reduced production of PGE2 from PBL of NSCLC patients (Fig. 6D) was observed.

Similar set of experiments were carried out with unstimulated PBL of NSCLC patients. As seen in Figure 7, significant increase in lymphocyte proliferation was observed when MAPK inhibitors were used along with rofecoxib. Significant decrease in IL-10 levels was observed when PD-98059 or SB-203580 was used with rofecoxib. Combination of SB-203580 with rofecoxib showed a marked increase in IFN-γ levels over that observed with PD-98059, SB-203580 or rofecoxib used alone (Fig. 7C).

Thus, our data confirms that COX-2, p38 and ERK pathways appear to be involved in high IL-10 production in NSCLC patients, p38 appears to play a dominant role. The effect was more prominently observed in unstimulated PBL of NSCLC patients.

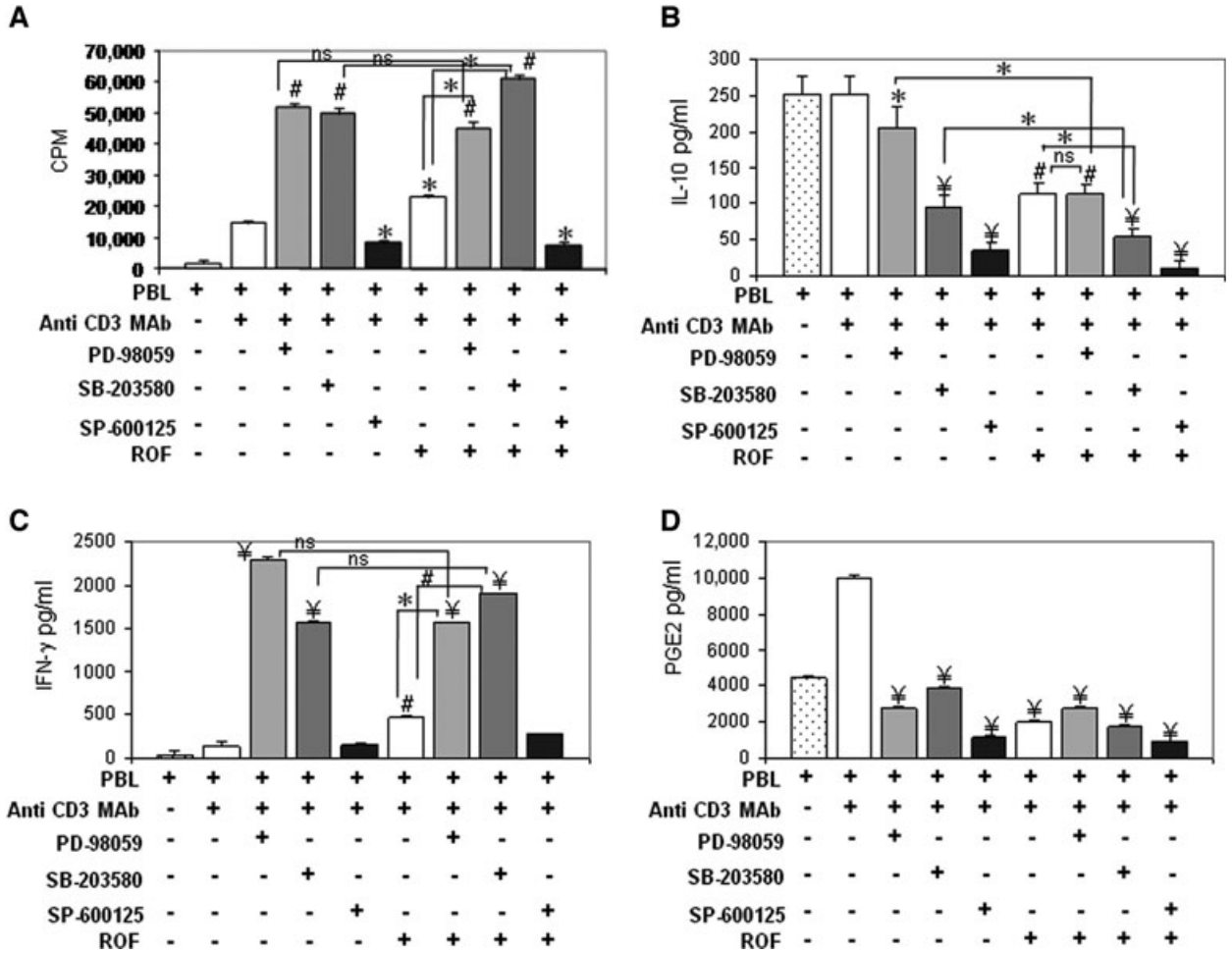

Fig. 6 (**A**) Proliferative response, (**B**) IL-10, (**C**) IFN-γ and (**D**) PGE2 production from PBL of NSCLC patients ($n = 5$) on anti-CD3 MAb stimulation in presence of inhibitors of ERK (PD-98059), p38 (SB-203580), JNK (SP-600125) and COX-2 rofecoxib (ROF). '+' indicates presence whereas '−' indicates absence of inhibitor. Graph represents mean cpm values or pg/ml from five samples. $^{*}P \leq 0.05$, $^{\#}P \leq 0.01$ and $^{¥}P \leq 0.001$ when results of anti-CD3 MAb-stimulated PBL were compared with PBL treated with inhibitors (symbols on data bars). Significance between various treatment groups compared are indicated in the graph as connecting bars with symbols as $^{*}P \leq 0.05$, $^{\#}P \leq 0.01$, $^{¥}P \leq 0.001$ and ns indicates non-significant.

Discussion

Although the role of PGE2 is pivotal in IL-10 production in NSCLC patients, the molecular mechanism is not well-understood [3, 10, 21]. This study focuses on further understanding the involvement of downstream MAPK and COX-2 pathways in IL-10– mediated immune dysfunction observed in NSCLC patients.

In this study, comparative analysis of lymphocyte proliferative response to anti-CD3 MAb stimulation in NSCLC patients and HI showed significant hyporesponsiveness in PBL of NSCLC patients. We observed increased levels of T regulatory cells in NSCLC patients that may contribute to decreased immune responses in these patients. Recent study also showed that TGF-β can induce regulatory T cells through a mechanism dependent on COX-2/PGE2 signalling. Pharmacologic inhibition of COX-2 suppressed the TGF-β–induced PGE2 production and the immunosuppressive activity of regulatory T cells [22]. Similarly, we observed that addition of rofecoxib caused a decrease in level of regulatory T cells in NSCLC patients (Table 2).

In lymphocytes of these patients reduced intracellular generation of calcium and high level of ROS on TCR/CD3 activation was observed. It has been reported that generation of an oxidative environment in the tumour has a strong influence on peripheral T lymphocytes leading to decreased expression of CD3-ζ chain in T cells and suppression of antigen specific T cells. In non-malignant conditions such as rheumatoid arthritis, the ROS generated by *in vitro* cultured lymphocytes from synovial fluid rendered a hyporesponsive state in T cells [23]. Hyporesponsive T cells showed

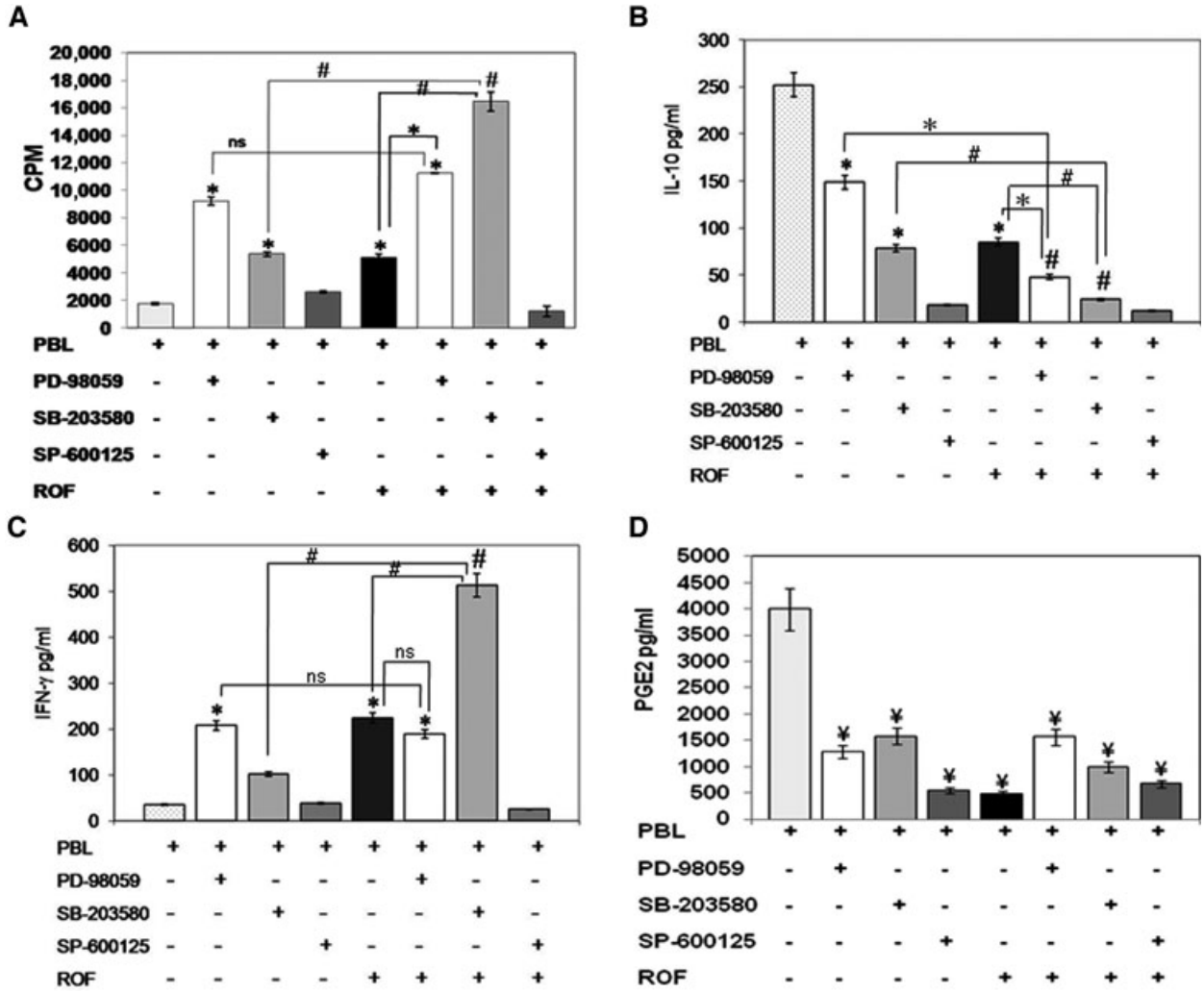

Fig. 7 (**A**) Proliferative response, (**B**) IL-10, (**C**) IFN-γ and (**D**) PGE2 production from unstimulated PBL of NSCLC patients ($n = 5$) in presence of inhibitors of ERK (PD-98059), p38 (SB-203580), JNK (SP-600125) and COX-2 rofecoxib (ROF). '+' indicates presence whereas '−' indicates absence of inhibitor. This graph represents mean cpm values or pg/ml from five samples. $^{*}P \leq 0.05$ and $^{\#}P \leq 0.01$ when results of untreated PBL were compared with PBL treated with inhibitors (COX-2 or MAPK) (symbols on data bars). Significance between various treatment groups compared are indicated in the graph as connecting bars with symbols as $^{*}P \leq 0.05$, $^{\#}P \leq 0.01$, $^{¥}P \leq 0.001$ and ns indicates non-significant.

defective TCR-dependent tyrosine phosphorylation of PLC-γ1 leading to a strongly decreased calcium flux [24].

Our data on cytokine analysis revealed high serum IL-10 with significantly low IFN-γ in NSCLC patients. Supernatants of unstimulated and anti-CD3 MAb-stimulated PBL of NSCLC patients showed increased IL-10 with decreased IFN-γ production. Our results are in accordance with earlier studies showing up-regulation of IL-10 and down-regulation of IL-12 from PBL of NSCLC patients [4]. In addition, we report high GATA-3 and low T-bet activity in PBL of NSCLC patients compared to HI. GATA-3 and T-bet are critical regulatory transcription factors for IL-10 and IFN-γ, respectively. Recent study has identified GATA-3 as a modulator which switches and stabilizes the IL-10 locus into a transcriptionally competent status independent of IL-4 in $CD4^{+}$ lymphocytes [20]. IL-10 is also known for its inhibitory effect on T-bet, thereby inhibiting anti-tumour responses and Th1 differentiation in T lymphocytes [25]. On the other hand, the expression and activity of T-bet is essential for anti-tumour immunity [19]. T-bet not only regulates IFN-γ production, but is known to determine cytotoxic responses of T cells and NK cells as it plays a key role in regulation of perforin and granzyme B [19, 26–29]. The increased expression and activity of GATA-3 with high IL-10 production observed in NSCLC patients indicates an immunosuppressive state in these patients.

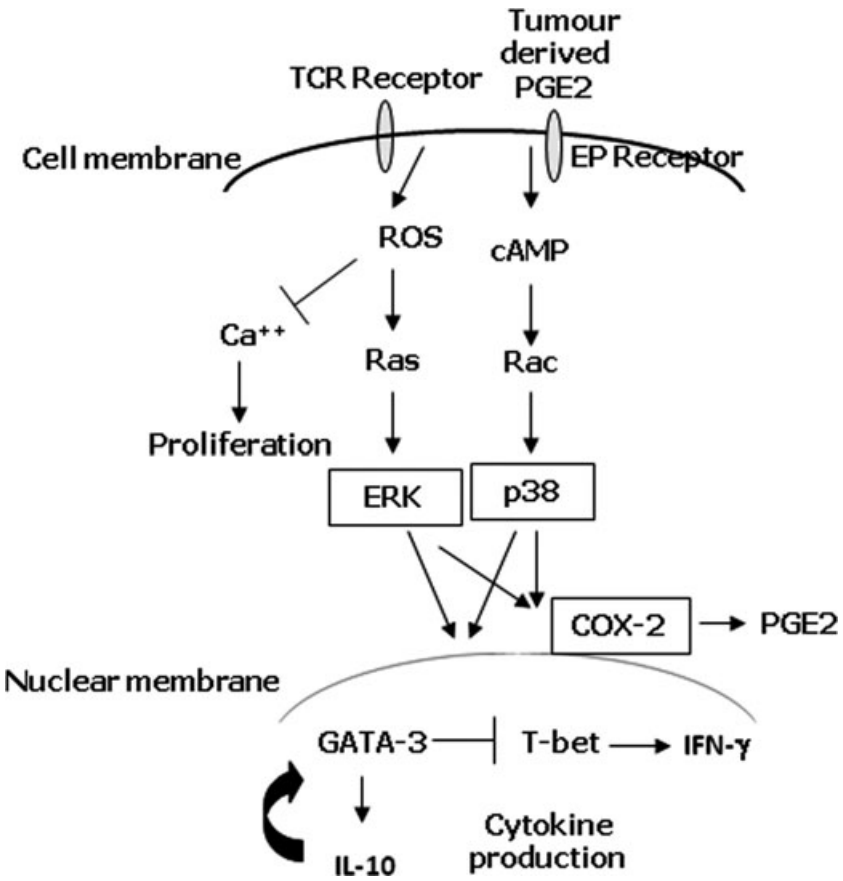

Fig. 8 Schematic representation of PGE2-mediated activation of MAPK and COX-2 leading to production of IL-10.

Isoforms of COX are central in T cell activation and cytokine production [1, 4, 16, 30]. Non-steroidal anti-inflammatory drugs (NSAIDs) selectively inhibit COX-2 and prevent inflammation [16]. These drugs are also used as immune modulators [21, 31]. Rofecoxib is one such NSAID known to inhibit selectively COX-2 activity and PGE2 production. PGE2, primarily product of COX-2 is an essential mediator of inflammation. PBL of NSCLC patients showed overexpression of COX-2 compared to that of HI. Also the PGE2 levels in serum and in culture supernatants from unstimulated and anti-CD3 MAb-stimulated PBL of NSCLC patients was significantly high compared to HI. The intervention of PGE2 in activation and function of PBL of NSCLC patients was confirmed by using rofecoxib. Treatment of PBL of NSCLC patients with rofecoxib resulted in marked restoration in lymphocyte proliferative response and intracellular calcium mobilization on TCR/CD3 stimulation. Also the high levels of ROS generation observed in PBL of NSCLC patients were found to be decreased after rofecoxib treatment. PGE2 is known to induce IL-10 production by activating cAMP with involvement of phosphotyrosine kinase (PTK) and protein kinase A (PKA) [10, 32]. The functional cross-talk between cAMP signalling and the Jak-STAT signalling pathway induced due to PGE2 was also reported [10]. In this study, treatment of PBL of NSCLC patients with rofecoxib showed marked decrease in IL-10 with concomitant increase in IFN-γ production. On TCR/CD3 stimulation, significant decrease in GATA-3 and increase in T-bet transcriptional activity was observed in PBL of NSCLC patients after rofecoxib treatment.

To further dissect the signalling network involved in IL-10 production regulated by PGE2, we analysed MAPK pathways in PBL of NSCLC patients. Our data revealed that p38 and ERK were activated and JNK was repressed in PBL of NSCLC patients. Moreover, we show that inhibition of p38, ERK and COX-2 activity by selective inhibitors restored IFN-γ production and proliferative response in anti-CD3 MAb-stimulated PBL of NSCLC patients. A marked increase in lymphocyte proliferative response to anti-CD3 MAb on treatment with ERK or p38 inhibitors was observed compared to treatment with rofecoxib alone. Inhibition of COX-2 and p38 together showed maximum restoration of lymphocyte proliferative response when stimulated with anti-CD3 MAb in NSCLC patients. The IL-10 production was strongly inhibited with appreciable induction of IFN-γ when COX-2 and p38 pathways were inhibited together. High IL-10 production depends more on activation of p38 and COX-2 pathways albeit with involvement of ERK pathway. Inhibition of MAPKs (ERK, p38, JNK) and COX-2

abrogated production of PGE2 from PBL of NSCLC patients indicating co-operative activity of these pathways in PGE2 production.

Several mechanisms have been reported to play a role in high IL-10 production [33–35]. We observed high ERK expression and ROS in unstimulated lymphocytes of NSCLC patients. Hyporesponsive T lymphocytes were reported to exhibit activation of ERK due to high ROS generation [24]. Activation of JNK pathway is an essential requisite for induction of IFN-γ, T cell effector functions and cytotoxic ability of T cells [36–38]. Our data reveal inactivation of JNK pathway in PBL of NSCLC patients which may lead to decrease in IFN-γ production and low effector functions of lymphocytes in NSCLC patients. Incubation of PBL of NSCLC patients with rofecoxib revealed significant increase in JNK activity with increase in IFN-γ production. Thus, COX inhibitor, rofecoxib showed profound effect on MAPKs pathways which are fundamental regulators of T cell activation, functions and cytokine production. Although it appears that COX-2, p38 and ERK are involved in high IL-10 production in NSCLC patients, p38 emerges as an important signalling intermediate that regulates IL-10 production with concomitant increase in IFN-γ production in NSCLC patients irrespective of the state of activation of the lymphocytes.

To conclude, our study demonstrates that lung tumours produce high PGE2 along with other tumour-derived immunosuppressive factors in NSCLC patients. In addition to conferring the malignant and metastatic phenotype in lung cancer, PGE2 binds to EP receptors present on lymphocytes of NSCLC patients. Based on the observations we proposed a model (Fig. 8) which explores the mechanism of PGE2-mediated IL-10 production in NSCLC patients that leads to observed hyporesponsiveness in NSCLC patients. Constitutive activation of ROS due to high PGE2 levels may cause defect in TCR-ζ chain expression and inhibition of PLCγ1 leading to inhibition of intracellular calcium release resulting in reduced proliferative and activation response on TCR stimulation. Independent of TCR activation, ROS and cAMP are known to activate ERK pathway. Also constitutive activity of COX-1 and cAMP activates p38 MAPK pathway. The activation of both these pathways induces COX-2 expression and PGE2 production which stabilizes ROS and cAMP activation. Activation of ERK and p38 pathways for extended time period leads to IL-10 production which activates GATA-3 and inhibits T-bet activity resulting in impaired IFN-γ production. Production of PGE2 and IL-10 may further inhibit JNK activity which is known to be involved in IFN-γ production. Treatment of lymphocytes with NSAIDs primarily inhibits COX activity and PGE2 production; this inhibits ERK and p38 activation resulting in reduced GATA-3 and IL-10 levels which results in improved T-bet, IFN-γ and anti-tumour responses.

Our studies reveal the important role of cyclooxygenase enzymes and PGE2 in lymphocyte dysfunction and cytokine imbalance in NSCLC patients. Present findings provide an insight into the molecular mechanism of PGE2 and COX-2 mediated regulation of signalling events in lymphocytes of NSCLC patients. The study not only demonstrates ability of COX inhibitors to restore these defects but also shows the potential of COX-2, ERK and p38 inhibitors as therapeutic tools in management of lung cancer.

Conflict of interest

The authors confirm that there are no conflicts of interest.

Supporting information

Additional Supporting Information may be found in the online version of this article:

Fig. S1 Comparison of SI values from paired samples HI ($n = 17$) and NSCLC patients ($n = 17$) before and after *in vitro* treatment with rofecoxib (ROF).

Fig. S2 Intracellular calcium levels in PBL of NSCLC patients ($n = 3$) on anti-CD3 MAb stimulation before and after treatment with different concentrations of rofecoxib (ROF) (100, 200 and 400 μM).

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