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ATRA reduces inflammation and improves alveolar epithelium regeneration in emphysematous rat lung

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ABSTRACT

Introduction: Pulmonary emphysema characterized by alveolar wall destruction is resultant of persistent chronic inflammation. All-trans retinoic acid (ATRA) has been reported to reverse elastase-induced emphysema in rats. However, the underlying molecular mechanisms are so far unknown.
Objective: To investigate the therapeutic potential effect of ATRA via the amelioration of the ERK/JAK-STAT pathways in the lungs of emphysematous rats.
Methods: In silico analysis was done to find the binding efficiency of ATRA with receptor and ligands of ERK & JAK-STAT pathway. Emphysema was induced by porcine pancreatic elastase in Sprague-Dawley rats and ATRA was supplemented as therapy. Lungs were harvested for histopathological, genomics and proteomics analysis.
Results and Discussion: In silico docking, analysis confirms that ATRA interferes with the normal binding of ligands (TNF-α, IL6ST) and receptors (TNFR1, IL6) of ERK/JAK-STAT pathways respectively. ATRA restored the histology, proteases/antiproteases balance, levels of inflammatory markers, antioxidants, expression of candidate genes of ERK and JAK-STAT pathways in the therapy group.
Conclusion: ATRA ameliorates ERK/JAK-STAT pathway in emphysema condition, resulting in alveolar epithelium regeneration. Hence, ATRA may prove to be a potential drug in the treatment of emphysema.

1. Introduction

Chronic Obstructive Pulmonary Disorder (COPD) is characterized by long-term poor airflow due to persistent lung inflammation that strengthens disease progression. COPD comprises two pathological conditions i.e. chronic bronchitis and emphysema. Pulmonary emphysema is characterized by the destruction of alveolar septa, decreased pulmonary elastic recoil, thus forming large airspace which leads to shortness of breath, accumulation of mucus, long-term fatigue and cough [1,2]. The pathogenesis of emphysema chiefly involves chronic inflammation, which arises due to the continuous exposure to cigarette smoke or to long-term exposure to toxic gases and particles, due to which a rapid up-regulation of innate immunity could occur. Such conditions in the lung may recruit a large influx of neutrophils into the airways, which further may increase the levels of oxidant-antioxidant imbalance, protease-antiprotease imbalance, ineffective repair, and apoptosis, leading to tissue remodeling [3].

The increased inflammatory response may be mediated indirectly through the activation of signal transduction pathways such as mitogen-activated protein (MAP) kinase [4] and extracellular regulated kinases (ERK) pathways that are involved in regulating the expression of many inflammatory genes [5]. ERK pathway is chiefly involved in processes like cell growth and proliferation; however, it is also involved in several inflammatory processes [6,7]. ERK pathway may be activated by proinflammatory cytokine i.e. TNF-α, and its subsequent activation plays an important role in innate immunity and inflammation [8]. Several reports have been suggested that the ERK pathway was deregulating in emphysema condition [9,10].

The other pathway widely known to be involved in the inflammation process is Janus kinase (JAK)–signal transducer and activator of

Abbreviations: COPD, chronic obstructive pulmonary disease; ATRA, all-trans retinoic acid; DI, destructive index; WLT, whole lung tissue; AMC, Alveolar macrophage cells; SS, saline + olive oil (control group); ES, elastase + olive oil (emphysema group); EA, elastase + ATRA (therapy group); SA, saline + ATRA (therapy control group)

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transcription (STAT) pathway (JAK-STAT) [11,12]. The JAK-STAT pathway is a classical signal transduction pathway for numerous cytokines and growth factors. The binding of ligands to their receptors leads to JAK activation, which in turn phosphorylates and activates STATs. It has been reported that JAK-STAT pathway, is activated by proinflammatory cytokines TNF-α and IL6 [13], thus regulates inflammation, apoptosis, protease expression, which is critical processes and are associated with the airways injury and lung tissue destruction [14]. JAK-STAT pathway is found to be activated in tissues of COPD patients [15] and in acute lung injury [16]. JAK's are activated in response to IL6 via IL6ST, which further activates STAT3 [11]. STAT3 is chiefly involved in processes like chronic inflammation and apoptosis [17,18]. STAT3 inhibition leads to reduced inflammation in acute lung injury model & acute respiratory distress syndrome patients via inhibition of macrophage and inflammatory cell infiltration [19]. All these findings suggest that JAK- STAT pathway activation is believed to be a central factor in the induction of airways inflammatory response. There is a possible cross-talk between the pathways as TNF-α is the common activator of both the pathways. TNF-α induces IL6 (interleukin 6) [20] which further activates STAT3, via the activation of the JAK-STAT pathway. Similarly, TNF-α activates ERK pathway which is known to be involved in the inflammation process [8]. The same has been validated by us using in silico approach (Fig. 1).

Nevertheless, the precious role of other target genes involved in above-mentioned pathways is needed to be traced out for a better understanding of emphysema pathogenesis and subsequently for its improvement. The current treatment options for emphysema aim at easing and preventing the disease, but none of them work towards the reversal of disease to normal. Therefore there is an urgent requirement for such a therapy option which would target towards the regeneration of the lost alveolar septa as well in reducing the inflammation by the amelioration of ERK & JAK-STAT pathways, thus easing as well as curing the disease.

All-trans-retinoic acid (ATRA) is the biologically active metabolite of vitamin A and possesses anti-inflammatory property [21]. Other than having an anti-inflammatory property, ATRA has been found to involve in a variety of processes such as proliferation, differentiation, survival, and apoptosis [22]. ATRA works as an anti-inflammatory molecule via the repression of inflammatory molecules such as IL6, TNF-α etc [23]. Interestingly, ATRA supplementation reversed the deleterious effects of elastase-induced emphysema in an animal model [24]. Massaro and Massaro for the first time analyzed the potential and promising effects of exogenous application of ATRA to promote alveolarisation in the elastase-induced experimental model (rat) of emphysema [25]. After Massaro and Massaro, various other research groups have shown the beneficial effects of ATRA in elastase or dexamethasone models of lung damage [26,27]. In a study conducted by Paiva et al., exogenously supplemented vitamin A has been found to improve pulmonary function in patients with moderate to severe emphysema [28]. Also, ATRA has been found to attenuate the protease/anti protease imbalance in the bronchoalveolar lavage (BAL) cells from patients with COPD and patients with other lung diseases [29].

Although potential mechanisms underlying the beneficial effects of ATRA in dexamethasone-induced impairment of alveolarisation have been suggested [26,30–32], only little is known about the molecular mechanisms contributing to its potential regenerative effects in smoke- or elastase-induced emphysema [25,33]. Hence, in this current study we have addressed a question i.e. does ATRA have a tendency to minimize the ongoing inflammation in elastase induced emphysema in rat lungs and eventually its potential role in alveolar epithelium regeneration? To validate this hypothesis, we have investigated the potential effect of ATRA via amelioration of the ERK/JAK-STAT pathways. Nevertheless, the potential of ATRA was further evaluated using in vivo model for emphysema.

2. Material & methods

2.1. Gene interaction

An online tool, GeneMANIA was used to predict the gene

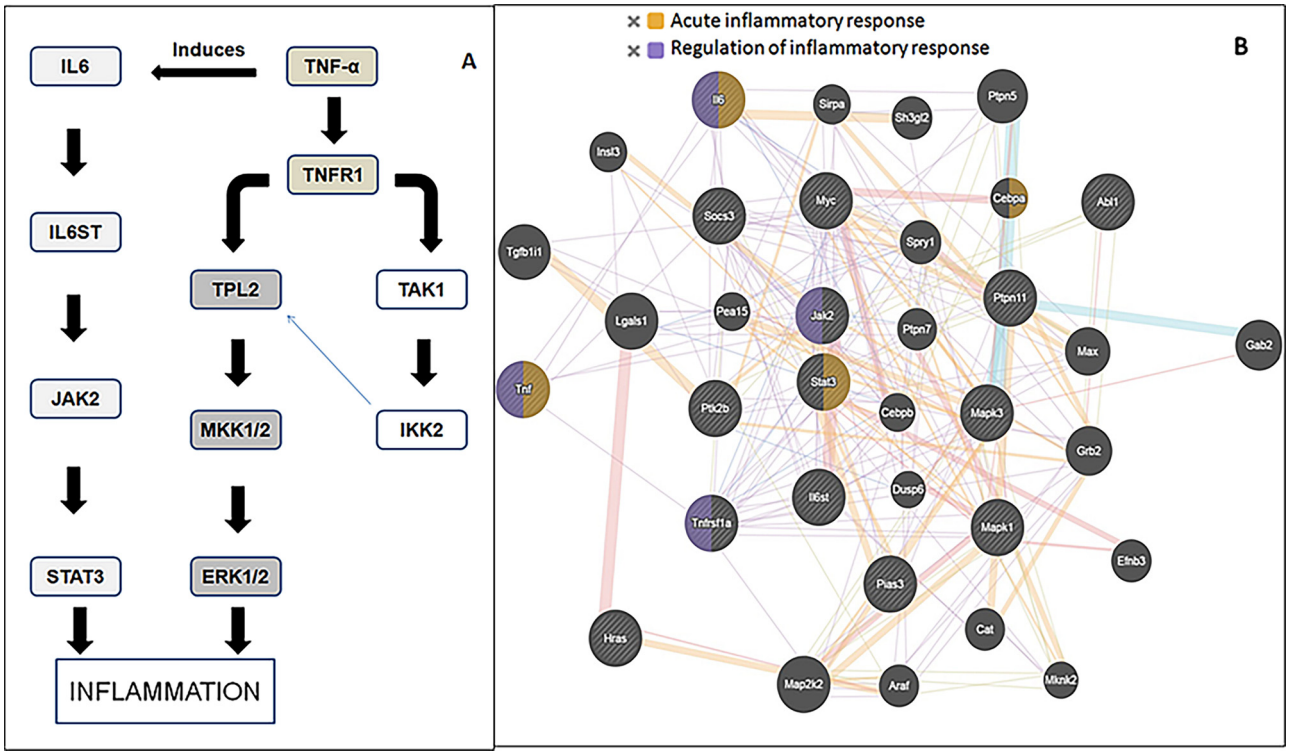

Fig. 1. A: Schematic representation of induction of JAK-STAT pathway and ERK pathway by TNF-α. 1B: gene interaction network for candidate genes of ERK as well as JAK-STAT pathway as obtained from GeneMANIA showing involvement of TNFα and IL6 in inflammation.

interaction. Candidate genes of ERK and JAK-STAT were given as input and their interactions were predicted by GeneMANIA on the basis of co-expression, predicted, pathway, physical interactions, shared protein domains and co-localization. Functions such as acute inflammatory response and regulation of inflammatory response were selected and the genes involved were highlighted with yellow and purple colour respectively.

2.2. In silico analysis

AutoDock version 4.0 suite was used for the docking studies of ATRA with all target proteins (IL6, IL6ST, TNF α1 & TNFR1) [34]. PatchDock (geometry based molecular docking algorithm tool) was used to execute protein–protein interaction simulation study [35]. IL6 & TNFR1 were docked with IL6ST & TNFalpha1, as well as IL6ST + ATRA & TNFalpha1 + ATRA complex which were the resultant of the AutoDock execution. IL6ST & IL6ST + ATRA complex *pdb* file was docked with IL6 & TNFalpha1. At last TNFalpha1 + ATRA complex was docked with TNFR1. After whole protein-protein interactions by PatchDock, refinement process was done for the better and improved results of protein–protein interaction by FireDock server [36] which was in build refinement option of PatchDock.

2.3. Preparation of animal models

All experimental models were prepared according to the procedure described by Seifart C et al. [27] and were in compliance with National and International guidelines approved by the regional government (IAEC, Ministry of Environment and Forests, India. INM/IAEC/2012/05). The models were prepared at Institute of Nuclear Medicine and Allied Sciences, DRDO, New Delhi under the supervision of Dr. Amit Tyagi. Pathogen-free eight weeks old male, Sprague Dawley rats (approximately 150 g body weight) were randomly assigned to four different experimental models (n = 6 per group), i.e. control (SS), Emphysema (ES), ATRA therapy (EA) and therapy control (SA). Details regarding the preparation of experimental rat models were summarized in Table 1. Rats were maintained under anesthesia by isoflurane and were given either elastase/saline oropharyngeally or ATRA/olive oil intraperitoneally. Animals were sacrificed on day 38 by cervical dislocation. Blood was removed by performing ventilation/perfusion with sterile PBS. Before harvesting the lungs bronchoalveolar lavage fluid (BALF) was collected by injecting 1 ml of PBS twice per injection into the lungs. Alveolar macrophage (AM) cells were collected from the BALF by centrifugation of the same at 1500 rpm for 20 min. at 4 °C. Lungs were harvested from each rat. For molecular analysis purpose, the left lung from each rat was stored at −80 °C until analyzed and the right lung from each rat was stored in paraformaldehyde for histopathological analysis.

2.4. Elastase activity assay

EnzChek Elastase Assay Kit (Molecular Probes, New Delhi, India) was used to estimate the elastase activity in lung tissue. The kit worked on the principle of digestion of non-fluorescent elastin substrate (BODIPY® FL conjugated with DQ™ elastin) to highly fluorescent fragments by elastase. The fluorescent products are then measured by using a fluorescence microplate reader (Synergy H1 Hybrid Multi-Mode Microplate Reader; BioTek Instruments, Inc., Mumbai, India). Lung tissue homogenates were mixed with DQ-elastin labeled with BODIPY FL dye in the presence or absence of 30 mM of the selective inhibitor (MeOsuc-Ala-Ala-Pro-Val-chloromethyl ketone). Fluorescence intensity was measured following incubation of the reaction in the dark for 1 h at room temperature at excitation/emission of 485/530 nm. Inhibitor helps in confirming the identity of the protease responsible for substrate digestion [37,38].

Table 1
Animal model preparation.

S.No	Group	At Day 0 & 10	From Day 26 to 37
1.	SS (control)	Saline (180ul/day)	Olive oil (300 µl/kg b.w./day)
2.	ES (emphysema)	Elastase (25 U/kg b.w./day)	Olive oil (300 µl/kg b.w./day)
3.	EA (therapy)	Elastase (25 U/kg b.w./day)	ATRA (500 µg/kg b.w./day)
4.	SA (therapy control)	Saline (180ul/day)	ATRA (500 µg/kg b.w./day)

2.5. Lung fixation & histopathology

Lung tissues were fixed in 6% phosphate-buffered paraformaldehyde and stored in the refrigerator. Tissue processing was carried out in accordance to the standard protocols followed by preparation of tissue blocks using molten paraffin which was allowed to cool and solidify before making tissue sections. 5–10 µm thin tissue sections were cut using a microtome (Spencers rotary microtome, India). Subsequently, tissue sections were deparaffinized three times by Xylene, rehydrated with different concentrations of ethanol and stained with hematoxylin and eosin (H&E) stain.

2.6. Destructive index

The destructive index was calculated by counting the points overlapping alveolar and duct spaces as described by Muyal et al. [39]. From each lung specimen (total 6), 3 sections were taken (top, middle & lower), from each section, 3 non overlapping sections were selected. The stained sections were printed on A4 size sheet, which was overlapped by a transparent sheet with 80 counting points. The percentage of all points falling in destroyed/ normal air spaces were computed to obtain Destructive Index, using the formula [D/(D + N)] x100%, where D = destroyed, and N = normal. Differences in DI of emphysema and therapy groups were calculated with respect to control (100%).

2.7. Total RNA isolation and cDNA synthesis

To determine the relative mRNA expression in lung tissue, total RNA was extracted using RNeasy Mini Kit (Qiagen, New Delhi, India). The quantity and purity of total RNA were determined with Nanodrop spectrophotometer (Thermo Scientific, New Delhi, India) while the quality of total RNA integrity was assessed by analyzing 18S and 28S ribosomal RNA on 1.2% ethidium bromide stained agarose gel electrophoresis. First-strand cDNA was synthesized by introducing equal amounts of RNA (300 ng) from each sample in a total reaction volume of 20 ml using an Oligo dT primer (Qiagen, New Delhi, India) and Omniscript RT Kit (Qiagen, New Delhi, India) and their respective protocol. The reaction was incubated at 37 °C for 1 h in Thermoblock TB2 (Biometra, New Delhi, India).

2.8. Relative mRNA expression

The expression of key gene transcripts (see Table 2) were examined by quantitative RT-PCR using the biorad kit as per the manufacturer's protocol. The thermal cycle conditions used for all reactions were as follows: Step 1: 95 °C for 15 min; a 30 cycles of Step 2 (95 °C for 45 s), Step 3 (sequence-specific oligonucleotide primer's annealing temperature for 35 s) and Step 4 (72 °C for 45 s), followed by one time of step 5: 72 °C, 5 min. The quantitative real time PCR for determining the amplification factor of the target genes were performed via a Bio-Rad CFX96™ instrument (Bio-Rad Laboratories Inc., New Delhi, India) using the manufacturer's guidelines. The expressions of test genes were normalized by using endogenous control that is GAPDH.

Table 2
Genes for mRNA expression.

S.No	Gene	Forward primer	Reverse primer	Amplicon size (bp)
1.	TNFR1	CTGTTGCCCCTGGTTATCTT	CCAGCCTTCTCCTCTTTGAC	143
2.	RAS	GATGGTTTTCAGGGCCACTA	TTCCACTGGACTGTGCTCTG	130
3.	ERK1	CAGTCTCTGCCCTCGAAAAC	CCTCTACTGTGATGCGCTTG	124
4.	ERK2	CCCAAATGCTGACTCCAAAG	GTCGTCCAACTCCATGTCAA	175
5.	ELK1	GCACGTATATGCCGAGACCT	CCGCCTCCTCTTCTTTATCC	151
6.	c-MYC	CAACGTCTTGGAACGTCAGA	TCGTCTGCTTGAATGGACAG	153
7.	TNF-α	ACGTCGTAGCAAACCACCAA	AAGGTACAACCCATCGGCTG	128
8.	IL-10	TCAGCCAGGTGAAGACTTTC	CTGGATCATTTCCGATAAGG	122
9.	MMP2	ATGCCATCCCTGATAACCTG	CCCAGCCAGTCTGATTTGAT	145
10.	MMP7	ATCAGTGGGAACAGGCTCAG	TCCATGATGTAGGGGGAGAG	151
11.	MMP8	AGGGAGAAGCAGACATCAAC	GCATCTCCTCCAATACCTTG	124
12.	MMP9	CTGGACAGCCAGACACTAAG	CTCGCGGCAAGTCTTCAGAG	145
13.	MMP12	CTGCTCCCATGAATGACAGTG	AGTTGCTTCTAGCCCAAAGAAC	158
14.	TIMP1	ATCTGGCATCCTCTTGTTGC	GGGAACCCATGAATTTAGCC	125
15.	TIMP2	AAGCAGTGAGCGAGAAGGAG	GGGGGCCGTGTAGATAAACT	137
16.	A1AT	GGAATCACAGAGGAAAATGC	GGGCATAGACATAGGAACCA	129
17.	GAPDH	AATGGTGAAGGTCGGTGTGAAC	GAAGATGGTGATGGGCTTCC	226
18.	CRP	GGGTCAAGGGTTTAGTATTGC	GAGATAGCACAAAGTCCCACAT	296
19.	IL6	ATACCACCCACAACAGACCA	TCCAGAAGACCAGAGCAGATT	244
20.	IL6ST	AAGGAGAATGGGAAGGGCTA	TGCGAAACTGACTTGGACTG	227
21.	JAK2	CAGATTCCGCAGGTTCATTC	CCTTATGTTTCCCTCTTGACCA	221
22.	PTK2B	GAATCTTGACCACCCTCACA	GACACAGTTGATGCTCTCCA	202
23.	STAT3	AAGGAAGGAGGGGTCACTTT	TCGGGGCGACAATACTTT	227
24.	PTPN11	ATGATGTTGGTGGAGGAGAG	GCCCTGTTTGACTTTATCTGTG	203
25.	SOCS3	CAAGAACCTACGCATCCAGT	CGGTGGTAAAGAAAAGGAAG	151
26.	ABL1	TTCATCCACAGAGACCTTGCT	ATACTCCAAATGCCCAGACG	208
27.	PIAS3	GGTTTGAGGAAGCCCACTTT	ATTCTTGGTTGGAGGGAGGT	233
28.	PTPase	CACCTGCCTCTTTCCTCAAT	GCATCTCCAACAGCACTTTCT	201

RAS = Rat sarcoma; ERK = Extracellular Signal Regulated Kinase; ELK1 = E twenty-six (ETS)-like transcription factor 1; c-MYC = myelocytomatosis viral oncogene; TNFα = Tumor Necrosis Factor α; IL10 = Interleukin 10; MMP = Matrix Metalloproteinase; TIMP = Tissue Inhibitor of Metallo-Protease; A1AT = Alpha 1 Anti Trypsin; GAPDH = Glyceraldehyde 3-phosphate dehydrogenase; CRP = C reactive protein; IL6 = Interleukin 6; IL6ST = Interleukin 6 signal transducer; JAK2 = Janus kinase 2; PTK2B = protein tyrosine kinase 2 beta; STAT3 = signal transducer and activator of transcription 3; PTPN11 = protein tyrosine phosphatase, non-receptor type 11; SOCS3 = suppressor of cytokine signaling 3; ABL1 = ABL proto-oncogene 1, non-receptor tyrosine kinase; PIAS3 = protein inhibitor of activated STAT3; PTPase = protein-tyrosine-phospatase.

2.9. Zymography and reverse zymography for estimation of protease-antiprotease imbalance

Cell-free bronchiolavage fluid (BALF) was concentrated (10X) and used for Gelatin zymography and reverse zymography. The concentrate of BALF was prepared by using Amicon Ultra-4 Centrifugal Filter Unit with Ultracel-10 membrane columns (Merck Life Science Private Limited, Mumbai, India) with a molecular cut-off at 10 kDa. Zymography and reverse zymography was carried out as per the protocol described by Seifart C et al. [27].

Negative images of the zymograms and reverse zymograms were analyzed by Image J (image processing program) (www.rsbweb.nih.gov/ij/). Densitometry was performed on the bands obtained and the differences in optical density of bands were calculated with respect to control (100%),

2.10. Biochemical analysis for catalase and glutathione peroxidase

Lung tissue was homogenized in phosphate buffer (pH 7.4) and centrifuged at 12,000 × g at 4 °C for 30 min to get tissue homogenates which were further used for antioxidant activity determination. Tissue antioxidant status was determined by estimation of antioxidant activities of catalase (CAT) and Glutathione peroxidase (GPx) as described by Maehly et al. and Mohandas et al. respectively [40,41].

2.11. Western blotting

Lung tissue homogenate was prepared from 100 mg tissue (from each rat of the group, n = 6), from which total protein was extracted by total protein extraction kit (Biochem Life Sciences, New Delhi, India) as per the manufacturer's protocol. The isolated proteins were pooled together to eradicate biological variations within a group. 50 µg protein from each group, in duplicates, was resolved on SDS–PAGE and transferred onto nitrocellulose membrane. The membrane was blocked by 5% skimmed milk solution in PBST (Phosphate-buffered saline + Tween 20) for 3 h.

Membrane was incubated in corresponding primary antibody (listed in Table 3) (Neo Scientific, Neo Biolabs, Cambridge, MA, USA ; Merck Life Science Private Limited, Mumbai, India) at 4 °C for overnight followed by washing (with PBST) and incubation with an alkaline phosphatase (ALP)-labeled secondary antibody (Bio-Rad Laboratories India Pvt. Ltd., Gurgaon, India) for 1.5 h at 4 °C. The membrane was washed with PBST and incubated with substrate (BCIP/NBT) for 15–20 min. at

Table 3
List of primary antibodies used for protein expression study.

S.No	Antibody	Brand
1.	TNF-α	Neo Scientific, Neo Biolabs, Cambridge, MA, USA.
2.	CRP	Neo Scientific, Neo Biolabs, Cambridge, MA, USA.
3.	GSTA	Neo Scientific, Neo Biolabs, Cambridge, MA, USA.
4.	MMP2	Neo Scientific, Neo Biolabs, Cambridge, MA, USA.
5.	MMP7	Neo Scientific, Neo Biolabs, Cambridge, MA, USA.
6.	MMP8	Neo Scientific, Neo Biolabs, Cambridge, MA, USA.
7.	CTSE	Neo Scientific, Neo Biolabs, Cambridge, MA, USA.
8.	SERPINA1	Neo Scientific, Neo Biolabs, Cambridge, MA, USA.
9.	TIMP4	Neo Scientific, Neo Biolabs, Cambridge, MA, USA.
10.	GAPDH	Merck Life Science Private Limited, Mumbai, India.
11.	ERK1/ERK2	Sigma-Aldrich, India (kind gift by Dr. Amit Kumar Tyagi)

TNFα = Tumor Necrosis Factor α; CRP = C-Reactive Protein; GSTA = Glutathione S Transferase Alpha; MMP = Matrix Metalloproteinase; CTSE = Cathepsin E; SERPINA1 = Serpin Peptidase Inhibitor, Clade A; TIMP = Tissue Inhibitor of Metallo- Protease; GAPDH = Glyceraldehyde 3-phosphate dehydrogenase.

RT. The obtained bands were analyzed by densitometry using Image J (image processing program) (www.rsbweb.nih.gov/ij/).

2.12. Immunohistochemistry

Formalin fixed tissues were embedded in paraffin and blocks were prepared. Fine tissue sections were deparaffinized with xylene. 95%, 70%, and 50% alcohol (3 min each) gradient were used to rehydrate tissue. Sections were washed with phosphate-buffered saline (PBS) and immersed in 3% hydrogen peroxide in methanol for 20 min to block endogenous peroxidase activity. Sections were washed twice with PBS and incubated with 5% bovine serum albumin (BSA) in PBS for 1 h at room temperature to block non-specific binding sites followed by overnight incubation with primary antibodies (0.5% bovine serum albumin in PBS) at 4 °C. Sections were washed twice with PBS followed by incubation with secondary HRP-conjugated antibodies for 1 h at room temperature. After washing DAB (0.05% 3,3′-diaminobenzidine tetrahydrochloride and 0.015% hydrogen peroxide) stain was applied on tissue sections for 5–10 min followed by counterstain with Mayer's hematoxylin (Sigma-Aldrich, New Delhi, India). Sections were mounted with DPX mountant (Himedia, Delhi, India) and covered by coverslips. Slides were visualized using a light microscope and images were captured & analyzed by using ImageJ (www.rsbweb.nih.gov/ij/) as per described by Jensen EC et al [42].

2.13. Statistical analysis

mRNA levels of target genes were determined relative to the endogenous control GAPDH, according to the formula 2 to the power of delta cycle threshold (2ΔCt), where ΔCt = Ct, reference gene – Ct, test gene. Differences between experimental groups were tested for significance using Student's unpaired *t*-test used to determine the level of significance of differences between control versus emphysema and emphysema versus ATRA therapy, respectively using GraphPad Prism version 5, San Diego, USA. Levels of significance are indicated by * = $p < 0.05$; ** = $p < 0.01$; *** = $p <= 0.001$. The same statistical analysis was also used to evaluate the results of elastase assay, lipid estimation, and western blotting.

3. Results

3.1. Gene interactions by GeneMANIA

The results obtained from GeneMANIA show the interactions between candidate genes of ERK and JAK-STAT pathway. IL6 and TNF are commonly involved in inflammatory response as well as in its regulation (Fig. 1B).

3.2. Effect on the binding of ligand and receptor of ERK & JAK-STAT pathway in presence of ATRA

Prior to testing the proposed hypothesis, we have checked the potential of ATRA using *in-silico* approach. The comparative optimization was carried out to find the best-docked pose and the energy of all target proteins (IL6, IL6ST, TNF-α & TNFR1) with ATRA. The best 10 docked poses were identified and analyzed. Figures were generated using program DS Visualizer. Fig. 2 illustrates best docked pose of IL6, IL6ST, TNF-α & TNFR1 with ATRA that possessed binding energy or ΔG of -3.55, -4.78, -6.17 & -5.96 Kcal/Mol & Ki value 2.51 mM, 315.24 μM, 30.09 μM & 42.58 μM respectively (Table 4). In protein-protein interaction study, the FireDock dock score of, IL6ST with IL6 was -4.90, IL6ST + ATRA with IL6 was -0.95 and TNFR1 with TNF-α was -51.16 & TNFR1 with TNF-α + ATRA was -37.21 (Table 5).

3.3. Effects of elastase and ATRA on elastase activity

The elastase treated lungs show a significant induction of endogenous elastase activity in emphysema group (ES) as compared to control group (SS) (Fig. 3). Interestingly, in the therapy group (EA), the endogenous elastase activity was significantly reduced and was comparable to control group (SS), while, no changes were noticed in the control lungs instilled only with ATRA (SA) than a control group (SS). The assay of elastase in the presence of varying concentrations of its selective inhibitor (MeOsuc-Ala-Ala-Pro-Val-chloromethyl ketone) confirmed the proteolytic activity of elastase.

3.4. Effects of elastase and ATRA on tissue architecture

Oropharyngeal instillation of 2.25 mg PPE/kg b.w. on two occasions (Days 0, 10) resulted in severe pulmonary emphysema, as shown in Fig. 4A (ES) of photomicrograph generated from histopathology study. However, the photomicrograph for ATRA treated emphysematous lungs markedly depicted the recovery of lost alveolar septa in therapy group (EA, Fig. 4A) and was well comparable to control group (SS, Fig. 4A). Upon determination of destructive index (DI) of tissue slices from all four animal models (Fig. 4A), emphysematous mice model showed significantly higher DI values than a control group. However, the DI was significantly reduced in the case of therapy group (EA) (Fig. 4B).

3.5. Effects of elastase and ATRA on mRNA, protein expression and immunohistochemistry of inflammatory/ anti-inflammatory markers

The expression of TNF-α, CRP and IL10 was studied using whole lung tissue (WLT) and Alveolar macrophage cells (AMC). TNF-α was also studied at tissue level by IHC. The mRNA expression of TNF-α and CRP from WLT and AMC showed a significant up-regulation in lungs treated with elastase group (ES) as compared to the control group (SS). Here, again ATRA has shown its anti-inflammatory effect by reducing the expression of TNF-α and CRP in the therapy group (EA) than ES group. However, there are no changes in the mRNA expression of TNF-α and CRP in lungs received only ATRA (SA) than SS group. However, mRNA expression pattern for IL-10 from WLT and AMC was opposite to that of TNF-α and CRP as it is anti-inflammatory in nature. The mRNA expression of IL-10 was significantly down-regulated in emphysema group (ES) as compared to control group (SS) while, it was significantly up-regulated in therapy group (EA). In the control group with ATRA (SA) the mRNA expressions were similar to the control group (Fig. 5A, B). Interestingly upon validation on the protein level, the protein expression result of TNF-α, CRP and IL-10 also show a similar trend as shown for mRNA expression of TNF-α, CRP and IL-10 (Fig. 5C). Similarly, IHC results of TNF-α also complied with its mRNA and western blot result (Fig. 5D).

3.6. Effects of elastase and ATRA on ERK and JAK-STAT signalling pathways

mRNA expressions of genes involved in two signaling pathways, i.e. ERK and JAK-STAT, have been evaluated to examine their involvement during inflammation process in elastase treated group. The mRNA expressions of candidate genes (Fig. 6A) of overall ERK pathway i.e. TNFR1, RAS, ERK1, ERK2, ELK1 and c-MYC were significantly up-regulated in emphysema group (ES) as compared to control group (SS), while, they were significantly down-regulated in therapy group (EA). In the control group with ATRA (SA), the mRNA expressions of these genes were similar to that of the control group (SS). Further, validation of mRNA results on protein level was determined for ERK1 and ERK2. Our western blotting result confirms the potential effect of ATRA by reducing the expression of ERK1 and ERK2 in emphysematous lungs, which was induced due to elastase effect (Fig. 6B). Similarly, the mRNA expressions of candidate genes (Fig. 6C) of JAK/STAT pathway i.e. IL6,

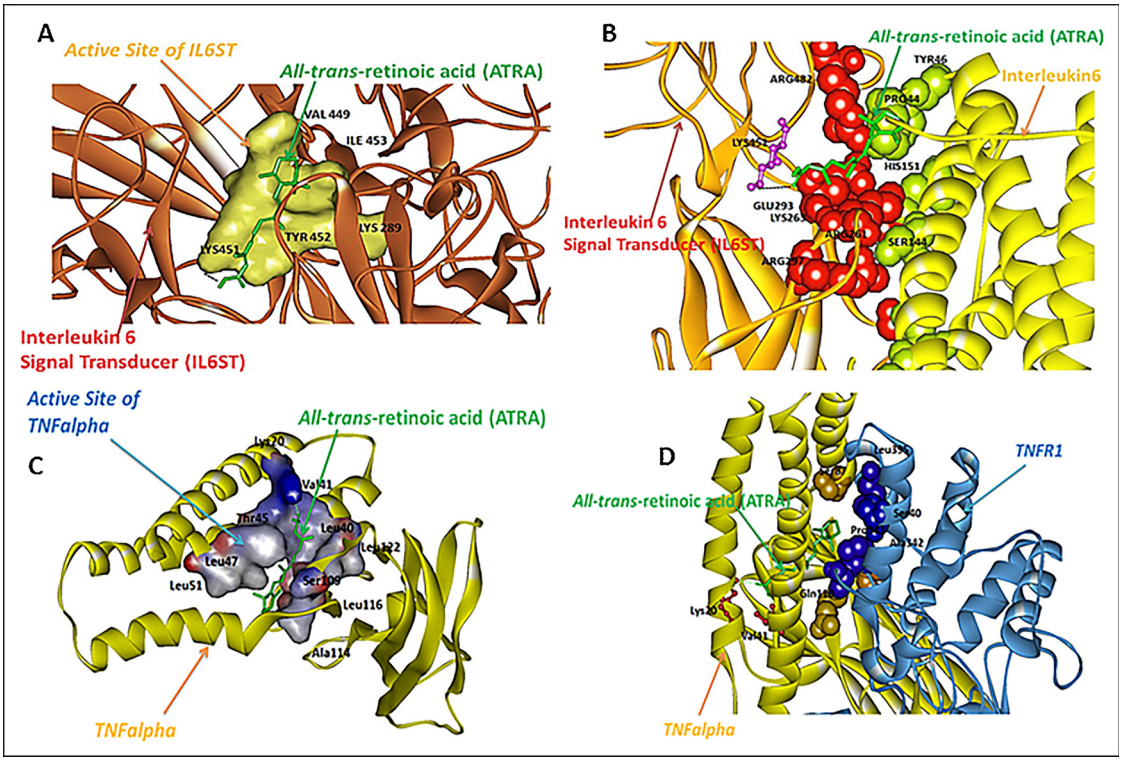

Fig. 2. *In silico* analysis: (A) interaction between ATRA and IL6ST (B) protein-protein interaction between IL6ST and IL6 in presence of ATRA (C) interaction between ATRA and TNF-α (D) protein-protein interaction between TNF-α and TNFR1 in presence of ATRA.

Table 4
Interaction of ATRA with IL6, IL6ST, TNFR1 & TNF-α.

S.No	Receptor	Ligand	Binding Energy (Kcal/Mol)	Ki (Inhibition constant)
1.	Interleukin-6 (IL6)	ATRA	−3.55	2.51mM
2.	Interleukin-6 Signal Transducer (IL6ST)	ATRA	−4.78	315.24μM
3.	TNFR1	ATRA	−5.96	42.58 μM
4.	TNF-α	ATRA	−6.17	30.09 μM

Table 5
Protein-Protein interaction in absence and presence of ATRA.

S. No.	Receptor	Ligand	Fire Dock Binding Score
1.	Interleukin-6 (IL6)	Interleukin-6 Signal Transducer (IL6ST)	−4.90
2.	Interleukin-6 (IL6)	Interleukin-6 Signal Transducer + ATRA (IL6ST + ATRA)	−0.95
3.	TNFR1	TNF-α	−51.16
4.	TNFR1	TNF-α +ATRA	−37.21

IL6ST, JAK2, PTK2B, ABL1 and STAT3 were significantly up-regulated in elastase group (ES) as compared to control group (SS). Interestingly, here, we have again found a potential role of ATRA (i.e. its anti-inflammatory property). The genes which were up regulated due to the elastase treatment were significantly down-regulated after supplementation of ATRA in the therapy group (EA). However, no change in the expression of these genes was obtained in the control group received ATRA (SA) alone. Nevertheless, the mRNA expressions of the inhibitors of STAT3 phosphorylation (PTPN11 & SOCS3) and the inhibitors of activated STAT3 (PIAS3 & PTPase) were found to be significantly down regulated in elastase treated group (ES) than control group (SS), while, the same were up-regulated in the therapy group (EA).

3.7. Effects of elastase and ATRA on antioxidant activity

Changes in lung activities of antioxidant enzymes (i.e. CAT and GPx) in all the experimental groups of the rat were noticed. Administration of elastase significantly depleted the activities of CAT and GPx in elastase group (ES) as compared to the control group (SS). Treatment with ATRA markedly alleviated the effects of elastase and restored the activities of CAT and GPx in the therapy group (EA). In the control group with ATRA (SA) the levels of these antioxidants were similar to that of the control group (SS) (Fig. 7A). Furthermore, the mRNA expression and western blot analysis revealed that the expression of GSTA1 (provides protection against oxidative stress), was significantly down-regulated in elastase group (ES) than to control group (SS). However, it was significantly up-regulated in therapy group (EA), and no change was noticed between control group with ATRA (SA) and control group (SS) (Fig. 7B).

3.8. Effects of elastase and ATRA on mRNA expression of proteases and anti-proteases

Using WLT and AMC, the mRNA expressions of proteases (MMP2, MMP7, MMP8, MMP9, MMP12) and anti-proteases (TIMP1, TIMP2, A1AT) were studied. The mRNA expression of proteases was significantly up-regulated in elastase treated group (ES) as compared to control group (SS), while they were significantly down-regulated in therapy group (EA), due to the effect of ATRA. However, no changes in mRNA expressions of these genes were noticed between control lungs supplemented with ATRA (SA) to that of control group (SS) (Fig. 8A, B).

However, the mRNA expressions of anti-proteases (TIMP1, TIMP2, A1AT) were significantly reduced in elastase treated group (ES) as compared to control group (SS), while, their mRNA expressions were significantly up-regulated in elastase treated lungs received ATRA

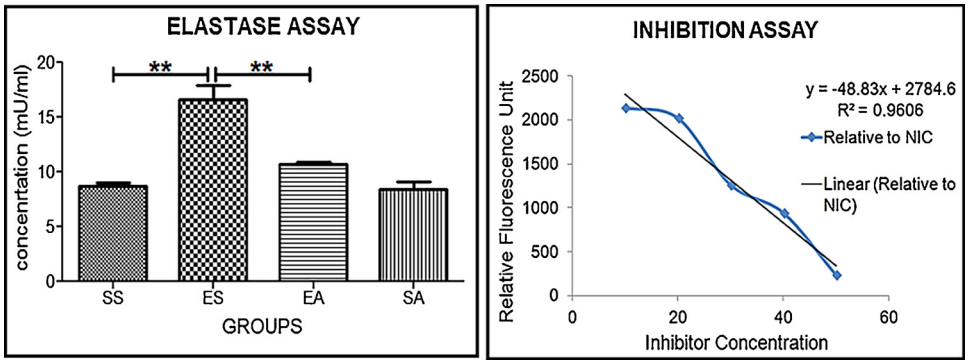

Fig. 3. Elastase activity assay: Elastase treatment significantly induced the endogenous elastase activity in ES group as compared to SS group. ATRA has shown its potential effect in reducing endogenous elastase activity in elastase treated group supplemented with ATRA and is well comparable to SS. While no change in the activity of elastase was seen between SA and SS (Fig. 3A). The inhibition assay in the presence of varying concentrations of its selective inhibitor confirms the proteolytic activity is due to elastase (Fig. 3B). Representative graphs of pooled tissue homogenate samples (n = 6) from each group in triplicates. Data were analyzed by means of unpaired *t*-test to test for the effect of ATRA and elastase, respectively. *p < 0.05; **p < 0.01; ***p < 0.001.

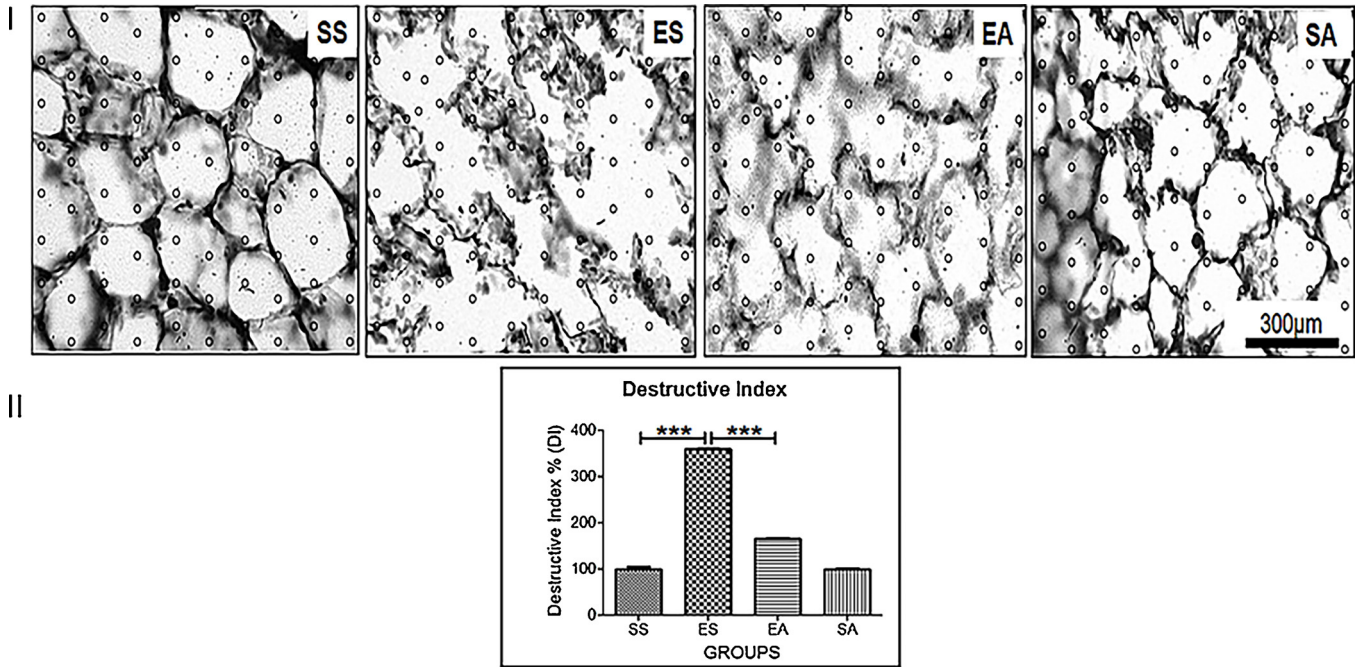

Fig. 4. (I) Histopathology of lung tissue and destruction index analysis: Hematoxylin and Eosin staining of lung tissue sections show normal histology in control group (SS), rarefaction of alveolar septa with dilated airspaces in ES group. Increased number of airspaces with thickened alveolar septa in the therapy group (EA) normal lungs treated with ATRA (SA) alone shows normal histology. All micrographs were captured at identical magnification. From each lung specimen (total 6), an average of 3 different sections was used, and in the sections generally, 3 representative non-overlapping fields were selected. (II) Statistical analysis of the DI. Destructive Index was calculated by laying a transparent sheet over the printed digitised image of an HE-stained section and marking 80 equally distributed points onto it. In comparison to the control group (SS), a significant increase in percentage DI was observed in the group of elastase treated lungs, while the same was reduced upon supplementation of ATRA in ES treated lungs. No change in DI was seen in the control group received ATRA alone. Graphs indicate mean values with standard deviation. Data were analyzed by means of unpaired *t*-test to test for the effect of ATRA and elastase, respectively. *p < 0.05; **p < 0.01; ***p < 0.001.

group (EA), and with no change in mRNA expressions of anti-proteases between healthy lungs received ATRA (SA) and control group (SS) (Fig. 8A, B).

3.9. Effects of elastase and ATRA on protein expression of proteases and anti-proteases

The western blot analysis of proteases (MMP2, MMP7, MMP8, CTSE) reveals that their protein expressions were remarkably induced in elastase treated group (ES) as compared to control group (SS). Nevertheless, ATRA treatment brought their expressions down to the normal in therapy (EA) group than elastase treated group (ES). While no changes in protein expressions were noticed between control group with ATRA (SA) and control group (SS) (Fig. 9).

The western blot analysis of anti-proteases (SERPINA1, TIMP4) revealed that their expressions were significantly down-regulated in emphysema group (ES) as compared to control group (SS), while, they were significantly up-regulated in therapy group (EA). In the control group with ATRA (SA) the expressions were similar to that of a control group (SS) (Fig. 9).

3.10. Effects of elastase and ATRA on enzymatic activity of MMP-2 and TIMP-1 in BAL fluid

In BAL fluid, enzymatic activity was detectable for MMP-2 (MW-72 kD) by zymography (Gel 1) and TIMP-1 (MW-23 kD) by reverse zymography (Gel 2), respectively (Fig. 10A, B). Densitometry based quantitative analysis of gels revealed that the activity of MMP-2 (Graph 1) was significantly increased and activity of TIMP-1 (Graph 2) was significantly reduced in emphysematous group (ES) when compared to control group (SS). However, significantly reduced activity of MMP-2 and significantly increased the activity of TIMP-1 was observed in therapy group (EA) when compared to emphysematous group (ES). In the control group with ATRA (SA) the enzyme activities were

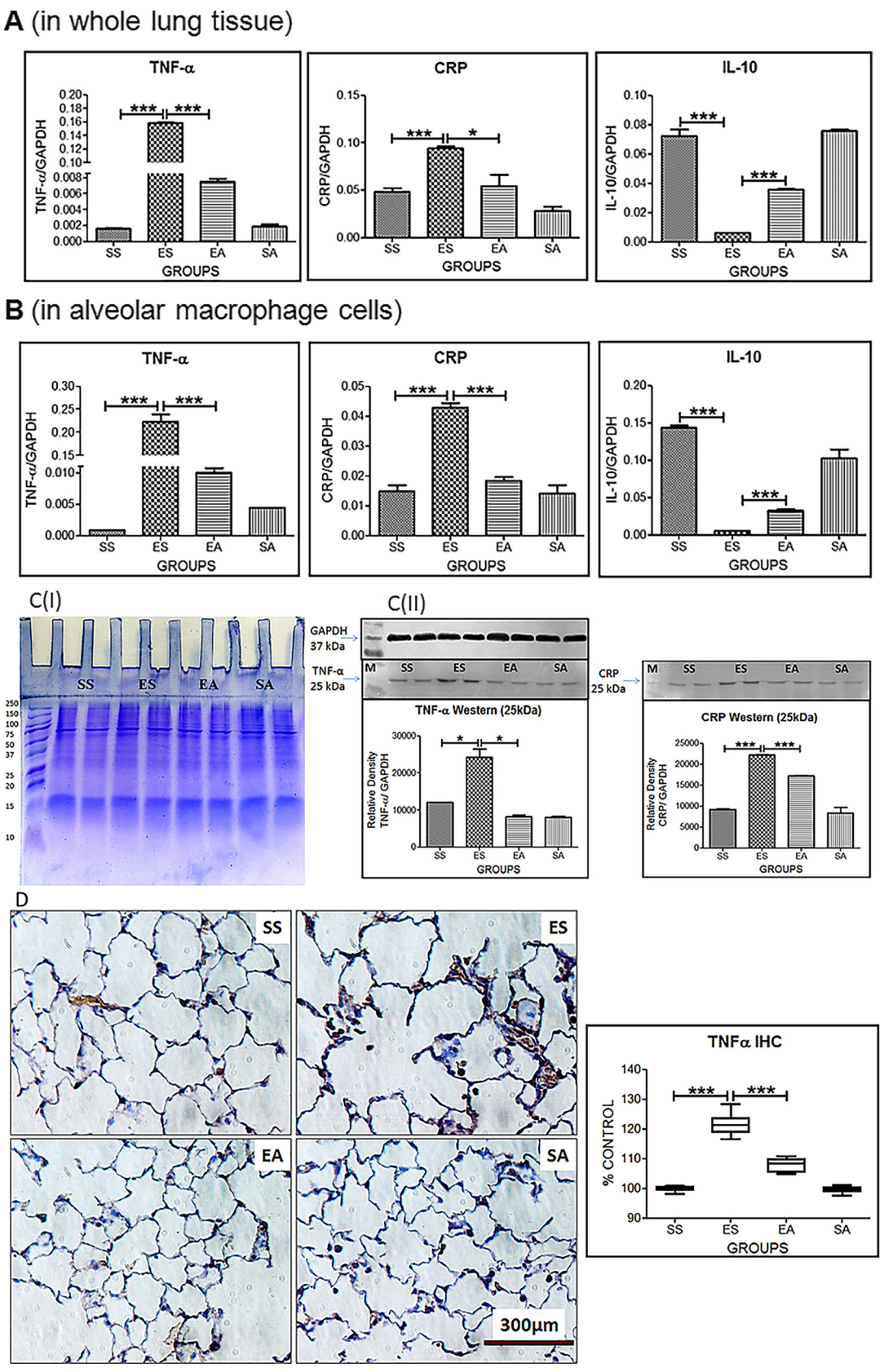

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Fig. 5. A, B: Relative mRNA expression of TNF-α, CRP and IL-10: In WLT and AM cells: The mRNA expression of inflammatory marker TNF-α and CRP was up-regulated and anti-inflammatory molecule IL-10 was down regulated in ES group as compared to SS group. The expression of TNF-α and CRP was down-regulated, while for IL10 it was increased, in therapy group (EA) than ES group and were comparable to the SS group. In the SA group, the mRNA expressions of these genes were similar to the SS group. Representative graphs of pooled cDNA samples (n = 6) from each group in triplicates. Graphs indicate mean values with standard deviation. Data were analyzed by means of unpaired *t*-test to test for the effect of ATRA and elastase, respectively. *p < 0.05; **p < 0.01; ***p < 0.001. C (I) SDS page loaded with equal (50ug) amount of protein and stained by coomassie blue stain. (II) Protein expression of TNF-α and CRP in WLT: The western blot analysis represents a significant increase in the protein expression of TNF-α and CRP in ES group as compared to the SS group. However, in the EA group, a significant decrease in the protein levels of TNF-α and CRP was noticed. Representative blots of pooled protein samples (n = 6) from each group in duplicates. Graphs indicate mean values with standard deviation. Data were analyzed by means of unpaired *t*-test to test for the effect of ATRA and elastase, respectively. *p < 0.05; **p < 0.01; ***p < 0.001. D. Immuno-histochemistry: The immunohistochemistry revealed induced DAB positive staining for TNFα in the alveolar epithelium in emphysema group when compared to control group. ATRA supplementation reduced the expression of TNFα in the alveolar epithelium in the therapy group. No significant changes were observed in the group receiving ATRA only. Data were analyzed by means of unpaired *t*-test to test for the effect of ATRA and elastase, respectively. *p < 0.05; **p < 0.01; ***p < 0.001.

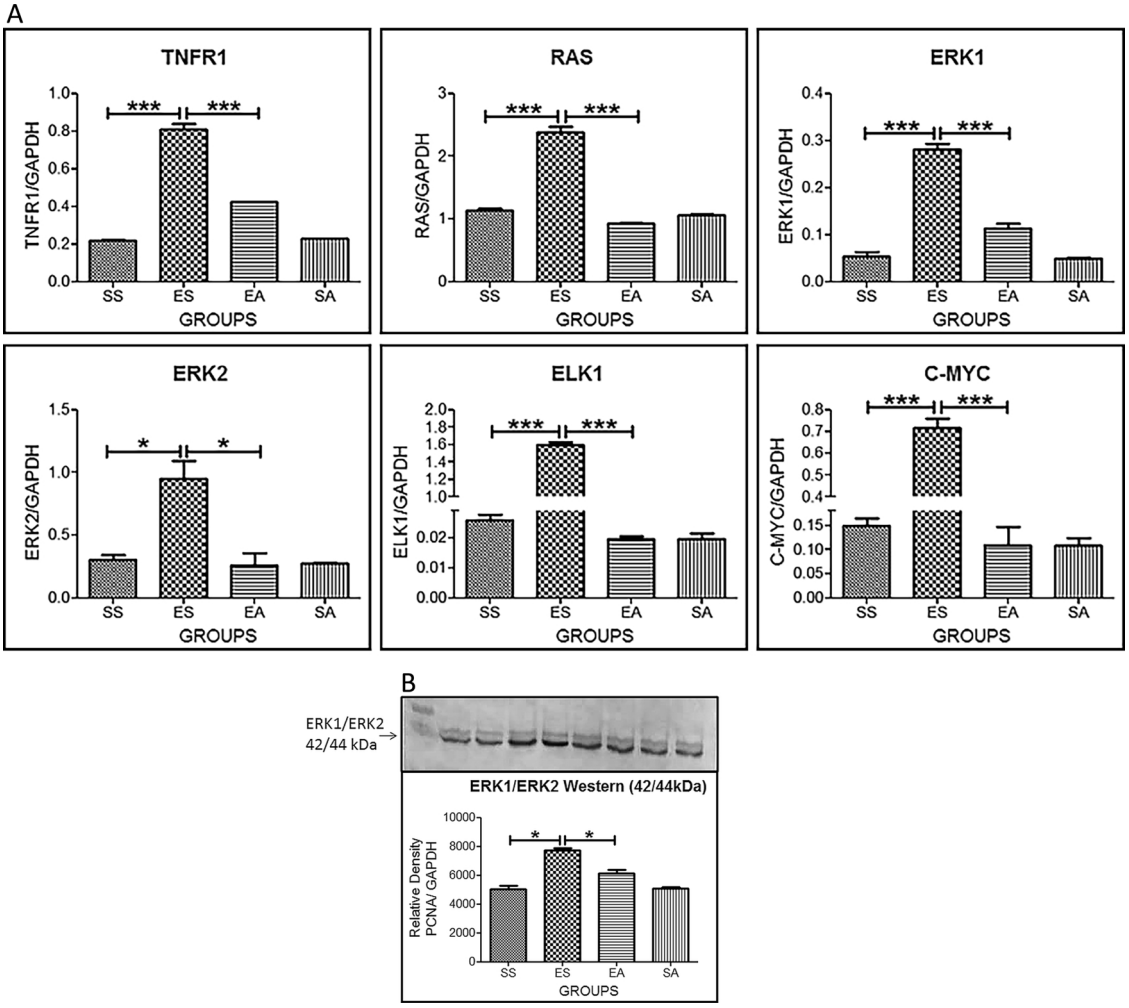

Fig. 6. A: Relative mRNA expression of key gene transcripts of ERK pathway: The mRNA expression of TNFR1, RAS, ERK1, ERK2, ELK1 and c-MYC were up-regulated in ES group compared to SS group. The expressions of these genes were down-regulated in EA group and were comparable to SS group. In SA group, the mRNA expression of these genes was almost similar to SS group lungs. Graphs indicate mean values with standard deviation. Representative graphs of pooled cDNA samples (n = 6) from each group in triplicates. Data were analyzed by means of unpaired *t*-test to test for the effect of ATRA and elastase, respectively. *p < 0.05; **p < 0.01; ***p < 0.001. B: Protein expression of ERK1/ERK2: The western blot analysis represents a significant increase in the expression of ERK1/ERK2 in ES group than the SS group. In EA group, a significant decrease in the protein expression level was noticed. Graphs indicate mean values with standard deviation. Representative blots of pooled protein samples (n = 6) from each group in duplicates. Representative graphs of pooled tissue homogenate samples (n = 6) from each group in duplicates. Data were analysed by means of unpaired *t*-test to test for the effect of ATRA and elastase, respectively. *p < 0.05; **p < 0.01; ***p < 0.001. Protein was normalized with GAPDH. C: Relative mRNA expression of key gene transcripts of the JAK-STAT pathway: The mRNA expression of IL6, IL6ST, JAK2, PTK2B, ABL1, and STAT3 were increased in ES group compared to SS group, and the same were decreased in EA group and were comparable to SS group. While, the mRNA expression of inhibitors (PTPN11, SOCS3, PIAS3 & PTPase) were down-regulated in the ES group compared to SS group and were up-regulated in EA group and were comparable to the SS group. Graphs indicate mean values with standard deviation. Representative graphs of pooled cDNA samples (n = 6) from each group in triplicates. Data were analyzed by means of unpaired *t*-test to test for the effect of ATRA and elastase, respectively. *p < 0.05; **p < 0.01; ***p < 0.001.

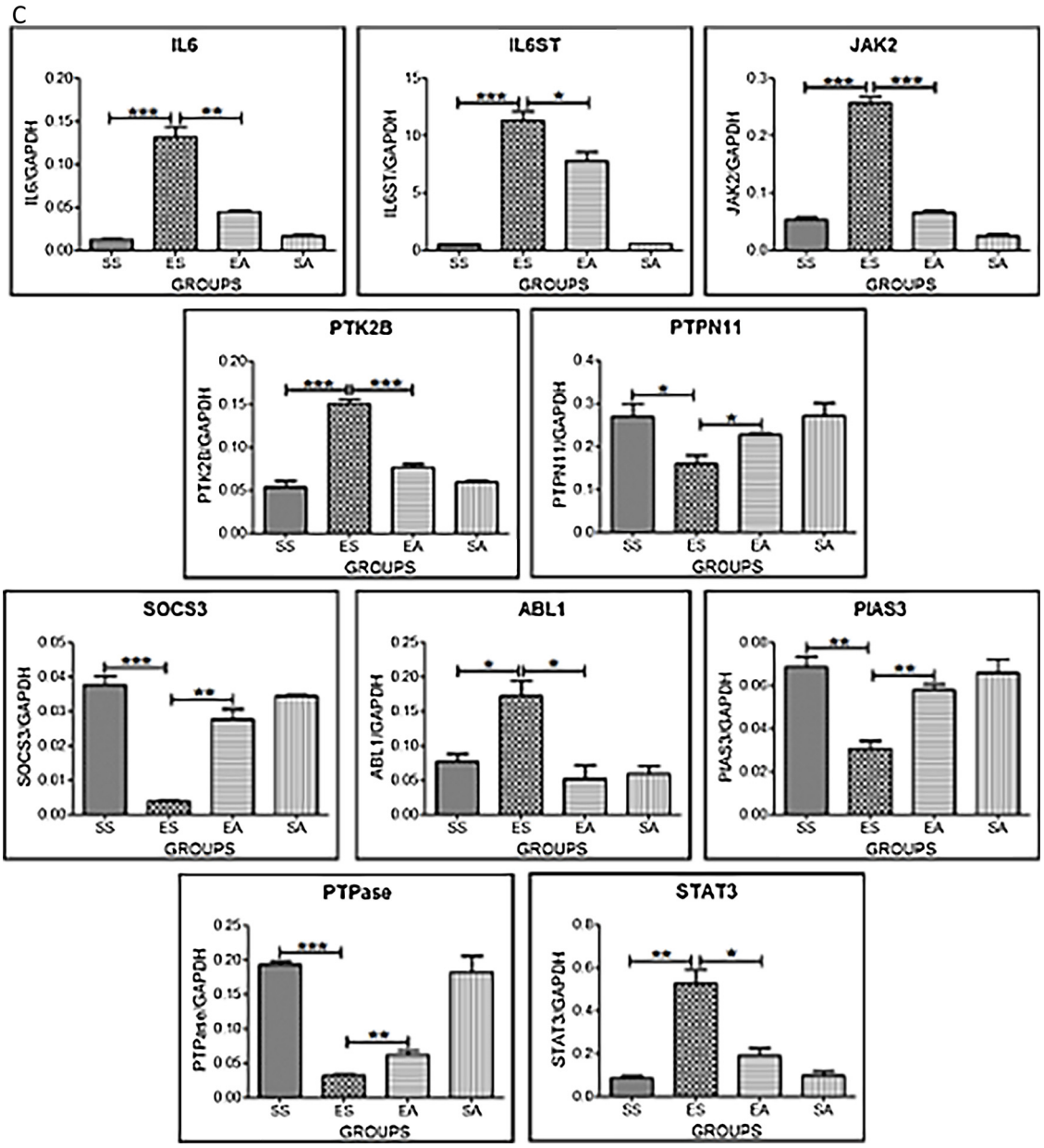

Fig. 6. (*continued*)

comparable to control group (SS).

4. Discussion

To investigate the anti-inflammatory and regenerative potential of ATRA, emphysema and ATRA therapy models were developed. Our *in-silico* results (Fig. 2), provided us the first clue regarding the potential binding of ATRA with those possible receptors, which are essential for triggering signaling mechanisms of ERK and JAK-STAT pathways. *In silico* analysis revealed that ATRA binds to both to the receptor (TNF-α, IL6ST) and ligand (TNFR1, IL6) for both the pathways, ERK and JAK-STAT respectively. However, ATRA shows more binding efficiency towards TNF-α and IL6ST. Also, it was evident from the *in silico* analysis that ATRA interferes between the normal binding of a ligand with the receptor (TNF-α with TNFR1 & IL6 with IL6ST) as the binding energy got reduced in presence of ATRA. Thus suggesting that ATRA has the potential to hinder these pathways at the initial steps and regulate the entire pathway. To validate our *in silico* findings, we further elucidated ATRA's potential role in ERK and JAK-STAT signaling pathways as these pathways are involved in initiating inflammation [8,13], using emphysema's animal models.

Here, emphysema was developed in the lungs of rats by the oropharyngeal instillation of porcine pancreatic elastase (PPE), which is a low-cost approach and its single administration may rapidly result in histological and morphological characteristics compatible with those of panacinar emphysema [43,44]. In the current study, the foremost step is to confirm the efficacy of PPE towards elastolytic damage in the lungs. Using lungs tissue homogenate, an elastase assay was performed. The result of elastase activity assay reveals an increased elastase activity in the ES group as compared to the SS group. We have reported similar findings in the lungs of eight weeks old C57BL/6 male mice [39]. However, the important finding here is that ATRA reduces the elastase activity in EA group, which might be due to the potential of ATRA to prevent neutrophil influx into the lungs, thus preventing the elastolytic damage in lungs [45,46]. Nevertheless, a study conducted by Frankenberger et al., ATRA supplementation has been found to reduce the levels of elastase in the sputum of patients with severe emphysema [47]. Along with elastase assay, an inhibition assay was carried out

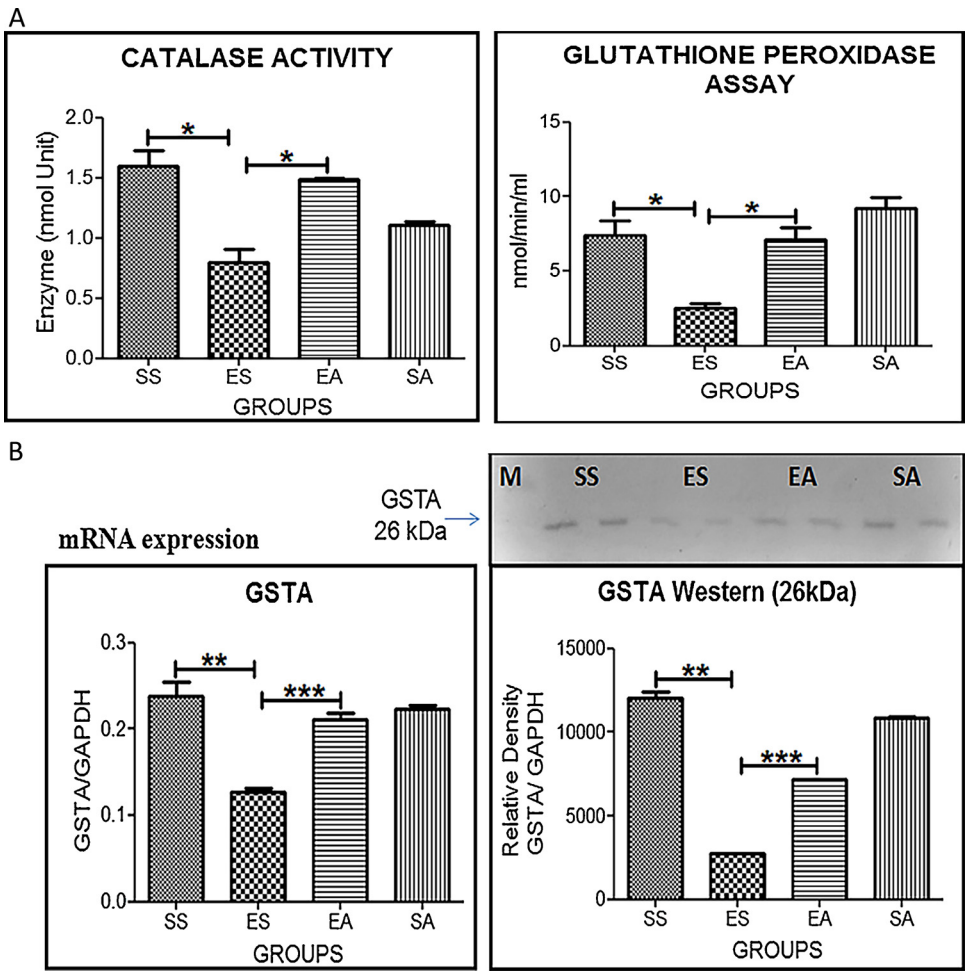

Fig. 7. A: Effects of elastase and ATRA on antioxidant level: Significant depleted levels of CAT and GPx can be seen in ES group in contrast to SS group. Upon ATRA supplementation a rise of CAT and GPx levels can be observed in EA group, which are well comparable to SS group. The CAT and GPx activity levels in SA group were similar to SS group. Graphs indicate mean values with standard deviation. Representative graphs of pooled tissue homogenate samples (n = 6) from each group in triplicates. Data were analysed by means of unpaired *t*-test to test for the effect of ATRA and elastase, respectively. *p < 0.05; **p < 0.01; ***p < 0.001. B: mRNA and protein expression of GSTA1: The western blot analysis and mRNA expression represents a significant decrease in the expression of antioxidant marker GSTA1 in ES group than SS group. In EA group, significant increase in the protein expression level was noticed. Graphs indicate mean values with standard deviation. Representative blots of pooled protein samples (n = 6) from each group in duplicates. Representative graphs of pooled tissue homogenate samples (n = 6) from each group in duplicates. Data were analysed by means of unpaired *t*-test to test for the effect of ATRA and elastase, respectively. *p < 0.05; **p < 0.01; ***p < 0.001. Protein was normalized with GAPDH.

using a selective inhibitor of elastase to confirm that the elastase activity was due to elastase and not any other protease.

The inflammatory responses are involved in triggering proteases in emphysema condition. Our results are in accordance with the general perception theory of proteases/anti-protease imbalance in emphysema condition. The proteases levels were induced while antiproteases levels were reduced in elastase treated lungs. Interestingly, ATRA has shown its potential towards reciprocation of proteases and antiproteases imbalance. MMP2, MMP7, MMP9, and MMP12 are the chief metalloproteases involved in the pathogenesis of emphysema [48]. Protease inhibitors (TIMP1 & TIMP2) maintains the levels of proteases [49], however, due to increased protease burden, the antiproteases are neutralized leading to the protease- antiprotease imbalance, while A1AT is a potent inhibitor of neutrophil elastase. When the levels of A1AT decrease significantly, it fails to protect the lower respiratory tissue from neutrophil elastases, leading to alveolar destruction and ultimately emphysema [50]. Here, the mRNA and protein levels of A1AT were decreased in elastase treated lungs as compared with the control ones. In a study conducted by Zhang et al., increased expression of CTSE, a protease was found to promote emphysema in the lung tissue of COPD patients and upon inhibition of CTSE, emphysema was also inhibited [51]. Similarly in our study elevated level of CTSE was found in emphysema group which was reduced in the ATRA supplemented group.

The potential anti inflammatory role of ATRA has been further studied in TNF-α, CRP and IL-10 expressions. TNF-α is an inflammatory cytokine is predominately produced by and involved in the upregulation of inflammatory reactions. CRP is associated with emphysema and is known to activate pro-inflammatory cytokines such as TNF-α [52]. We found expressions of TNF-α and CRP upregulated in elastase treated lungs while ATRA has shown its positive signature by reducing the expression of TNF-α and CRP & brought it to the normal level. On the other hand, mRNA and protein expression of IL-10, which an important anti-inflammatory molecule, found to be reduced in elastase treated lungs than healthy lungs. The expression of IL-10 has been reported significantly reduced in COPD patients [53]. Here, such a reduction in the mRNA and protein expression of IL-10 due to elastase treatment has been induced by ATRA supplementation. This result further suggests us that ATRA has a potential characteristic through which the expressions of these genes were ameliorated and were comparable to our normal group. Wu J et al. have previously also shown in their study that ATRA treatment moderately increased the mRNA levels of IL-10 as well as attenuating the airway inflammation in experimental allergic asthmatic BALB/c mice [54].

During inflammation, there is activation of leukocytes and neutrophils that release reactive oxygen species (ROS) which ultimately leads to oxidative stress. Oxidative stress is a well recognized feature of COPD [55]. Anti-oxidants play a crucial role in protecting the tissues from the oxidative damage. Glutathione peroxidase (GPx) and catalase (CAT) present in the epithelium lining of lungs and alveolar cells protect the lung tissue from oxidative damage [56]. To evaluate the oxidative stress we performed biochemical assays for glutathione and catalase activity. Our results suggested a depleted activity of GPx as well as of CAT in the elastase treated group when compared to the control group. Reduced GPx activity is in direct proportion to the severity of COPD [57]. Catalase activity has also been reported to be reduced in COPD patients [58]. However, after ATRA supplementation the activities of GPx and CAT increased significantly in ATRA supplemented group as compared to the emphysematous group. Glutathione S

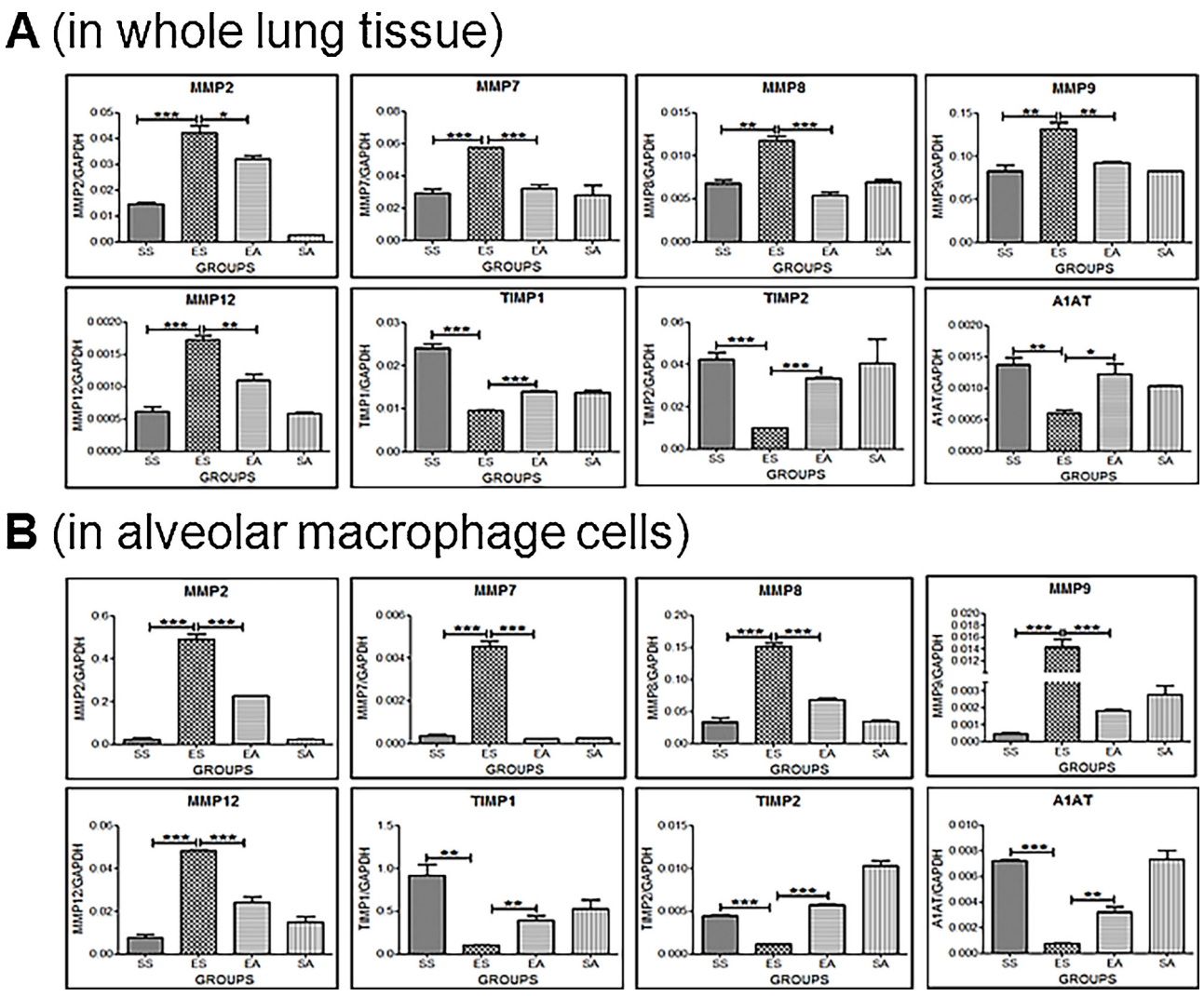

Fig. 8. Relative mRNA expression of proteases and anti-proteases: In WLT (A) and AMC (B), the mRNA expression of proteases MMP2, MMP7, MMP8, MMP9 and MMP12 were significantly up-regulated and anti-proteases TIMP1, TIMP2 and A1AT were significantly down-regulated in ES group as compared to SS group. In therapy group (EA), the mRNA expression of MMP2, MMP7, MMP8, MMP9 and MMP12 were down-regulated and TIMP1, TIMP2 and A1AT were up-regulated than ES group. In SA group, the mRNA expressions of these genes were similar to that of SS group. Graphs indicate mean values with standard deviation. Representative graphs of pooled cDNA samples (n = 6) from each group in triplicates. Data were analyzed by means of unpaired *t*-test to test for the effect of ATRA and elastase, respectively. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

Transferase (GSTA) belongs to an enzyme family which is crucial in protecting the lungs from oxidative injury and is studied as a marker of oxidative stress [59]. Our study reveals the reduced protein expression of GSTA1 in emphysematous lungs as compared to the control group lungs. GSTA1 has been reported to be downregulated in COPD patients [60]. However, after ATRA supplementation the levels of GSTA1 were significantly upregulated in the therapy group as compared to the emphysematous group. ATRA has previously shown to upregulate the levels of GSTA in lungs [61]. The possible reason might be that after ATRA supplementation the alveolar wall was regenerated (which is evident from histopathology result, Fig. 4) and the production of anti-oxidants was restored which was hindered due to alveolar wall destruction in the emphysematous condition. The above findings support the anti-oxidative property of ATRA [62].

Our above-mentioned findings made us believe that exogenous supplemented elastase has resulted in lung inflammation. Therefore, we further studied its effect on the molecular pathways which are known to be involved in the inflammation process underlying emphysema and the potential role of ATRA in ameliorating these pathways. ERK and JAK-STAT pathways were studied as they have been already reported to be involved in the inflammation process in various tissues [6,7,12]. Interestingly, our findings suggested that the ERK and JAK-STAT pathways might be associated with inflammation underlying emphysema. The candidate genes of these pathways were upregulated in ES group, but upon ATRA supplementation the pathways were down-regulated as compared to ES group. ERK pathway is known to be up-regulated in inflammatory conditions and is regulated by proinflammatory cytokine such as TNF-α [6,8]. Similarly, the JAK-STAT pathway is also activated by TNF-α and IL-6. The levels of IL-6 are known to be high in the sputum as well as in the lung tissue of COPD patients which in turn activates STAT3 [63–65]. Moreover, the JAK-STAT pathway is found to be elevated in lung parenchyma tissue of COPD patients [15]. Our study reveals the upregulation of TNF-α (at mRNA as well as at protein level) and IL-6 (at mRNA level) due to elastase treatment. Therefore, the upregulation of the candidate genes of ERK and JAK-STAT pathways might be due to their activation by the enhanced levels of TNF-α and IL-6. However, the anti-inflammatory response of ATRA [66,67] supplementation was able to significantly down-regulate the levels of TNF-α & IL-6 as well as overall ERK & JAK-STAT pathways in elastase induced emphysema. ATRA has been found to inhibit the production of TNF-α from macrophages and inhibits the production of IL-6 via inhibition of ERK pathway [68,69]. Such findings

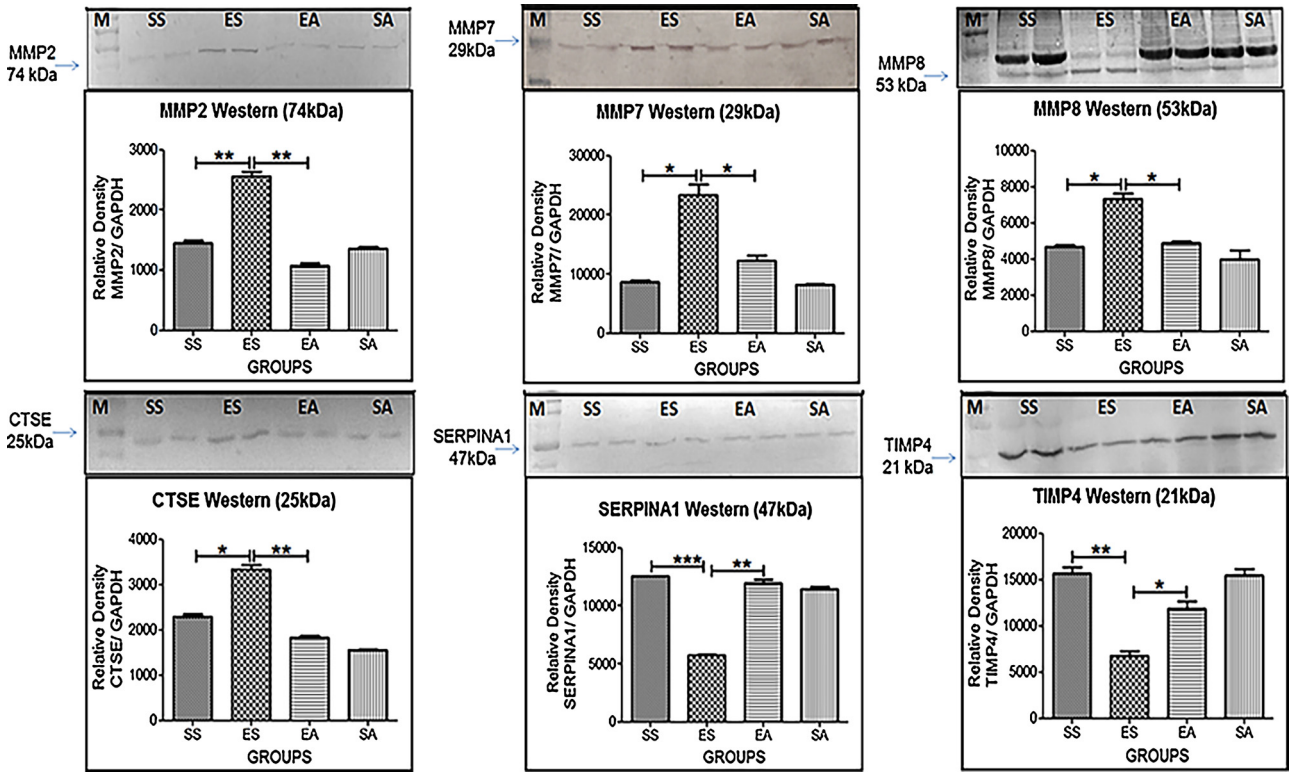

Fig. 9. Protein expression of proteases and anti-proteases: The western blot analysis represents a significant increase in the protein expression of MMP2, MMP7, MMP8 & CTSE, while a significant decrease in SERPINA1 and TIMP4, in ES group as compared to SS group. However, in EA group there is a significant decrease in the protein levels of MMP2, MMP7, MMP8 & CTSE, while a significant increase in SERPINA1 and TIMP4 was noticed. Graphs indicate mean values with standard deviation. No significant changes were observed in SA group and it was well comparable to SS group. Representative blots of pooled protein samples (n = 6) from each group in duplicates. Data were analysed by means of unpaired *t*-test to test for the effect of ATRA and elastase, respectively. *p < 0.05; **p < 0.01; ***p < 0.001.

regarding the involvement of ATRA in the regulation of ERK pathway may confirm our findings as we also obtained a significant down-regulation of ERK pathway due to ATRA supplementation. Furthermore, on the basis of above findings, it is also evident that there is a crosstalk between the ERK and JAK-STAT pathways as TNF-α is a common activator of both the pathways and downregulation of IL-6 expression is dependent on ERK inhibition by ATRA (Fig. 1). ERK and JAK-STAT pathways are also involved in oxidant anti-oxidant imbalance and protease-antiprotease imbalance along with inflammation. Cigarette smoke is one of the causative agents for induction of COPD. ERK pathway is activated by smoke derived oxidants [70]. Similarly, the JAK-STAT (JAK2/STAT3) pathway is also activated by oxidants like H2O2 in diseases like acute respiratory distress syndrome, Parkinson's disease, pulmonary fibrosis, and Alzheimer's disease [71,72]. In COPD, excessive activation of the JAK-STAT pathway leads to increased MMP9 expression and reduction in TIMP1 expression [73].

Lastly, the effect of elastase and the potential role of ATRA were observed on tissue architecture by histopathology and morphometry based DI analyses. The photomicrographs showed the destruction of alveolar septa in the lungs of elastase treated lungs as compared to the lungs of healthy rats. Amusingly, ATRA has shown its therapeutic potential role in elastase treated lungs (therapy group), as alveolar septa which has been lost on elastase treated group is now been regenerated and were well comparable to the lungs of the healthy group. Damage and recovery of alveolar septa in lungs was analyzed by DI based analysis tool, which is an alternative tool for morphological quantification [74]. The average value of DI was higher in case of emphysematous lungs as compared to healthy lungs which may be due to the destruction of alveolar septa and formation of large spaces. Massaro and Massaro have demonstrated that exogenous application of ATRA promotes alveolarisation in elastase-induced experimental model (rat) of emphysema [25] and ATRA induces the alveolar septal formation [30,75]. However, the underlying mechanism behind alveolar septal regeneration is still unknown. Our morphometry result is similar to the Massaro findings, where ATRA supplemented rat lungs represented the alveolar regeneration resulting in intact alveolar septa indicated by significantly reduced DI which was comparable to healthy lungs. The DI values of ATRA only group were similar to the control group.

5. Conclusion

The current study focused on the regenerative and anti-inflammatory potential of ATRA. Pathophysiologies involved in the progression of emphysema are interlinked. Inflammation further leads to protease-antiprotease imbalance and oxidative stress. It was evident from the results that ATRA was able to reduce inflammation via the amelioration of ERK and JAK/STAT pathway along with the down-regulation of inflammatory markers. Protease and antiprotease balance and levels of antioxidants were also restored. These results suggest that if inflammation is reduced in the emphysematous condition it can overall ameliorate the pathophysiologies involved. The histopathological results showed that exogenous supplemented ATRA maintained the tissue architecture, thus indicating the regenerative capacity of ATRA. However, elaborated studies need to be conducted to further explore the regeneration mechanism.

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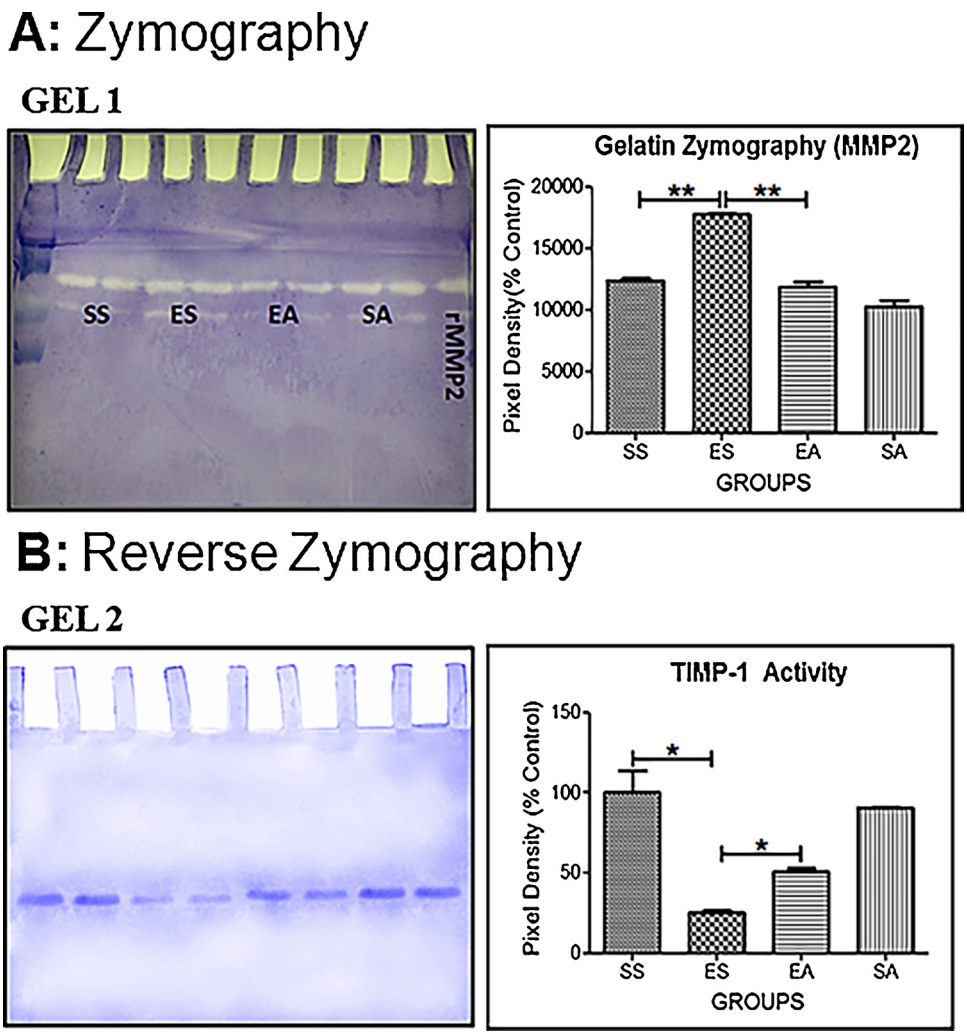

Fig. 10. Enzymatic activity of MMP2 and TIMP1: Using bronchoalveolar lavage fluid (BALF), MMP2 activity as assessed by zymogram gel electrophoresis (A). MMP2 activity was noticed highest in ES group as compared to SS group and was significantly reduced in EA group, as revealed by densitometry. Similarly, TIMP1 activity as assessed by reverses zymogram gel electrophoresis (B), which was significantly reduced in ES group as compared to SS group and was significantly up-regulated in EA group. No significant changes were observed in SA group and it was well comparable to SS group. Representative graphs of pooled BALF samples (n = 6) from each group in duplicates. Data were analysed by means of unpaired *t*-test to test for the effect of ATRA and elastase, respectively. *p < 0.05; **p < 0.01; ***p < 0.001.

Declarations of interest

None.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.biopha.2018.09.166.

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