Research Article   **Open Access**

Immuno-modulatory and Therapeutic Effect of Curcumin in an Allergen-sensitized Murine Model of Chronic Asthma

Pramathadhip Paul[1], Sourav Majhi[2], Shinjini Mitra[1] and Ena Ray Banerjee[1*]

[1]Deptartment of Zoology, Immunobiology and Regenerative Medicine Research laboratory, University of Calcutta, Kolkata, West Bengal, India

[2]Department of Zoology, Vidyasagar University, Midnapore, West Bengal, India

*Corresponding author: Ena Ray Banerjee, Deptartment of Zoology, Immunobiology and Regenerative Medicine Research laboratory, University of Calcutta, Kolkata, West Bengal, India, Tel: +913324615445; E-mail: enaraybanerjee@gmail.com

Abstract

Background: Asthma is a chronic allergic disease of the lung and airways, characterized by persistent inflammation and airway hyper-responsiveness. More than 200 million people are affected with severe asthma worldwide. T cells, especially Th2 cells, secreting IL-4, IL-5 and IL-13, are pivotal in orchestrating the disease process. We herein investigated the inhibition mechanism of curcumin, a polyphenol present in turmeric, and its ability to alleviate allergic asthma in C57BL/6J mice.

Methods: Mice were sensitized and challenged with Ovalbumin for 54 days. We investigated cellular infiltration in blood and BALF, assessed levels of serum IgE, assessed structural changes by histological analysis, and determined the levels of expression of relevant genes and proteins.

Results: Our data revealed that curcumin has some anti-inflammatory effect and is successful in ameliorating the disease. We found that curcumin most likely acts by inhibiting the activation of NFκB and its subsequent downstream processes. Our data provided evidence in support of therapeutic application of curcumin in allergic inflammation and airway remodeling.

Abbreviations Ova: Ovalbumin; AHR: Airway Hyper-responsiveness; PB: Peripheral Blood; BALF: Broncho-Alveolar Lavage Fluid; IL: Interleukin; CD: Cluster of Differentiation; IgE: Immunoglobulin E; iNOS: Inducible Nitric Oxide Synthase; TGF-β: Transforming Growth Factor β; NFκB: Nuclear factor κ-light chain enhancer of activated B cells; IFN-γ: Interferon γ; PBS: Phosphate Buffered Saline; PBST: Phosphate Buffered Saline with Tween-20; DMEM: Dulbecco's Modified Eagle Medium; HRP: Horse Radish Peroxidase; IMDM: Iscove's Modified Dulbecco's Medium; FBS: Fetal Bovine Serum; BSA: Bovine Serum Albumen; PCR: Polymerase Chain Reaction; JAK: Janus Kinases; STAT: Signal Transducer and Activator of Transcription

Introduction

Asthma is a major non-communicable disease that occurs throughout the world, regardless the level of development of the country. Over 200 million people suffering from asthma and failure to adhere to proper treatment may lead to death [1]. Chronic allergic asthma is characterized by airway hyper-responsiveness (AHR), broncho-constriction, over production of mucus, redness and breathing trouble. Asthma involves airway obstruction, leading to wheezing, which can vary from mild to life threatening. Preclinical studies reveal that the pathogenesis of allergic asthma involves activation of inflammatory cells, like eosinophils, mast cells, and T helper cells (Th2). Allergic asthma also involves changes in cytokine levels, mainly of interleukin 4 (IL-4), interleukin 5 (IL-5), and interleukin 13 (IL-13), which are secreted by CD4^{+} Th2 cells [2-4]. Studies have demonstrated a central role of the transcription factor GATA3 in the expression of Th2 cytokines, of IL-4 in the generation of IgE, of IL-5 in the promotion of airway eosinophilia, of IL-9 in the recruitment, proliferation and differentiation of mast cells and of IL-13 in the mucus hypersecretion and induction of expression of inducible nitric oxide synthase (iNOS) which generates nitric oxide [3-6]. Airway fibrosis is another characteristic of asthma, where the epithelial basement membrane gets thickened due to eosinophilic infiltration and deposition of collagen. Pro-fibrotic cytokines, like TGFβ, are involved in this process [3].

In the well-known murine model of ovalbumin (Ova)-induced asthma, sensitization and challenge with Ova, lead to increased AHR, increased Th2 cytokines, increased eosinophils, mucus hypersecretion and increased deposition of collagen in the airways. It has been found that exposure to Ova leads to activation of NFκB in the epithelial cells of the airways [7]. The NFκB localizes to the nucleus and activates the transcription of pro-inflammatory genes, like IL-4, IL-13 and iNOS.

Curcumin [(1E, 6E)-1, 7-Bis-(4-hydroxy-3-methoxyphenyl)-1, 6-heptadiene-3, 5-dione] is a curcuminoid present in turmeric (*Curcuma longa*), which belongs to the ginger family. A curcuminoid is a natural phenol-a linear diarylheptanoid with a yellow colour. Curcuminoids mainly consist of curcumin, demethoxycurcumin and bisdemethoxycurcumin. They have anti-oxidant properties, but are not very stable, and have to be stabilized using nanoparticles or micelles [8]. Of the three phenols, curcumin has the best anti-oxidant property. Studies have also shown that, apart from the anti-oxidant properties,

curcumin also has anti-inflammatory, anti-microbial, anti-parasitic and anti-cancer activities [9,10]. It has been reported that curcumin has the ability to inhibit bladder cancer progression and also to protect lung oxidative damage [11,12]. Curcumin is thought to exert its effects by down-regulating IgE, IL-4, IL-5 and IFN-γ [13]. When administered intranasally, curcumin has been found to improve lung function in a murine model of asthma [14].

In this study, we have induced chronic asthma in mice, by treating them with Ova for a period of 54 days, and then used curcumin, administered orally, as a therapeutic agent against asthma.

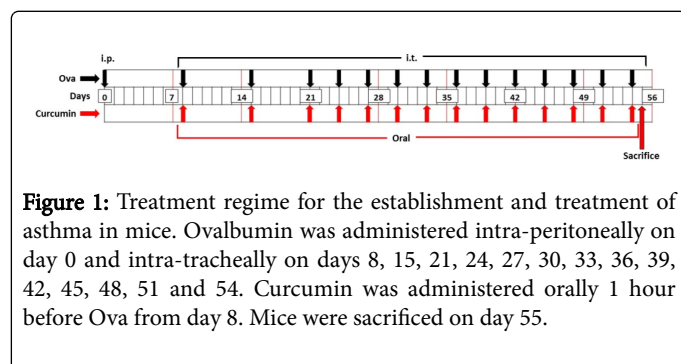## Materials and Methods

Ethical approval

All experiments have been performed according to rules laid down by the Institutional and departmental animal ethics committee, and the animals were housed under specific pathogen free conditions at the animal house of the Department of Zoology, University of Calcutta.

Mice

6-8 weeks old male C57BL/6J mice, weighing 20-25 gm, were divided into three groups, with n=5 for each group: (I) Control, (II) Ova and (III) Ova+C.

Induction of disease and treatment with curcumin

The mice were sensitized and challenged with 100 μg of Ovalbumin (OVA) grade-V, complexed with Al(OH)$_3$ in a 0.2 ml volume [13-15]. Intra-peritoneal injection was administered on day 0. On day 8 (250 μg OVA) and on days 15, 18, 21, 24, 27, 30, 33, 36, 39, 42, 45, 48, 51 and 54 (125 μg OVA) was administered [15]. The mice were anaesthetized briefly by isoflurane in a standard anesthesia chamber. Anaesthetized mice were placed in a supine position and the tongue was extended with lined forceps and 50 μl of Ova (in the required concentration) was placed at the back of its tongue [15]. 125 μg Ova was given intra-tracheally three times a week up to day 54 (chronic phase). Animals were sacrificed on day 55. Control group of this experiment received normal saline (Figure 1). For drug treated groups, 2 μM/Kg curcumin (50 nM), dissolved in DMSO, was given orally one hour before each intra tracheal challenge.

Figure 1: Treatment regime for the establishment and treatment of asthma in mice. Ovalbumin was administered intra-peritoneally on day 0 and intra-tracheally on days 8, 15, 21, 24, 27, 30, 33, 36, 39, 42, 45, 48, 51 and 54. Curcumin was administered orally 1 hour before Ova from day 8. Mice were sacrificed on day 55.

Sacrifice and collection of tissues

Mice were sacrificed on day 55 by cervical dislocation, and the following tissues were collected.

Peripheral blood (PB): 1 ml blood was collected by cardiac puncture in tubes containing EDTA as anticoagulant. A drop of blood was placed on a microscope slide and, using another slide, a smear was prepared.

Bronchoalveolar lavage fluid (BALF): BALF was collected from both lungs, by injecting 1 ml of cold PBS slowly into the lungs, and drawing it out [16]. The BALF was centrifuged at 200 g for 10 minutes at 4°C. The cell pellet was resuspended in fetal bovine serum and a smear was made on a microscope slide.

Lung parenchyma: Immediately after collecting the BALF, the lungs were washed with PBS.

- **For assays:** One lung was taken in a Petri dish and chopped well into digestible pieces. The lung was then digested using a 1X collagenase/hyaluronidase cocktail (Stem Cell Technology) overnight at 37°C. It was then filtered through a no. 60 sieve (Sigma Aldrich) to get a single cell suspension. For estimation of collagen content by hydroxyproline assay, lungs were washed, minced and dried in a speed-vacuum.
- **For gene and protein expression:** Whole lung was taken, washed with PBS and stored at -80°C for further use.
- **For histology:** One lung was collected whole in 10% buffered formalin.

Total cell (TC) count

PB/BALF was mixed with an equal volume of Trypan Blue dye (Himedia, India), and the cell viability was determined using a haemocytometer.

Differential cell (DC) count

The PB/BALF smears were air dried and fixed with methanol (SRL, India). They were stained with Wright-Geimsa stain (SRL, India) for 15 minutes, washed and observed under a light microscope (Dewinter Fluorex LED) at 40X magnification [17]. The cell types were distinguished on the basis of their nuclear morphology.

Ova-specific serum IgE

The levels of Ova-specific IgE in the serum was determined using ELISA, according to the datasheet (BD OptEIA Mouse IgE ELISA Set). Wells of a 96 well, high-binding flat bottom plate (Corning, USA) were coated with 50 mg/ml ovalbumin in sterile PBS overnight at room temperature. Next day, plates were washed three times with PBST (PBS +0.05% Tween 20). The wells were then blocked with 5% skimmed milk in PBS for 1 hour at room temperature, and washed four times with wash buffer. 50 μl plasma samples (1:10 dilution in PBS) were added per well and incubated for 90 minutes at 37°C, then washed four times with wash buffer, and blotted dry by inverting over paper towels. 2 μg of biotin-conjugated rat anti-mouse IgE (clone R35-118, BD Biosciences) was added to each well and incubated overnight at 4°C, then washed four times with wash buffer and blotted dry. 100 μl avidin-HRP (1: 1000 in PBS) (BD Biosciences) was added in each well and incubated at 37°C for 90 minutes, then washed four times with wash buffer and blotted dry. 100 μl of substrate (3, 3', 5, 5', Tetramethylbenzidene; Sigma Aldrich) was added to each well for 30 minutes at room temperature. Absorbance was measured at 405 nm by using ELISA plate reader (Thermo Scientific). The concentration of IgE in each well was calculated using a standard curve prepared using purified IgE (BD Biosciences).

CFU-c assay

Colony forming unit-cell assay was performed using methylcellulose semisolid media (Himedia, India) to evaluate clonogenic potential of the cells of the tissues. Semisolid media was prepared with IMDM (Himedia, India), 30% FBS (Himedia, India), 20 mg/ml BSA (Biosera), 1% Pen-Strep (Himedia, India) and finally 5 ng/ml stem cell factor (Biovision). All constituents, except methyl cellulose, were added in a 50 ml centrifugation tube and vortexed. Finally 1.5% methyl cellulose (Himedia, India) was added to this mixture. 1 ml of the semisolid medium was added to the wells of a 24 well plate (Nest Biotech). 106 cells were added to each well according to treatment groups, and incubated at 37°C in a 5% CO_2 humidified chamber (Thermo Fisher) for 7 days. Generated total colonies were counted per organ against number of plated cells to the total number of cells in the organ.

Flow cytometry

Cells from lung parenchyma were stained with suitable antibodies. Flow cytometry was done on BD FACSVerse (BD Biosciences, USA), and results were analyzed using the FACSSuite (BD Biosciences, USA) software for analysis. Cell surface staining was performed using the following antibodies: CD45-PerCPCy5.5 (BioLegend), CD3e-PE (BD Biosciences, USA), CD4-V450 (BD Biosciences, USA), CD8a-Alexa Fluor 488 (BD Biosciences, USA), B220-FITC (BD Biosciences, USA), GR-1-FITC (MACS), and F4/80-PE (eBiosciences). Among the CD45$^+$ hematopoietic cells, CD45$^+$CD3$^+$ are T cells, and CD45$^+$B220$^+$ are B cells. Of the CD3$^+$ cells, CD3$^+$CD4$^+$ are helper T (TH) cells, and CD3$^+$CD8$^+$ are cytotoxic T (TC) cells. Neutrophils are CD45$^+$Gr1$^+$ and macrophages are CD45$^+$F4/80$^+$ [15].

Gene expression

Total RNA was extracted from lung tissues with TRIzol reagent according to manufacturers' instructions (Life Technologies, California, cat. no. 15596018). Lung tissue was homogenized in 1 ml TRIzol reagent, incubated at room temperature for 5 minutes, 0.2 ml chloroform was added and shaken vigorously for 15 seconds. After incubation at room temperature for 2-3 minutes, it was centrifuged at 12000 g for 15 minutes at 4°C, and the upper aqueous layer transferred to a new tube. 0.5 ml of 100% isopropanol was added to it, incubated at room temperature for 10 minutes, and centrifuged at 12000 g for 10 minutes at 4°C. The pellet was washed with 1 ml of 75% ethanol, vortexed briefly, centrifuged at 7500 g for 5 minutes at 4°C, and the pellet dried, before resuspending it in RNase-free water. It was incubated at 55°C-60°C for 10-15 minutes, before proceeding to cDNA preparation.

cDNA was synthesized from isolated RNA using SuperScript-III Reverse Transcriptase (Life Technologies, California). 50-250 ng of random hexamer primer was mixed with the isolated RNA, 10 mM dNTPs and water were added, and heated to 65°C for 5 minutes, and then incubated on ice for at least 1 minute. To this, 5X buffer, 0.1 M DTT and 200 units/μl SuperScript III RT were added, mixed by gentle pipetting, and incubated at 50°C for 30-60 minutes, after which the enzyme was inactivated by heating at 70°C for 15 minutes.

Real time (qRT)-PCR: Real time PCR was performed by the semi-quantitative SYBR Green assay (Applied Biosystem, USA) using specific primer. Primers were designed according to the RNA sequence published on Gene Bank. The primers used are listed in Table 1A, with β-actin as the internal control.

Reverse transcriptase (RT)-PCR: Gene expression was also analyzed by RT-PCR using gene specific primer (Table 1B). We used different inflammatory cytokines such as IL-5, IL-13, and TGF-β; GAPDH was used as the housekeeping gene. PCR products were run in 1% agarose gel to detect expression of inflammatory genes.

	Method	Gene	Type	Primer sequence	
A.	qRT-PCR primers	β-actin	House-keeping gene	Forward	5'GTGGGCCGCTCTAGGCACCAA
				Reverse	5'CTCTTTGATGTCACGCACGATTTC
		IL-3	Cytokine genes	Forward	5'CCGTTTAACCAGAACGTTGAA
				Reverse	5'CCACGAATTTGGACAGGTTT
		IL-4		Forward	5'GGCATTTTGAACGAGGTCAC
				Reverse	5'AAATATGCGAAGCACCTTGG
		IL-5		Forward	5'ATGGAGATTCCCATGAGCAC
				Reverse	5'AGCCCCTGAAAGATTTCTCC
B.	RT-PCR primers	GAPDH	House-keeping gene	Forward	5'TGTGATGGGTGTGAACCACGA
				Reverse	5'TGCTGTTGAAGTCGCAGGAGAC
		IL-4	Cytokine genes	Forward	5'TCGGCATTTTGAACGAGGTC
				Reverse	5'GAAAAGCCCGAAAGAGTCTC
		IL-5		Forward	5'TCACCGAGCTCTGTTGACAA

				Reverse	5'CCACACTTCTCTTTTTGGCG
		IL-13		Forward	5'GGTCCCTGGCAATATTACTGTAT
				Reverse	5'CCCATTCACTACACATCACCTT
		IFN-γ		Forward	5'CCTCTGAGACAATGAACGCT
				Reverse	5'AAAGAGATAATCTGGCTCTGC
		TGF-β		Forward	5'ACCGCAACAACGCCATCTAT
				Reverse	5'GTAACGCCAGGAATTGTTGC
		iNOS		Forward	5'CCCTTCCGAAGTTTCTGGCAGCAGC
				Reverse	5'GGCTGTCAGAGCCTCGTGGCTTTGG

Table 1: List of primers used for determination of gene expression by real time PCR (A) and reverse transcriptase PCR (B).

Protein expression by Western blot

Lungs were homogenized and total cellular protein was isolated in RIPA buffer containing Tris (pH 7.4) (Amresco), 150 mM NaCl (SRL, India), 1% Triton-X-100 (SRL, India), 0.5% Sodium deoxycholic acid (SRL, India), 0.1% SDS (Merck, USA), 1mM EDTA (SRL, India), 10 mM sodium fluoride (SRL, India), 1 mM PMSF (SRL, India) and 1X protease inhibitor cocktail (Cell Signaling Technology, USA). Cell lysate was incubated on ice for 30 minutes, and residual tissue was removed by centrifugation at 10000 rpm at 4°C for 20 minutes. Supernatant was taken and stored at -80°C for future use. Total protein in the supernatant was estimated by Bradford reagent (Himedia, India). Concentration of total lung protein was determined from a standard graph prepared using BSA. Equal amounts (30 µg) of the lung protein from each group were run in a 12% SDS gel with appropriate marker and transferred onto PVDF membrane (Merck, USA). The membrane was blocked with 5% nonfat dried milk in PBST for 2 hours at room temperature, hybridized overnight with the primary antibody {1:1000 rabbit anti-mouse NFκB (Santa Cruz), 1:2000 rabbit anti-mouse TGFβ (Elabscience), 1:1000 rabbit anti-mouse STAT6 (Santa Cruz) and 1:1000 rabbit anti-mouse GAPDH (Santa Cruz)} at 4°C, and incubated with the secondary antibody {1:10000 goat anti-rabbit IgG-HRP (Santa Cruz)} at room temperature for 2 hours. The membrane was washed with PBST in between hybridizations. The blot was developed using Western ECL substrate (BioRad), and visualized using Azure chemidoc system.

Hydroxyproline assay

The lungs were washed with PBS, minced, processed and hydroxyproline content was assessed, following the protocol by Hofman et al. [18]. Chopped lungs were dried in a speed vacuum and weighed. Dried samples were hydrolyzed overnight in 6N HCl at 110°C. The hydrolyzed sample was dried, and re-suspended in citrate-acetate buffer (sodium acetate trihydrate, citric acid, acetic acid, sodium hydroxide; pH 6.5). Freshly prepared Chloramine-T solution (1.27 gm chloramine T dissolved in 20 ml water, diluted with 30 ml n-propanol and 50 ml citric-acetate buffer) was added to the sample and incubated for 20 minutes, at room temperature. 1.88M perchloric acid was added for 5 minutes at room temperature. Freshly prepared Ehrlich's solution (p-dimethylaminobenzaldehyde in n-propanol) was then added, and the mixture was heated at 65°C for 15 minutes. The samples were cooled and transferred to a 96-well plate. The absorbance of the samples was measured at 550 nm. From a standard graph, concentrations of hydroxyproline in the lung samples were determined.

Histology

Lung tissues were fixed with 10% formalin overnight at room temperature. Tissues were dehydrated because tissues are mainly composed of water. The tissues were passed through a series of increasing concentrations of ethanol, beginning with 50% alcohol and progressing in graded steps to 100%. They were then treated with xylene, a chemical that is miscible with molten paraffin. Tissues were then embedded in paraffin and cut in to 5 µm section. The intensity of cellular infiltration around pulmonary blood vessels was assessed by hematoxylin and eosin staining. Subepithelial pulmonary fibrosis and subsequent collagen depositions were visualized by Masson's Trichrome staining [6].

Statistical analysis

All data were analysed using GraphPad Prism 6. Data are represented as Mean ± SEM, and p values less than 0.05 have been considered to be significant. Statistical significance has been calculated by t-test.

Results

Curcumin inhibited the infiltration of cells in the blood, BALF and lung

Increase in total cell (TC) count in any tissue is an indication of inflammation (Figure 2). The TC of blood increased 5.15 fold (p<0.05) after 54 days of treatment with Ova, which reduced by 1.80 fold with curcumin (Figure 2A). The TC of BALF also increased significantly, by 6.01 fold (p<0.05) with Ova treatment. Curcumin successfully reduced the inflammation by 5.32 fold (p<0.05) (Figure 2B). This was supported by histological analysis of lung sections, stained with hematoxylin and eosin. As compared to Ova treated lung (Figure 2D), curcumin treatment reduced the cell infiltration (Figure 2E).
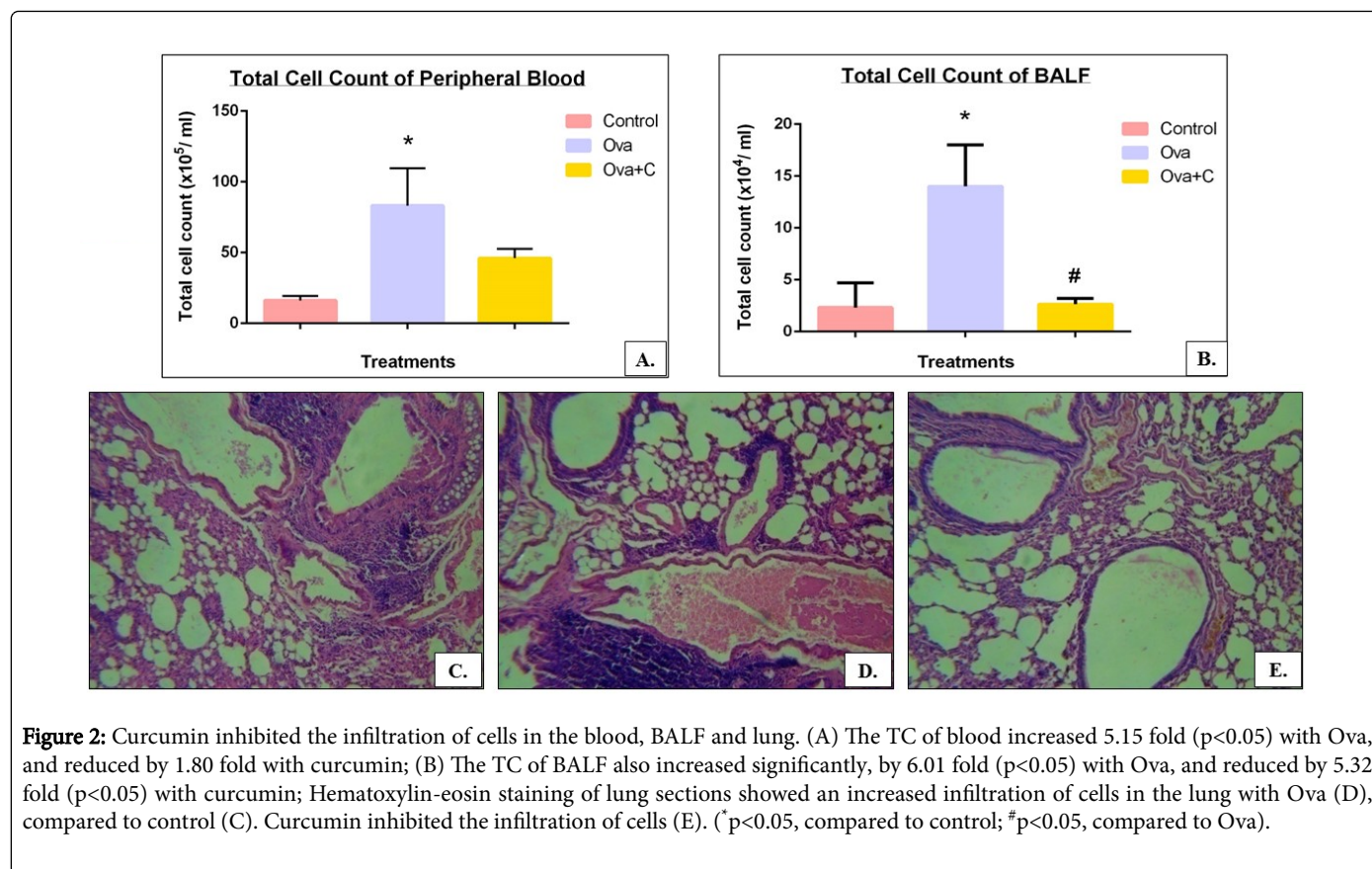
Figure 2: Curcumin inhibited the infiltration of cells in the blood, BALF and lung. (A) The TC of blood increased 5.15 fold (p<0.05) with Ova, and reduced by 1.80 fold with curcumin; (B) The TC of BALF also increased significantly, by 6.01 fold (p<0.05) with Ova, and reduced by 5.32 fold (p<0.05) with curcumin; Hematoxylin-eosin staining of lung sections showed an increased infiltration of cells in the lung with Ova (D), compared to control (C). Curcumin inhibited the infiltration of cells (E). (*p<0.05, compared to control; $^\#$p<0.05, compared to Ova).

Curcumin inhibited eosinophilic infiltration in the blood and BALF

Ovalbumin treatment led to an increase in the count of eosinophils, both in the blood (Figure 3A) and in the BALF (Figure 3B). The count increased by 3.50 fold (p<0.05) in the blood and by 2.50 fold (p<0.05) in the BALF. Curcumin successfully reduced the infiltration of eosinophils, by 1.44 fold (p<0.05) in the blood and by 1.52 fold in the BALF.
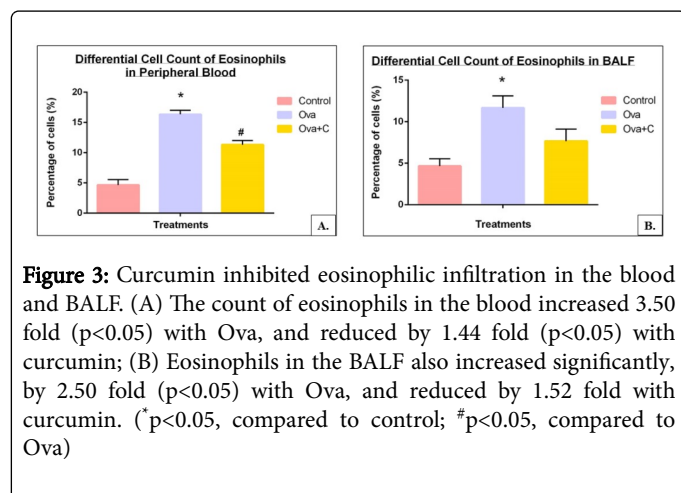

Figure 3: Curcumin inhibited eosinophilic infiltration in the blood and BALF. (A) The count of eosinophils in the blood increased 3.50 fold (p<0.05) with Ova, and reduced by 1.44 fold (p<0.05) with curcumin; (B) Eosinophils in the BALF also increased significantly, by 2.50 fold (p<0.05) with Ova, and reduced by 1.52 fold with curcumin. (*p<0.05, compared to control; $^\#$p<0.05, compared to Ova)

Curcumin successfully reduced levels of Ova-specific IgE in serum

Increase in levels of serum IgE is one of the main symptoms of asthma. Treatment with Ova led to a 63.88 fold increase (p<0.05) in the levels of Ova-specific IgE in the serum, compared to the control. Curcumin successfully reduced the level by 1.80 fold (p<0.05) with respect to Ova (Figure 4).

Curcumin restored the clonogenic potential of cells

Clonogenic potential, the ability of cells to proliferate and form colonies, was assessed by the CFU-c assay of PB (Figure 5A), bone marrow (Figure 5B) and lung (Figure 5C). Ova treatment led to a decrease in the clonogenic potential, by 1.95 fold, by 1.40 fold and by 1.20 fold in the PB, BM and lung respectively. While curcumin restored the clonogenic potential by 1.29 fold in the blood, and by 1.16 fold in the bone marrow, the restoration was significantly higher in the lung (1.48 fold, p<0.05).

Curcumin reduced the population of immune cells in the lung

Of the total number of cells in the control lung, the population of CD45$^+$ increased by 1.12 fold with Ova treatment, and decreased by 1.80 fold with curcumin. Of these CD45$^+$, 12.59% were also B220$^+$ (B cells), 1.06% were also CD3$^+$ (T cells), 12.08% were also GR1$^+$ (neutrophils) and 7.35% were also F4/80$^+$ (macrophages) in the control.

Ova treatment led to a 1.13 fold increase in the CD45$^+$B220$^+$ population, and to a 2.07 fold increase in the CD45$^+$CD3$^+$ T cell population (Figure 6A). The CD45$^+$GR1$^+$ population increased by 1.27 fold and the CD45$^+$F4/80$^+$ population increased by 1.45 fold (Figure 6B). Curcumin treatment successfully reduced all the populations. CD45$^+$B220$^+$ decreased by 1.37 fold, CD45$^+$CD3$^+$ decreased by 1.11 fold, CD45$^+$GR1$^+$ decreased by 1.99 fold and CD45$^+$F4/80$^+$ decreased by 1.68 fold.
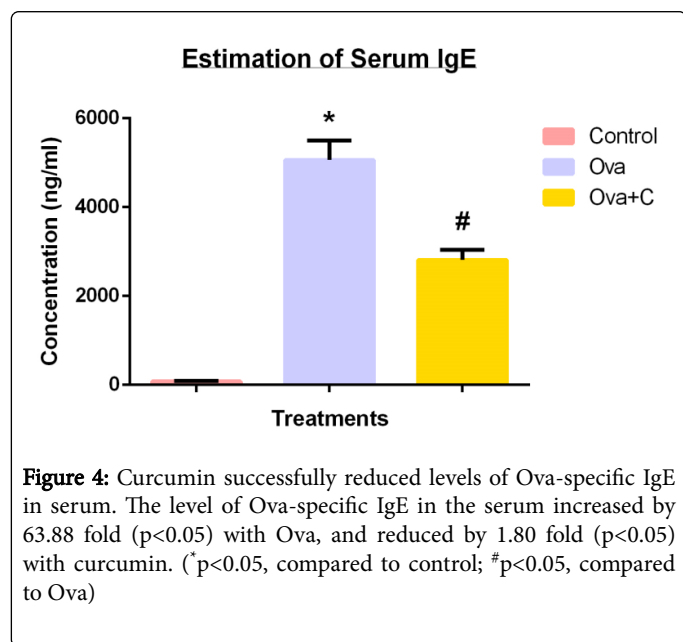
Figure 4: Curcumin successfully reduced levels of Ova-specific IgE in serum. The level of Ova-specific IgE in the serum increased by 63.88 fold ($p<0.05$) with Ova, and reduced by 1.80 fold ($p<0.05$) with curcumin. ($^*p<0.05$, compared to control; $^#p<0.05$, compared to Ova)
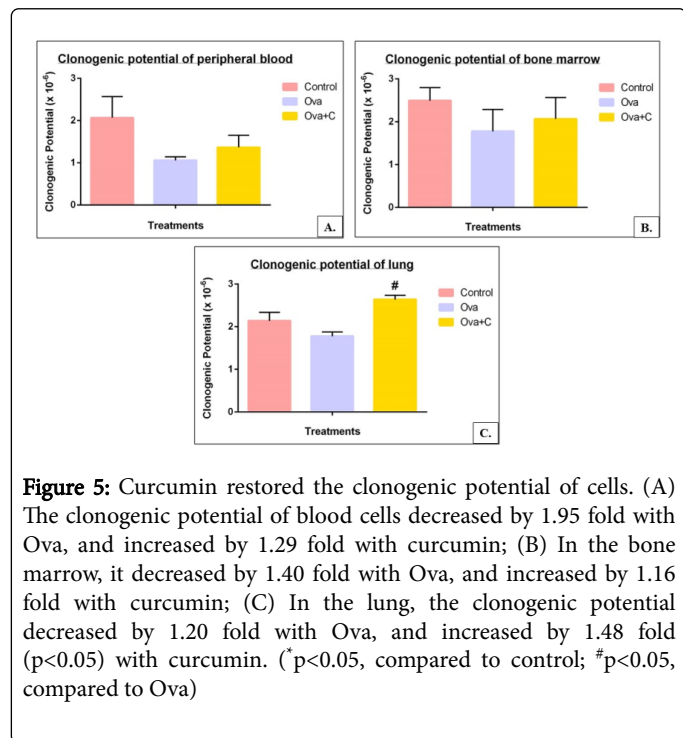

Figure 5: Curcumin restored the clonogenic potential of cells. (A) The clonogenic potential of blood cells decreased by 1.95 fold with Ova, and increased by 1.29 fold with curcumin; (B) In the bone marrow, it decreased by 1.40 fold with Ova, and increased by 1.16 fold with curcumin; (C) In the lung, the clonogenic potential decreased by 1.20 fold with Ova, and increased by 1.48 fold ($p<0.05$) with curcumin. ($^*p<0.05$, compared to control; $^#p<0.05$, compared to Ova)
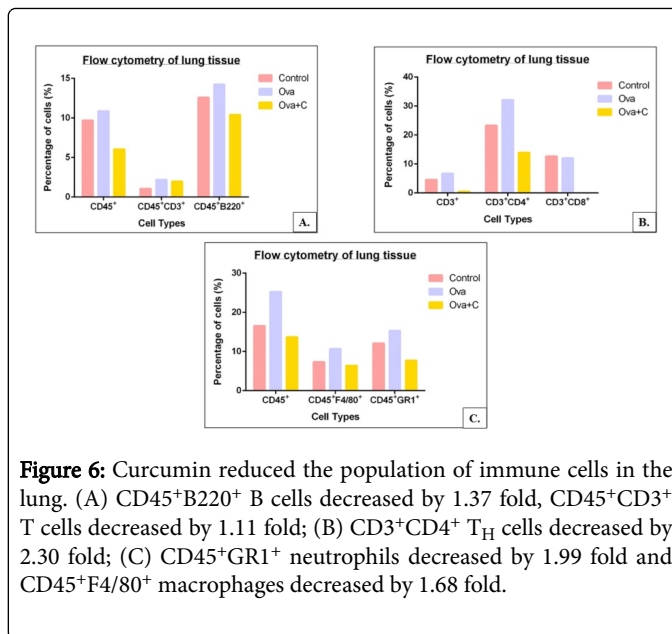

Figure 6: Curcumin reduced the population of immune cells in the lung. (A) CD45$^+$B220$^+$ B cells decreased by 1.37 fold, CD45$^+$CD3$^+$ T cells decreased by 1.11 fold; (B) CD3$^+$CD4$^+$ T$_H$ cells decreased by 2.30 fold; (C) CD45$^+$GR1$^+$ neutrophils decreased by 1.99 fold and CD45$^+$F4/80$^+$ macrophages decreased by 1.68 fold.
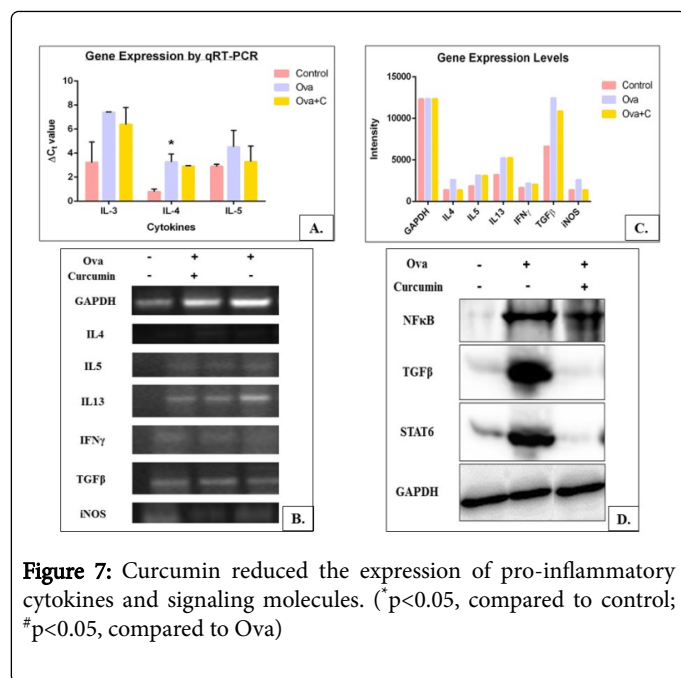
Of the CD45$^+$CD3$^+$ cells, 23.33% were also CD4$^+$ and 12.66% were also CD8$^+$ in the control (Figure 6C). The CD3$^+$CD4$^+$ T$_H$ cell population increased by 1.38 fold with Ova, and decreased by 2.30 fold with curcumin. There was not much change in the population of CD3$^+$CD8$^+$ T$_C$ cells.

Curcumin reduced the expression of pro-inflammatory cytokines and signaling molecules

Real time (qRT)-PCR: The relative expression of the target gene versus β-actin was determined using "$2^{-\Delta CT}$" where ΔCT represents the difference between the CT (threshold cycle) value of the target gene and the CT value of β-actin (Figure 7A). With Ova treatment, the levels of IL-3, IL-4 and IL-5 increased by 2.30 fold, 4.14 fold ($p<0.05$) and 1.56 fold respectively, as compared to the control. The expressions of IL-3, IL-4 and IL-5 decreased by 1.16 fold, by 1.13 fold, and by 1.38 fold respectively, with curcumin treatment, as compared to Ova treatment.

Reverse transcriptase (RT)-PCR: GAPDH, as a housekeeping gene, showed no change in expression with either Ova or curcumin treatment. The levels of IL-4, IL-5, IL-13, IFN-γ, TGF-β and iNOS increased by 1.89 fold, by 1.71 fold, by 1.63 fold, by 1.33 fold, by 1.88 fold and by 1.89 fold respectively. Curcumin successfully reduced the expression levels of IL-4, TGF-β and iNOS, by 1.91 fold, by 1.15 fold and by 1.91 fold respectively. Curcumin did not have much effect on the expression of IL-5, IL-13, and IFN-γ, as compared to the Ova treated group (Figures 7B and 7C).
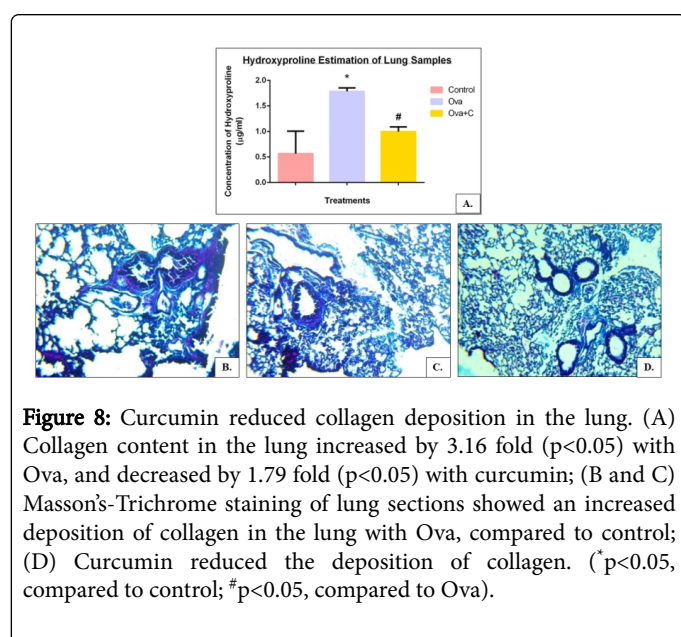
Western blot: Our protein expression studies supported the observations from the gene expression experiments. The levels of NFκB, TGFβ and STAT6 increased with Ova treatment, and decreased with curcumin (Figure 7D).

Figure 7: Curcumin reduced the expression of pro-inflammatory cytokines and signaling molecules. (*p<0.05, compared to control; $^#$p<0.05, compared to Ova)

Curcumin reduced collagen deposition in the lung

Collagen content in the lung and airways increases due to asthma. Ova treatment increased the collagen content in the lung by 3.16 fold (p<0.05). Curcumin significantly reduced it by 1.79 fold (p<0.05) (Figure 8A).

This was supported by histological analysis of lung sections stained with Masson's trichrome stain, which facilitates visualization of changes in lung collagen content. Control group (Figure 8B) showed no significant deposition of collagen as compared to Ova-induced group (Figure 8C). Curcumin treated lung (Figure 8D) showed minimal amount of collagen deposition.

Figure 8: Curcumin reduced collagen deposition in the lung. (A) Collagen content in the lung increased by 3.16 fold (p<0.05) with Ova, and decreased by 1.79 fold (p<0.05) with curcumin; (B and C) Masson's-Trichrome staining of lung sections showed an increased deposition of collagen in the lung with Ova, compared to control; (D) Curcumin reduced the deposition of collagen. (*p<0.05, compared to control; $^#$p<0.05, compared to Ova).

Discussion

Asthma is a global respiratory disease, with over 200 million people suffering worldwide. India itself has over 15-20 million asthmatics, and these numbers are rising with time [1]. Till date, asthma cannot be completely cured, but it can be controlled by medications like corticosteroids [1]. Poor control of asthma is mainly due to insufficient access to medications and health services [1]. Our study to assess the use of curcumin, a natural product, was done with this drawback of asthma medications in mind. Natural products like curcumin are easily available, and thus, may be more readily available to the public. Studies have shown that curcumin exerts its effects by regulating transcription factors, like signal transducer and activator of transcription proteins (STAT), Janus kinases (JAK) and peroxisome proliferator-activated receptor-γ (PPAR-γ), and downregulating Th2 cytokines, most likely by suppressing the activation of NFκB and its subsequent pathways [19-22]. It also prevents accumulation of inflammatory cells in, and thickening of, the airways [20]. The effect of curcumin in lung diseases has not been studied extensively [19]. When administered intranasally, it has been found to be effective in restoring lung function and reducing inflammation [14,20].

In this study, we induced asthma in mice, and then treated them orally with curcumin. Our assays showed that curcumin is quite successful in alleviating the symptoms of asthma. Curcumin inhibited the infiltration of cells into the blood (Figure 2A), BALF (Figure 2B) and lung (Figures 2C-2E). Eosinophilic infiltration (Figure 3), CD45$^+$B220$^+$ B cell populations (Figure 6A), CD45$^+$CD3$^+$ T cell populations (Figure 6A), CD45$^+$GR1$^+$ neutrophil populations (Figure 6C), CD45$^+$F4/80$^+$ macrophage populations (Figure 6C) and CD3$^+$CD4$^+$ TH cell populations (Figure 6B) were also inhibited. Levels of Ova-specific IgE in the serum (Figure 4), as well as collagen deposition in the lung and airways (Figure 8), were also reduced significantly. Curcumin restored the clonogenic potential of blood (Figure 5A), bone marrow (Figure 5B), and especially, the lung (Figure 5C). Cytokines and signaling molecules, like NFκB, IL-3, IL-4, TGF-β, STAT6 and iNOS, which over-express in asthmatic conditions, were also reduced (Figure 7).

In inflammation, functional NFκB localizes to the nucleus after its inherent inhibitor, IκB, is phosphorylated and degraded by the action of IKK (IκB Kinase complex) [21,22]. In ovalbumin-induced asthma, on treatment with Ova, mast cells get activated, leading to the production of IL-4 [14]. IL-4 production leads to synthesis of IgE by activated B cells. This promotes activation of CD4$^+$Th2 cells and inhibition of Th1 cells [14]. IL-4 and IL-13 also bind to their receptors and activates JAK, which in turn leads to the activation of STAT6 by phosphorylation and dimerization. STAT6 promotes the activation of Th2 cytokines, synthesis of IgE and induction of airway hyper-responsiveness [23]. The mechanism by which Ova acts to establish asthma is summarized in Figure 9.

From these studies, and from our observations, we hypothesized a possible mechanism of action of curcumin (Figure 10). We hypothesized that orally-administered curcumin prevents formation of functional NFκB. Curcumin probably acts by blocking the activity of IKK, thereby blocking the formation of functional NFκB. This leads to inhibition in transcription of genes activated by NFκB, like IL-4, IL-13 and iNOS. Downregulation of IL-4 leads to inhibition of reduced production of IgE and CD4$^+$ cells. Downregulation of iNOS also leads to reduction in the production of nitric oxide. Inhibition of IL-4 and IL-13 also leads to downregulation of STAT6, and subsequent reduction in CD4$^+$ T$_H$ cells, IgE and airway hyper-responsiveness.

STAT6 may also be downregulated by the direct action of curcumin on JAK. According to Zhang et al. [24], curcumin promotes the expression of SOCS1 (suppressor of cytokine signaling 1), which inhibits the JAK/STAT pathway in a murine model of colitis. It is possible that curcumin acts similarly in the lung, and inhibits the JAK/STAT pathway.
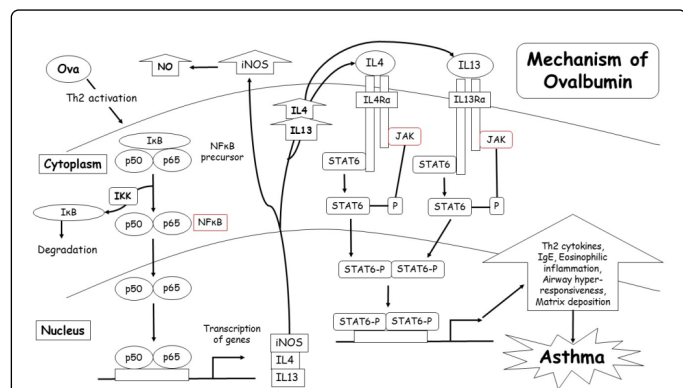

Figure 9: Mechanism of action of ovalbumin in establishing asthma. Ovalbumin activates Th2 response, which in turn leads to activation of pro-inflammatory cytokines, and subsequent synthesis of IgE, airway hyper-responsiveness and collagen deposition.
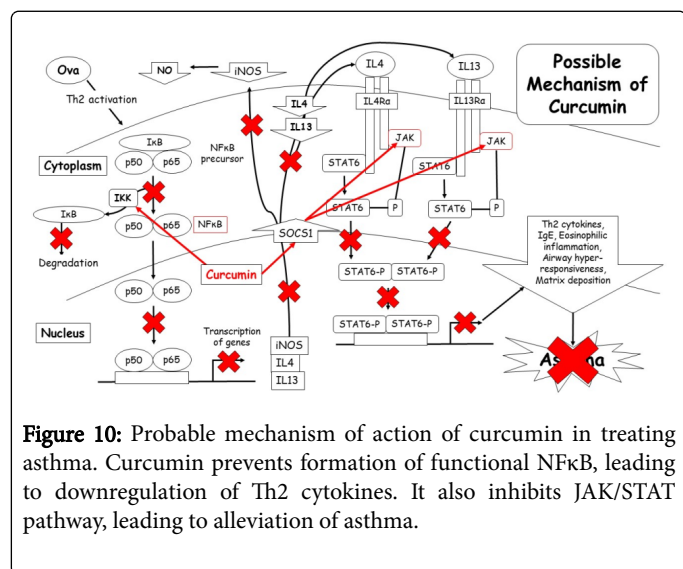

Figure 10: Probable mechanism of action of curcumin in treating asthma. Curcumin prevents formation of functional NFκB, leading to downregulation of Th2 cytokines. It also inhibits JAK/STAT pathway, leading to alleviation of asthma.

The main limitation of curcumin is the fact that it is poorly soluble in water and has limited oral bioavailability. This can be overcome by loading the curcumin in colloidal systems or coating it with nanoparticles [8,25]. It can also be encapsulated in liposomes [26]. Curcumin can also be administered as a nanoemulsion powder, which ensures shorter release time and better oral bioavailability [27].

While our studies have shown that curcumin is effective as an anti-inflammatory agent, and can be used as a therapeutic agent in asthma, studies to determine its ADMET (absorption, distribution, metabolism, excretion and toxicity) properties are yet to be done. Whether we can modify the curcumin in such a way as to enhance its effects and its bioavailability, is also yet to be seen. Studies will also be done using human cells, so that our findings can be validated in a human clinical setting.

Acknowledgements

Competing Interests

There is no conflict of interest among the authors.

Contribution of Authors

PP performed the experiment, did the assays, analyzed the data and wrote the manuscript. SM[2] performed some of the assays. SM[1] analyzed the data and wrote the manuscript. ERB initiated the project, designed the experiments, analyzed the data and wrote the manuscript.

References

1. World Health Organization (2017).

2. Mitra S, Paul P, Mukherjee K, Biswas S, Jain M, et al. (2015) Mesoporous Nano-carbon particle Loaded Fisetin has a Positive Therapeutic Effect in a Murine Preclinical Model of Ovalbumin Induced Acute Allergic Asthma. J Nanomedine Biotherapeutic Discov 5: 132.

3. Barnes PJ (2017) Cellular and molecular mechanisms of asthma and COPD. Clin Sci 131: 1541-1558.

4. Ray A, Raundhal M, Oriss TB, Ray P, Wenzel SE (2016) Current concepts of severe asthma. J Clin Invest 126: 2394-2403.

5. Henderson WR Jr, Chi EY, Maliszewski CR (2000) Soluble IL-4 receptor inhibits airway inflammation following allergen challenge in a mouse model of asthma. J Immunol 164: 1086-1095.

6. Cho JY, Miller M, Baek KJ, Han JW, Nayar J, et al. (2004) Inhibition of airway remodeling in IL-5-deficient mice. J Clin Invest 113: 551-560.

7. Janssen-Heininger YM, Poynter ME, Aesif SW, Pantano C, Ather JL, et al. (2009) Nuclear factor kappa B, airway epithelium, and asthma: avenues for redox control. Proc Am Thorac Soc 6: 249-255.

8. Tiyaboonchai W, Tungpradit W, Plianbangchang P (2007) Formulation and characterization of curcuminoids loaded solid lipid nanoparticles. Int J Pharm 337: 299-306.

9. Jayaprakasha GK, Rao LJ, Sakariah KK (2006) Antioxidant activities of curcumin, demethoxycurcumin and bisdemethoxycurcumin. Food Chemistry 98: 720-724.

10. Jacob A, Wu R, Zhou M, Wang P (2007) Mechanism of the Anti-inflammatory Effect of Curcumin: PPAR-gamma Activation. PPAR Res 89369.

11. Shi J, Wang Y, Jia Z, Gao Y, Zhao C, et al. (2017) Curcumin inhibits bladder cancer progression via regulation of β-catenin expression. Tumour Biol 39: 1010428317702548.

12. Hassani S, Sepand MR, Jafari A, Jaafari J, Rezaee R, et al. (2015) Protective effects of curcumin and vitamin E against chlorpyrifos-induced lung oxidative damage. Hum Exp Toxicol 34: 668-676.

13. Shakeri F, Boskabady MH (2017) Anti-inflammatory, antioxidant, and immunomodulatory effects of curcumin in ovalbumin-sensitized rat. Biofactors 43: 567-576.

14. Subhashini, Chauhan PS, Dash D, Paul BN, Singh R (2016) Intranasal curcumin ameliorates airway inflammation and obstruction by regulating MAPKinase activation (p38, Erk and JNK) and prostaglandin D2 release in murine model of asthma. Int Immunopharmacol 31: 200-206.

15. Banerjee ER, Jiang Y, Henderson WR Jr, Latchman Y, Papayannopoulou T (2009) Absence of alpha 4 but not beta 2 integrins restrains development of chronic allergic asthma using mouse genetic models. Exp Hematol 37: 715-727.

16. Han H, Ziegler SF (2013) Bronchoalveolar Lavage and Lung Tissue Digestion. Bio Protoc 3: e859.

17. Sood R (2009) Medical Laboratory Technology: Methods and Interpretations. 6th ed. Jaypee Brothers Medical Publishers Pvt Ltd, India, 288.

18. Hofman K, Hall B, Cleaver H, Marshall S (2011) High-throughput quantification of hydroxyproline for determination of collagen. Anal Biochem 417: 289-291.

19. Lelli D, Sahebkar A, Johnston TP, Pedone C (2017) Curcumin use in pulmonary diseases: State of the art and future perspectives. Pharmacol Res 115: 133-148.

20. Chauhan PS, Subhashini, Dash D, Singh R (2014) Intranasal curcumin attenuates airway remodeling in murine model of chronic asthma. Int Immunopharmacol 21: 63-75.

21. Abidi A, Gupta S, Agarwal M, Bhalla HL, Saluja M (2014) Evaluation of Efficacy of Curcumin as an Add-on therapy in Patients of Bronchial Asthma. J Clin Diagn Res 8: 19-24.

22. Jobin C, Bradham CA, Russo MP, Juma B, Narula AS, et al. (1999) Curcumin blocks cytokine-mediated NF-kappa B activation and proinflammatory gene expression by inhibiting inhibitory factor I-kappa B kinase activity. J Immunol 163: 3474-3483.

23. Goenka S, Kaplan MH (2011) Transcriptional regulation by STAT6. Immunol Res 50: 87-96.

24. Zhang X, Wu J, Ye B, Wang Q, Xie X, et al. (2016) Protective effect of curcumin on TNBS-induced intestinal inflammation is mediated through the JAK/STAT pathway. BMC Complement Altern Med 16: 299.

25. Peng S, Li Z, Zou L, Liu W, Liu C, et al. (2018) Enhancement of Curcumin Bioavailability by Encapsulation in Sophorolipid-Coated Nanoparticles: An in Vitro and in Vivo Study. J Agric Food Chem 66: 1488-1497.

26. Ibrahim S, Tagami T, Kishi T, Ozeki T (2018) Curcumin marinosomes as promising nano-drug delivery system for lung cancer. Int J Pharm 540: 40-49.

27. Hu L, Hu Q, Yang C (2018) Spray-dried curcumin nanoemulsion: A new road to improvement of oral bioavailability of curcumin. Pak J Pharm Sci 31: 169-173.