

ORIGINAL ARTICLE

Experimental Allergy and Immunology

Modulation of allergic responses by mitochondrial STAT3 inhibitors

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Abstract

Background: Recently, we have shown that mast cell mitochondrial STAT3 could serve as a new target for the regulation of the allergic response as it plays an essential role in immunologically mediated degranulation of mast cells. In the present work, we explored how two recently developed mitochondrial STAT3 inhibitors (Mitocur-1 and Mitocur-3) modulate the allergic response.

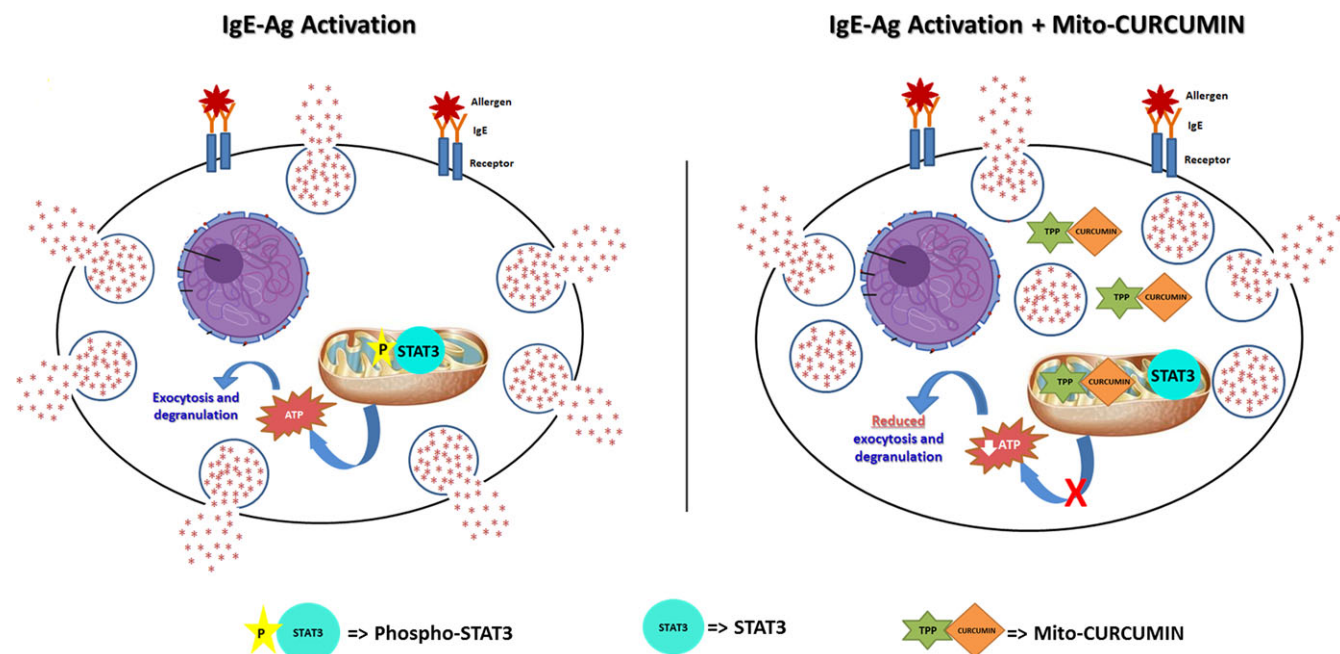
Methods: Experiments were performed both in vitro in cultured human/mouse mast cells and with rat basophilic leukemia (RBL) cells and also in vivo in mice. The effect of mitochondrial STAT3 inhibition on mast cell function was determined via checking degranulation and several cytokines secretion levels.

Results: Here, we show that treatment of rodent and human cultured mast cells with low concentrations of mitochondrial STAT3 inhibitors had no effect on STAT3 target gene expression. However, these inhibitors caused a significant reduction in mast cell exocytosis and cytokine release, due to a decrease in OXPHOS activity and STAT3 serine 727 phosphorylation. It was also observed in an OVA mouse model of allergic asthma that one of the inhibitors used significantly reduced eosinophilia and neutrophilia compared to the control mice group. Furthermore, it was observed that treatment with this inhibitor resulted in a significant reduction in blood histamine levels in mice after IgE-Ag challenge.

Conclusion: The present data strongly suggest that the development of mitochondrial STAT3 inhibitors could serve as a potential treatment for allergy-associated diseases.

KEYWORDS

asthma, degranulation, mast cells, mitochondria, STAT3



GRAPHICAL ABSTRACT

Both mitocur-1 and mitocur-3 are targeted to the mitochondria where they disrupt ATP production and oxygen consumption as well as STAT3 S727 phosphorylation. This disruption of the mitochondrial parameters lead to reduced degranulation and cytokine secretion in cultured rodent and human mast cells in addition to in-vivo inhibition in mice models.

1 | INTRODUCTION

We have previously shown that oxidative phosphorylation (OXPHOS)-mediated ATP production is essential for mast cell function. Pyruvate dehydrogenase is activated following immunological stimulation of mast cells to produce NADH and FADH₂ via the Krebs cycle.¹ These cofactors are necessary for the electron transport chain (ETC), which is positively regulated by mitochondrial signal transducer and activator of transcription 3 (STAT3) during mast cell activation to produce more ATP for the degranulation process.²

STAT3 is a transcription factor involved in signal transduction pathways that are activated by the IL-6 family of cytokines. STAT3 signaling has been shown to prevent programmed cell death and enhance cell proliferation through regulating genes such as Bcl-2,³ Mcl-1,⁴ and caspase3.⁵ Recently, it has been shown in a variety of cell types that STAT3 has a non-canonical role in mitochondria, whereby it regulates OXPHOS by modulating ETC activity.⁶⁻⁸ The mechanism by which mitochondrial STAT3 regulates ETC activity is still unknown. Direct protein-protein interactions with different ETC complexes depending on cell type have been suggested,⁸⁻¹⁰ as have the involvement of STAT3 in mitochondrial gene transcription.¹¹ We have shown in mast cells that mitochondrial STAT3 regulates complex3 of the ETC. Unlike nuclear STAT3, which is activated by IL-6 and tyrosine 705 phosphorylation,¹² mitochondrial STAT3 is activated by the MAPK pathway which triggers the phosphorylation of mitochondrial STAT3 on serine 727.²

These results, together with our previous finding that mitochondrial STAT3 plays a major role in IgE-antigen-mediated mast cell

exocytosis,² suggest that this protein could serve as a new target for the manipulation of allergic diseases.

In this study, we present our findings using two mitochondrial STAT3 inhibitors that are based on curcumin, a plant-derived polyphenolic compound naturally present in turmeric (*Curcuma longa*).¹³ Curcumin is a well-known inhibitor of STAT3,^{14,15} and here, we have conjugated it to a mitochondrial-targeting small molecule.¹⁶ Although STAT3 inhibitors such as ISIS-STAT3 Rx are already being tested in clinical trials for the treatment of cancer,¹⁷ it is impossible to use these drugs for the treatment of allergy due to their effects on nuclear STAT3. Therefore, it was essential to modify curcumin in such a way that it will specifically target mitochondrial STAT3. Triphenylphosphonium (TPP) is a lipophilic cation that targets small molecules specifically to the mitochondria based on its attraction to the mitochondrial membrane potential.¹⁶ This TPP moiety has been successfully used in the past to target a wide variety of compounds into the mitochondria.¹⁸⁻²⁰ Our assumption was that such selective targeting to the mitochondria by conjugation of the inhibitors to TPP should decrease the external dose required, limit toxic side reactions and minimize metabolism of the compound.¹⁶ In this study, we have used two inhibitors: curcumin conjugated to TPP and a curcumin analog with a deletion of a methoxy substitution on the aryl groups conjugated to TPP. The deletion of the methoxy substitution could enhance free radical scavenging activity,²¹ an important feature when inhibiting mitochondrial activity. Reddy et al were the first to describe these molecules for cancer treatment and named the curcumin conjugated to TPP mitocur-1 and the curcumin analog conjugated to TPP mitocur-3.²²

In the present work, we show that at low concentrations both mitocur-1 and mitocur-3 are targeted to the mitochondria with no detected effect on STAT3 target genes. These inhibitors reduced degranulation and cytokine secretion in cultured rodent and human mast cells and reduced allergic parameters as measured in two different *in vivo* models. Therefore, these inhibitors and the approach of targeting mast cell mitochondrial STAT3 could be used as the basis for future development of drugs to modify the allergic response.

2 | MATERIALS AND METHODS

2.1 | Mice

Male and female C3H wild-type mice aged 6 to 7 weeks and Balb/c OlaHsd male mice aged 8 to 10 weeks were purchased from Jackson Laboratories (Cambridge, Mass). Mice were fed a regular diet and given drinking water *ad libitum*. Experiments were approved by the Hebrew University Ethical Committee for Animal Experimentation.

2.2 | Antibodies

Mouse antiactin antibody was purchased from Sigma-Aldrich (St Louis, Mo). Rabbit anticapase3 antibodies were purchased from Cell Signaling Technologies (Beverly, Mass). Rabbit anti-STAT3 and -pS727-STAT3, rabbit anti-Mcl-1, as well as rabbit anti-Bcl2 antibodies, were purchased from Santa Cruz Biotechnologies (Santa Cruz, Calif). Rabbit anti-Bcl2 for BMMC Western blot was purchased from Cell Signaling Technologies (Beverly, Mass).

2.3 | Chemical inhibitor treatment

Two curcumin derivatives, mitocur-1 and mitocur-3, which are conjugated to the mitochondrial-targeting molecule TPP, were synthesized by Enamine Ltd. Kiev, Ukraine. The inhibitors were dissolved in 100% DMSO (Sigma-Aldrich) for *in vitro* experiments and in saline for *in vivo* experiments.

2.4 | Cell culture

The rat basophil leukemia (RBL) cell line RBL-2H3 was maintained in RPMI 1640 medium without sodium pyruvate or L-glutamine, supplemented with 10% FBS, 2 mol L⁻¹ L-glutamine, 100 U/mL penicillin, and 100 µg/mL of streptomycin (Biological Industries, Beit Haemek, Israel), as previously described.² Bone marrow-derived mast cells (BMMCs) were cultured in RPMI 1640 conditioned medium, as previously described.²

2.5 | qReal-time PCR

Total RNA was isolated using GENEzol™ TriRNA Pure Kit from Genaid Biotech (New Taipei, Taiwan) according to the manufacturer's instructions. The quality of total RNA was tested by 1% agarose gel

electrophoresis. Equal amounts of RNA (1 µg) were used to synthesize single-strand cDNA using qScript cDNA Synthesis Kit from Quantabio (Beverly, MA). The resulting cDNA was used as a template for PCR using gene-specific primers:

Actin: forward primer, 5'-AAGACCTCTATGCCAACAC-3'; reverse primer, 5'-TGATCTTCATGGTGCTAGG-3';

Bcl-2 (RBL): forward primer, 5'-GATTGTGGCCTTCTTTGAG-3'; reverse primer, 5'-CAAAGTCTGAGAGAGTCTTC-3'.

Bcl-2 (BMMC): forward primer: 5'-CTGGTGGACAACATCGCTCTG-3'; reverse primer, 5'-GGTCTGCTGACCTCACTTGTG-3'.

The PCR amplification was performed using Fast SYBR™ Green Master Mix from Applied Biosystems Inc. (Foster City, CA). The transcript of rat β-actin was used as an internal RNA control, and each sample was normalized based on its β-actin content. The thermal cycling conditions involved an initial denaturing step at 94°C for 45 seconds, followed by annealing at 60°C for 45 seconds and 72°C for 45 seconds and an annealing extension step at 72°C for 10 minutes.

2.6 | Trypan blue exclusion

RBL cells were cultured on 6-well plates and incubated for 3 hours with 3, 6, 12 or 25 µmol L⁻¹ mitocur-1 or Mitocur-3, 0.05% (v/v). DMSO was used as control. The cells were then washed once with PBS and incubated with complete RPMI media containing 0.05% Trypan blue for 20 minutes at 37°C. Dye-containing media was then removed by washing three times with ice-cold PBS. Visual analysis of the cultures revealed an admixture of live cells (trypan blue negative) and dead cells (trypan blue positive) in each well. The cells were then lysed with 200 µL 1% SDS. Finally, 180 µL of the SDS/trypan blue solution was transferred to a 96-well Nunc Maxisorp™ plates and analyzed by a plate reader at 590 nm.²³

2.7 | Human cord blood-derived mast cell (CBMC) culture and activation

CBMCs were prepared from umbilical cord blood of healthy donors according to a well-described method.²⁴ CBMCs were sensitized by the addition of human myeloma IgE (0.3 µg/mL; Calbiochem-Merck, Schwalbach, Germany) and human IL-4 (0.10 ng/mL, PeproTech) to cell cultures for 4 days in a humidified incubator (37°C at 5% CO₂). CBMCs were washed in PBS and pre-incubated with 12 µmol L⁻¹ mitocur-1 or -3 or DMSO vehicle for 3 hours at 37°C. Then, cells were centrifuged, washed in PBS, and activated by addition of rabbit α-human-IgE (5 µg/mL; Dako, Glostrup, Denmark) for 30 minutes (β-hexosaminidase assay) or overnight (IL-6 release). Supernatants and cell pellets were collected for assessment of β-hexosaminidase release and supernatants for IL-6 release.

2.8 | β-hexosaminidase release assay (degranulation assay)

The β-hexosaminidase release assay was performed as has been previously described.^{1,2}

2.9 | ATP determination

RBL cells were cultured on black 96-wells plates with complete medium or OXPHOS-dependent medium. Three hours prior to the assay, the cells were incubated with various concentrations of either inhibitor. Mitochondrial ATP levels were measured using the ATPlite luminescence-based assay according to the manufacturer's instructions (Perkin Elmer, Waltham, MA, USA) and were expressed as levels relative to control cells that were treated with DMSO.

2.10 | Oxygen consumption

RBL cells were cultured on Seahorse XF 24-well plates with complete medium. Three hours prior to the oxygen measurement, the cells were incubated with various concentrations of either inhibitor. The oxygen consumption rate was measured with an XF24 extracellular flux analyzer (Seahorse Biosciences, North Billerica, Mass.)

2.11 | Gel electrophoresis and Western blot analysis

Gel electrophoresis and Western blot analysis were performed as has been previously described.^{1,2}

2.12 | Passive systemic IgE-Ag-mediated anaphylaxis

Wild-type C3H mice were sensitized with 3 μ g murine IgE anti-DNP mAb SPE-7 by means of intravenous injection to the tail vein. Three days prior to the challenge, mice were injected ip once per day with 7 mg/kg mitocur-1 or 20 mg/kg curcumin dissolved in saline or saline alone as control, and then the mice were challenged with an i.v. injection of DNP (250 μ g/mouse). 100 μ L blood was collected immediately and added to an equal volume of pre-cooled Alsever solution. Blood was centrifuged at 2000 g for 20 minutes, and the plasma supernatant was collected. Plasma histamine levels were determined using a competitive ELISA (IBL International GmbH, Hamburg, Germany).

2.13 | Allergen challenge protocol

Balb/c OlaHsd male mice aged 8-10 weeks were sensitized and challenged with OVA to induce allergic inflammation. Mice received ip injections of 10 μ g ovalbumin (OVA) and 1 mg Al(OH)₃ in 0.5 mL saline on days 1 and 10. One week after the last sensitization (on days 14-18), the mice were challenged with OVA aerosols (2% in saline) for 20 minutes each day for 3 days while animals of the control group were challenged with saline. Mice were challenged with 0 to 64 mg/mL methacholine (MCH) nebulized directly into the ventilatory circuit using an AeroNebLab nebulizer (SCIREQ). Mice were fed ad libitum with standard mouse chow. Mitocur-1 was administered as a single ip injection once a day for 3 days prior to the end of the experiment.

2.14 | Analysis of the bronchoalveolar lavage fluid (BALF)

BALF was centrifuged at 200 g for 10 minutes, and the supernatants were stored at -20°C for further examination. Cell pellets were resuspended in 1 mL saline and aliquots of 100 μ L were used for cytospin preparations using a Shandon cytospin (Thermo Fisher Scientific, Waltham, MA, USA), and stained using Diffquik stain (Dade Diagnostica, Munich, Germany). A total of 200 cells were counted and classified for each slide. Total BALF cells were counted using a Neubauer chamber (Marienfeld, Lauda-Königshofen, Germany). Results are expressed as percent of different cell types in BALF.

2.15 | MCPT-1 secretion

MCPT-1 levels in serum and BALF were measured using an ELISA kit from Invitrogen eBiosciences (Carlsbad, CA, USA).

2.16 | IL-13 cytokine secretion

IL-13 levels in serum and BALF were measured using an ELISA kit from R&D Systems, Inc. (Minneapolis, MN, USA).

2.17 | Histology

Formalin-fixed, paraffin-embedded tissue samples were cut into 5 μ m sections, deparaffinized in xylene, and rehydrated through a series of decreasing concentrations of ethanol. Sections were stained with Toluidine blue (Sigma-Aldrich) according to the manufacturer's instructions. The sections were observed under Nikon light microscope and purple-stained cells were counted from three sections in each group.

2.18 | Statistical analysis

Either the 1-sample *t* test or the 2-tailed Student's *t* test was performed, when appropriate. Data are reported as means $\pm$ SEMs.

3 | RESULTS

3.1 | Effect of mitochondrial STAT3 inhibitors on STAT3 target genes, cleaved caspase 3, and toxicity

To determine whether mitocur-1 and 3 have an effect on STAT3 target genes, RBL cells and BMMCs were incubated with increasing concentrations of the inhibitors or DMSO as control for 3 hours. The levels of the STAT3 target genes Bcl-2, Mcl-1, and caspase3 were analyzed by Western blot. In both cell types, 25 μ mol L⁻¹ mitocur-1/3 reduced the levels of Bcl-2, Mcl-1, and caspase3 (Figure 1A-N). However, no effect on STAT3 transcriptional activity as measured both by protein levels (Figure 1A-N) and mRNA levels (Figure S1A,B) was observed in cells treated with ≤ 6 μ mol L⁻¹ mitocur-1/3. In addition, no effect on cleaved caspase 3 was observed in cells treated with

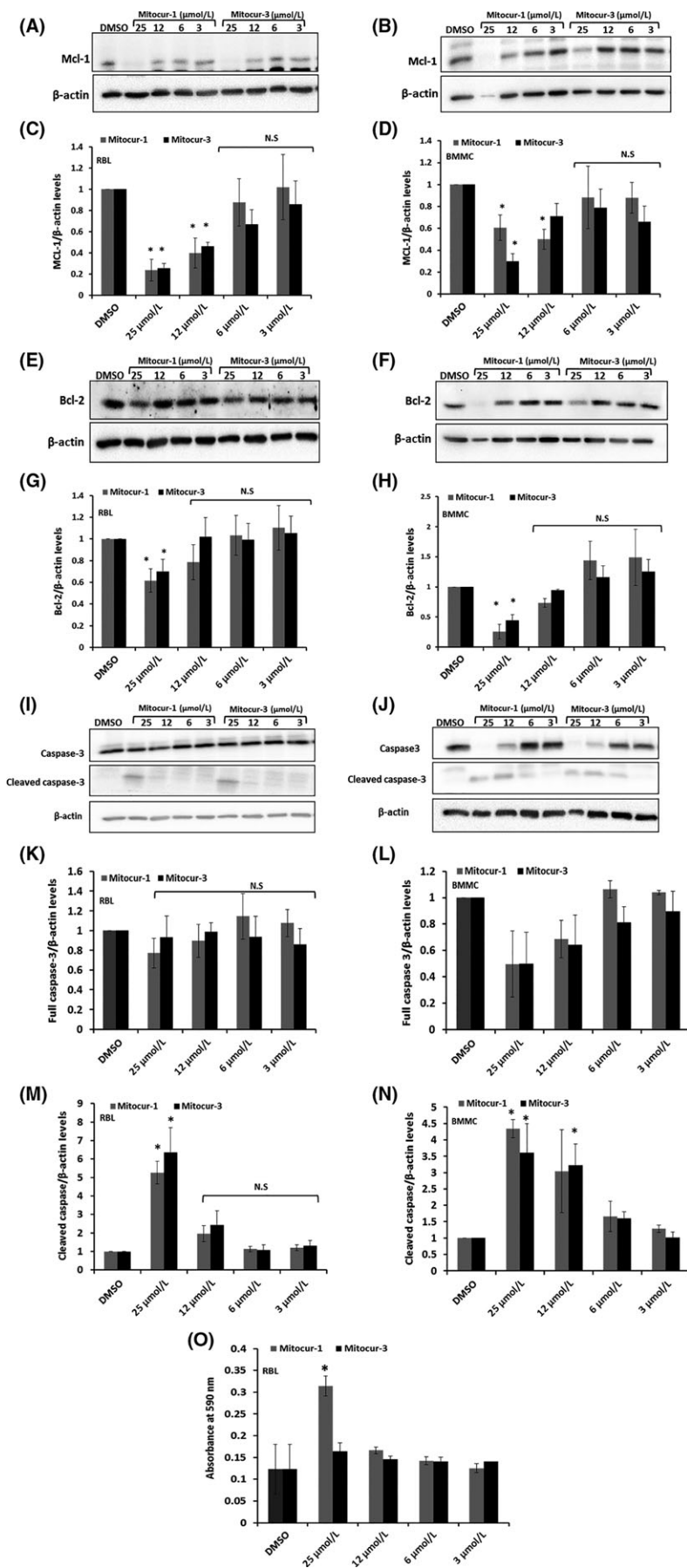


FIGURE 1 The effect of mitochondrial STAT3 inhibitors on STAT3 target genes, cleaved caspase3, and toxicity in cultured mast cells: RBL (A) or BMMC (B) cells were treated with mitocur-1 or mitocur-3 (3, 6, 12 or 25 $\mu\text{mol L}^{-1}$) for 3 h, and then the levels of β -actin and Mcl-1 were determined using Western blot analysis. The results are presented relative to the DMSO control (0.05% v/v). Densitometry of the blots is shown in (C) RBL and (D) BMMC. RBL (E) and BMMC (F) cells were treated with mitocur-1 or mitocur-3 (3, 6, 12 or 25 $\mu\text{mol L}^{-1}$) for 3 h, and the levels of β -actin and Bcl-2 were determined by Western blot analysis. For control, 0.05% (v/v) DMSO was used. Densitometry of the blots is shown in (G) RBL and (H) BMMC. One representative experiment of four is shown. RBL (I) and BMMC (J) cells were treated with mitocur-1 or mitocur-3 (3, 6, 12, or 25 $\mu\text{mol L}^{-1}$) for 3 h and the levels β -actin, full caspase-3 and cleaved caspase-3 levels were determined by Western blot analysis. 0.05% DMSO was used as control. Densitometry of the full caspase-3 blots is shown in (K) RBL and (L) BMMC, the densitometry of cleaved caspase-3 blots is shown in (M) RBL and (N) BMMC. One representative experiment of three is shown. (O) RBL cells were stained with Trypan blue after incubation with mitocur-1 or mitocur-3 (3, 6, 12, or 25 $\mu\text{mol L}^{-1}$), 0.05% DMSO was used as control. To determine the amount of Trypan blue stain captured by the cells, we lysed the cells using 1% SDS, and the absorbance was read at 590 nm. ($n > 3$; results represent means $\pm$ SEMs; * $P < .05$ compared to DMSO group; all other N.S.)

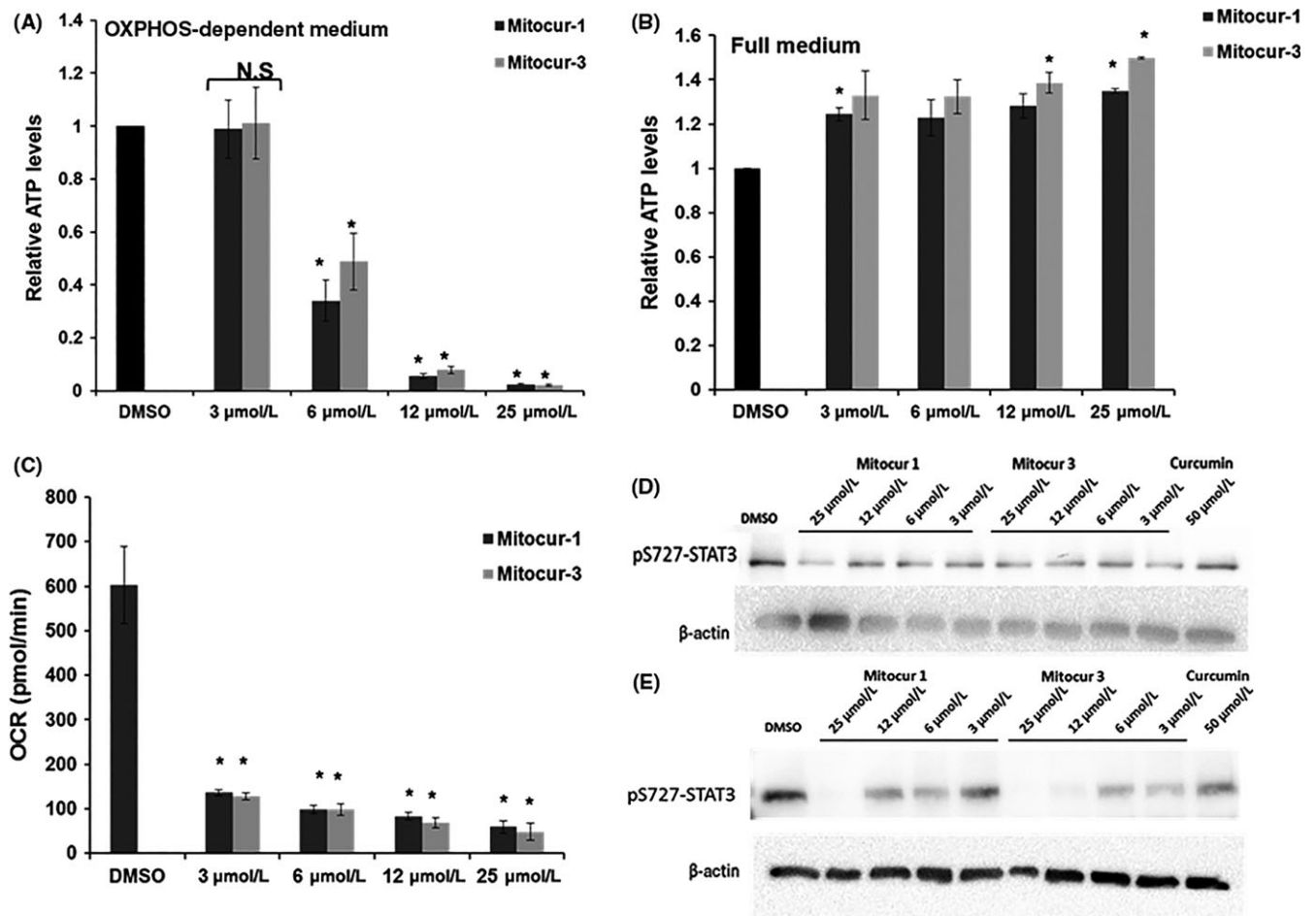


FIGURE 2 The effect of mitochondrial STAT3 inhibitors on mitochondrial function: (A) RBL cells were incubated with glucose-free RPMI medium for 24 h before the addition of mitocur-1 or -3 (3, 6, 12 or 25 $\mu\text{mol L}^{-1}$) or 0.05% (v/v) DMSO for 3 h. Mitochondrial ATP levels were measured using the ATP-lite kit. (B) RBL cells were incubated for 24 h in glucose supplemented RPMI 1640. The cells were then treated with mitocur-1 or -3 (3, 6, 12 or 25 $\mu\text{mol L}^{-1}$) or 0.05% (v/v) DMSO for 3 h. Total ATP levels were using the ATP-lite kit. (n = 3; results represent means $\pm$ SEMs; * P < .05 compared to DMSO group; all other N.S). (C) RBL cells were cultured overnight in a Seahorse XF 24-well plates overnight in glucose supplemented RPMI 1640 medium. The cells were then treated with mitocur1 or -3 (3, 6, 12 or 25 $\mu\text{mol L}^{-1}$) or 0.05% DMSO for 3 h. Oxygen consumption rate (OCR) was measured using the Seahorse XF24 device. (n = 3; results represent means $\pm$ SEMs; * P < .05 compared to DMSO group; all other N.S). RBL (D) and BMMC (E) cells were incubated with 0.05% DMSO, curcumin (50 $\mu\text{mol L}^{-1}$) or with mitocur-1 or -3 (3, 6, 12 or 25 $\mu\text{mol L}^{-1}$) for 3 h, then cells were lysed and the levels of β -actin and phospho-S727-STAT3 were estimated by Western blot analysis. One representative experiment of three is shown

$\leq 6 \mu\text{mol L}^{-1}$ mitocur-1/3 in RBL cells (Figure 1I,M). However, mitocur-3 enhanced cleaved caspase levels in BMMCs (Figure 1J,N). To determine whether mitocur-1/3 have any toxic effect on the cells, trypan blue staining was used as previously described.²³ As shown in Figure S1C and Figure 1O, no significant cell toxicity was observed after treatment with $\leq 12 \mu\text{mol L}^{-1}$ mitocur-1/3.

3.2 | Effect of mitochondrial STAT3 inhibitors on mitochondrial function

It has been previously shown that impairing mitochondrial STAT3 function reduces ATP levels and oxygen consumption.² To determine whether mitochondrial STAT3 inhibitors reduced mitochondrial ATP, RBL cells were cultured for 24 hours in a glucose-free, OXPHOS-dependent medium supplemented with dialyzed serum as an energy

source or in complete medium. Cells were then incubated with increasing concentrations of mitocur-1/3 for 3 hours. The ATP levels were determined by ATP-lite Luminescence Assay System. As shown in Figure 2A, mitocur-1/3 reduced mitochondrial ATP levels in a dose-dependent manner when incubated in an OXPHOS-dependent medium. In contrast, when the cells were incubated in complete medium, the total ATP levels were not reduced at the concentrations used of either inhibitor as can be seen in Figure 2B. Moreover, mitocur-1/3 reduced mitochondrial oxygen consumption (Figure 2C). To further evaluate the effect of the inhibitors on mitochondrial STAT3 function in RBL and BMMC cells, the levels of phosphorylation of STAT3 at serine 727, a residue important for STAT3 mitochondrial activity, were determined by Western blot analysis after the cells were incubated with increasing concentrations of mitocur-1 or 3. As shown in Figure 2D,E, in both cell types, mitocur-1 and 3 reduced the levels of serine 727 STAT3

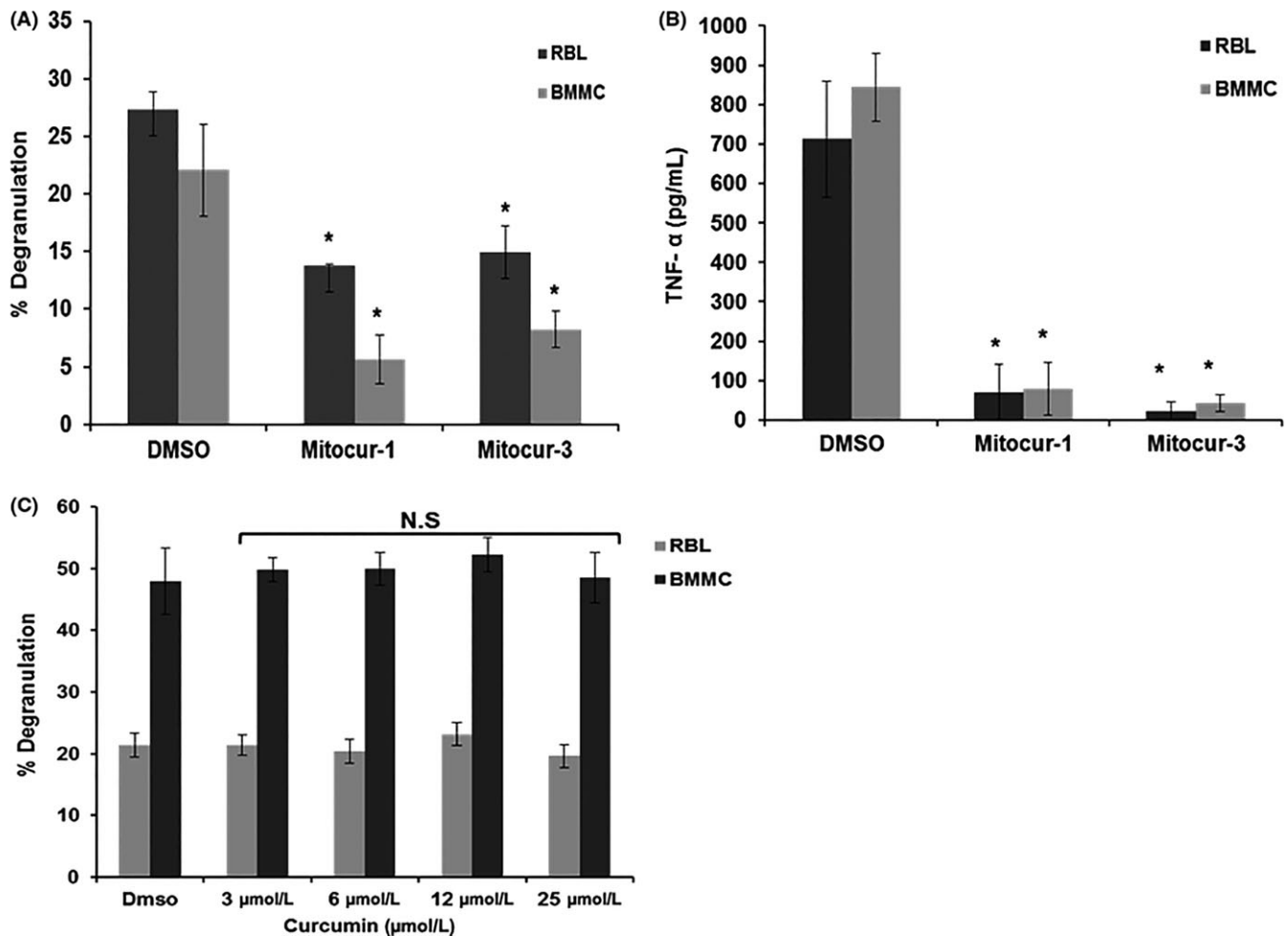


FIGURE 3 The effect of mitochondrial STAT3 inhibitors on mast cell function: IgE-sensitized RBL and BMMC cells were incubated with 0.012% DMSO or 6 $\mu\text{mol L}^{-1}$ of either mitocur-1 or -3 for 3 h, followed by 30-min DNP-BSA challenge. β -hexosaminidase release (A) as well as TNF- α cytokine secretion (B) was then measured. In (C), the cells were treated with (3, 6, 12 or 25 $\mu\text{mol L}^{-1}$) of non-targeted curcumin or 0.05% DMSO and degranulation levels were determined. ($n = 3$; results represent means $\pm$ SEMs; * $P < .05$ compared to DMSO group; all other N.S.)

phosphorylation in a dose-dependent manner (Figure S2). Thus, mitochondrial STAT3 is specifically affected by mitocur-1/3.

3.3 | Effect of mitochondrial STAT3 inhibitors on mast cell function

To explore the effect of mitochondrial STAT3 inhibitors on mast cell function, RBL and BMMC cells were incubated for 3 hours with 6 $\mu\text{mol L}^{-1}$ mitocur-1 or 3, or DMSO as control, followed by determination of the degranulation levels and TNF- α secretion. As shown in Figure 3A, β -hexosaminidase release in mitocur-1 and -3-treated RBL and BMMC cells was significantly reduced compared to the DMSO-treated cells. The optimal concentration was determined by a dose-response experiment shown in Figure S3.

In both RBL cells and BMMCs, treatment with mitocur-1 or mitocur-3 reduced β -hexosaminidase release by more than 50% ($P < .05$) (Figure 3A) and totally abolished TNF- α secretion (Figure 3B). Furthermore, non-targeted curcumin had no effect on mast cell

degranulation in RBL cells in concentrations between 3 and 25 $\mu\text{mol L}^{-1}$ (Figure 3C).

3.4 | Effect of mitochondrial STAT3 inhibitors on human primary mast cells

Next, we determined whether the effect of mitochondrial STAT3 inhibitors observed in murine mast cells is similar in human mast cells. IgE-sensitized human CBMCs were exposed to mitocur-1 or 3 or DMSO for 3 hours and then activated by rabbit α -human-IgE for 30 minutes. As shown in Figure 4, degranulation (A, B) was significantly reduced by each of the inhibitors ($P < .05$) while IL-6 release was completely abolished (Figure 4C).

3.5 | Effect of mitochondrial STAT3 inhibitors on passive systemic IgE-Ag-mediated anaphylaxis

The effect of mitochondrial STAT3 inhibitors in vivo was investigated by evaluating the response to allergic stimulation in mitocur-1-treated

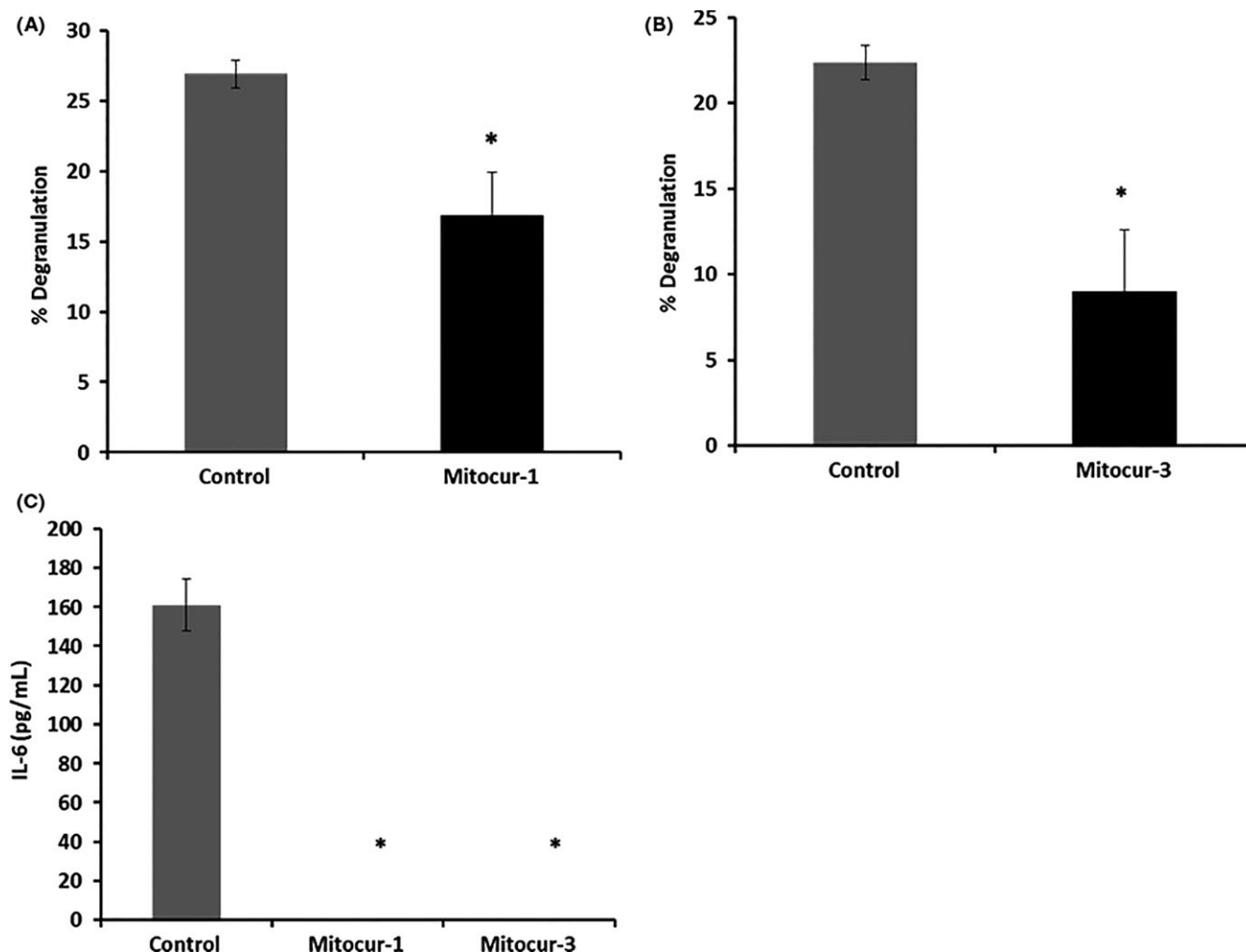


FIGURE 4 The effect of mitochondrial STAT3 inhibitors on human primary mast cell culture: IgE-sensitized CBMCs were incubated with either 0.025% DMSO or with $12 \mu\text{mol L}^{-1}$ mitocur-1 (A) or mitocur-3 (B) for 3 h and then activated with $5 \mu\text{g/mL}$ rabbit α -human-IgE for 30 min to measure β -hexosaminidase release. For the measurement of IL-6 release (C), the cells were incubated with $5 \mu\text{g/mL}$ rabbit α -human-IgE overnight, and the levels of the released IL-6 were determined using an ELISA. ($n = 3$; results represent means $\pm$ SEMs; $*P < .05$ compared to DMSO group; all other N.S)

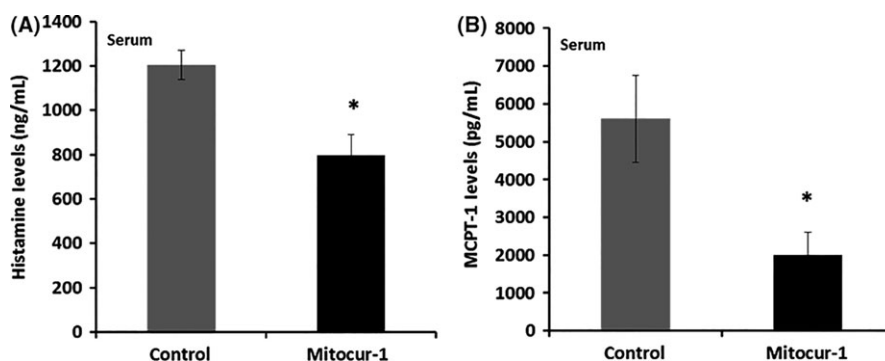


FIGURE 5 The effect of mitochondrial STAT3 inhibitors on passive systemic IgE-Ag-mediated anaphylaxis: Mice were injected (ip) once a day for 3 d with 7 mg/kg of mitocur-1 or saline for the control group. The mice were sensitized with IgE anti-DNP through injection to the tail vein. 24 h later, the mice were challenged with DNP-BSA. After 1.5 min of the challenge, blood was collected, and histamine levels (A) as well as MCPT-1 cytokine release (B) were measured by ELISA. ($n = 5$; results represent means $\pm$ SEMs; $*P < .05$ compared to DMSO group; all other N.S)

mice in comparison with vehicle-treated mice. The passive systemic IgE-mediated anaphylaxis model was used. Mice were injected (ip) with mitocur-1 (7 mg/kg) or saline for three consecutive days. On the

second day, mice were injected (i.v.) with IgE anti-DNP. Twenty-four hours later, they were challenged with DNP and killed 1.5 minutes after the challenge. Plasma was collected and histamine levels were

measured. As shown in Figure 5A, mitocur-1 reduced plasma histamine levels by 34% compared to saline-treated mice ($n = 5$; $P = .007$). In addition, to show that the reduction in histamine is due to mast cell inhibition, secretion of mast cell protease 1 (MCPT-1), a marker of mast cells, was also measured. As shown in Figure 5B, 7 mg/kg mitocur-1 reduced plasma MCPT-1 levels by 65% compared to control mice ($n = 7$; $P = .02$). To compare the effect of mitocur-1 to the non-targeted curcumin, we repeated the systemic anaphylaxis model with three groups of mice; the control group was injected with saline and the two other groups were injected with either 7 mg/kg mitocur-1 or 20 mg/kg non-targeted curcumin. As can be seen in Figure S4, curcumin reduced plasma histamine by 20% ($n = 3$; $P = .29$), while mice injected with a 3-time lower dose of mitocur-1 showed 40% reduction in plasma histamine levels ($n = 3$; $P = .03$). Thus, mitocur-1 is more effective than non-targeted curcumin *in vivo*.

3.6 | Effect of mitochondrial STAT3 inhibitors on an asthma model

The effect of mitochondrial STAT3 inhibition on inflammation in BALF and bronchial submucosa was investigated. Differential cell type counts were determined after methacholine challenge in OVA- or saline-treated mice that had been previously administered (ip) either mitocur-1 or saline once a day for three consecutive days. As shown in Figure 6A, OVA treatment increased the total cell number

in BALF from control and mitocur-1-treated mice. However, mitocur-1 reduced the total cell number compared to OVA challenge. Mitocur-1 also reduced eosinophils in the absolute cell count (Figure 6B) and differential cell count (Figure 6C). In addition, the levels of MCPT-1 and IL-13, a cytokine shown to be central to the pathogenesis of asthma,^{25,26} were measured in BALF from OVA-treated mice which were previously administered saline or mitocur-1 (3 mg/kg). As shown in Figure 6D,E, mitocur-1 reduced the levels of MCPT-1 by 44% ($n = 7$; $P = .12$) and IL-13 by 46% ($n = 7$; $P = .001$). To check if the inhibitor affected mast cell number in the lungs, we assessed the number of mast cells in the tissue of the mice that were not challenged with OVA using histological staining with toluidine blue, a mast cell-specific dye. As can be seen in Figure S5, there was no significant difference in mast cell number in lungs of mice treated with mitocur-1 compared to mice treated with saline.

4 | DISCUSSION

Allergies are among the most common medical disorders, affecting more than one in six people in the United States.²⁷ Today, allergies are managed by prevention, and by medication such as epinephrine, antihistamines, anti-inflammatory agents (corticosteroids), decongestants, leukotriene inhibitors, mast cell stabilizers, the anti-IgE antibody omalizumab and immunotherapy.²⁸ New therapeutic

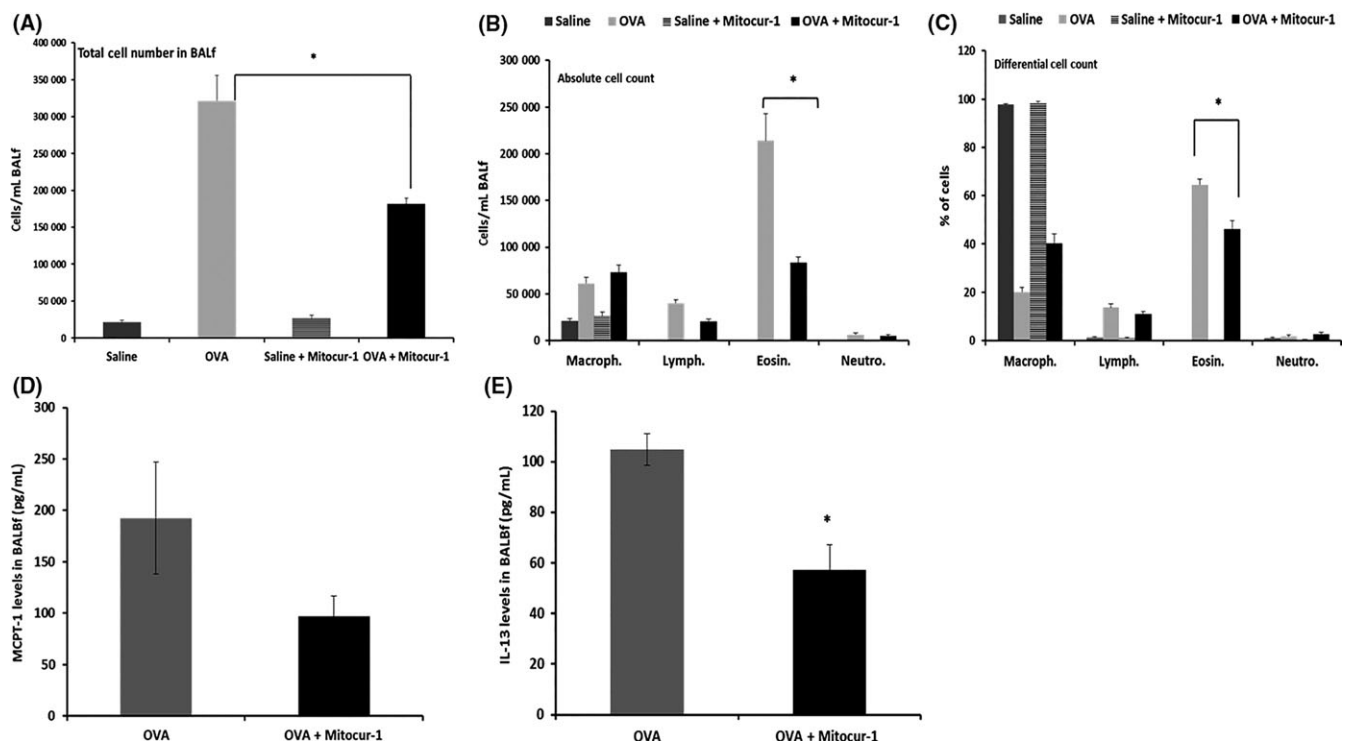


FIGURE 6 The effect of mitochondrial STAT3 inhibitors on an asthma model: (A–B) Inflammatory cell counts in OVA-induced allergic asthmatic mice administered mitocur-1, (3 mg/kg) or saline ip once a day for 3 d during the challenge period compared to those in non-sensitized mice (saline control). (A) Represents the total cell number/mL in the BALF, (B) represents the absolute count of the BALF cells and (C) represents the differential count of the BALF cells. MCPT-1 (D) and IL-13 (E) levels in BALF were determined using ELISA. ($n = 7$; results represent means $\pm$ SEMs; * $P < .05$ compared to OVA or saline group; all other N.S.)

approaches include Toll-like receptor agonists, cytokine blockers, specific cytokine receptor antagonists and transcription factor modulators targeting syk kinase, peroxisome proliferator-activated receptor gamma, and nuclear factor kappa B.

Anti-inflammatory medications usually inhibit mast cell degranulation indirectly by modulating T-cell activation and have an indirect effect on mast cell inhibition.²⁹ Omalizumab is an anti-IgE monoclonal antibody that binds free IgE in the serum and prevents IgE binding to the FcεRI. However, this drug takes weeks to be effective and it shows an effect through mast cell differentiation and not a direct effect on mast cell activation.³⁰ Immunotherapy, whereby low doses of allergens are injected over 12–24 months, is a long-term treatment that is not effective for all types of allergies.³¹ Therefore, there is a real need for an effective general treatment for the symptoms of allergy, and inhibition of mast cell exocytosis provides a promising approach. As we have recently observed, mitochondrial STAT3 is essential for mast cell exocytosis² and thus the development of a novel mitochondrial STAT3 inhibitor has the potential to be an effective treatment for all kinds of allergy-associated diseases.

In the present study, we have demonstrated the potential of developing mitochondrial STAT3 inhibitors for interfering with the allergic response. These inhibitors were produced based on curcumin, a known STAT3 inhibitor,¹⁵ and TPP, a moiety previously shown to target molecules to the mitochondria.¹⁶ Reddy et al²² used TPP to increase the bioavailability of curcumin for cancer treatment, without distinguishing between inhibiting STAT3 in the mitochondria or nucleus. As mitochondrial function and downstream targets of STAT3 were inhibited, their findings clearly show that both mitochondrial and nuclear STAT3 were inhibited.

We showed that low concentrations of both mitocur-1 and -3 did not affect the transcriptional role played by nuclear STAT3 as they did not inhibit STAT3 target genes such as Bcl-2, MCL-1, and caspase3 both in the RBL cell line and in BMMC, a primary mouse mast cell culture. In addition, low concentrations of these inhibitors did not induce apoptosis in these cells as assessed by cleaved caspase3 levels and were not toxic to the cells (Figure 1A–O). These results support the observation that inhibiting STAT3 in the mitochondria does not induce apoptosis.⁴

The role of STAT3 in the mitochondria is still unclear, making it difficult to develop an assay for measuring STAT3 mitochondrial activity. However, it has been shown that inhibition of STAT3 leads to a reduction in ATP levels and oxygen consumption^{5–8} and its phosphorylation on Ser727 is crucial for its mitochondrial activity.⁵ Thus, measuring these indirect effects of STAT3 could provide an assessment of its mitochondrial activity. Clearly, both mitocur-1 and -3, in glucose-free medium, reduced mitochondrial ATP in a dose-dependent manner. Interestingly, in complete medium, both mitocur-1 and -3 enhanced ATP production (Figure 2B). One possibility is that the loss of mitochondrial STAT3 activity could lead to activation of glycolysis to compensate for the loss of mitochondrial ATP. Similar results were observed when oxygen consumption, a marker of OXPHOS, was measured (Figure 2C). In addition, mitocur-1 and -3

decreased serine 727 phosphorylation of STAT3 in a dose-dependent manner (Figure 2C–D), indicating that the inhibition of mitochondrial activity was a result of impaired mitochondrial STAT3 activity. Reddy et al²² showed that the ratio of mitocur-1 and -3 in the cytosol to the mitochondria is 1:10, confirming that conjugation of TPP to curcumin successfully targeted the molecules to the mitochondria. It is important to emphasize that Reddy et al used TPP to increase the uptake of curcumin and not to specifically target the mitochondria.

After observing the effect of mitocur-1 and -3 on mitochondrial activities, we hypothesized that these inhibitors would affect mast cell function. Based on our observation of a significant reduction in mast cell degranulation and abolished cytokine secretion following treatment with mitocur-1 and -3 in rat as well as mouse and human primary mast cells (Figures 3 and 4), these molecules emerge as potent inhibitors of mast cell exocytosis. It is important to emphasize that this effect was not a result of apoptosis as no caspase3 cleavage was observed at the concentrations used (Figure 1A–B). Furthermore, the fact that mitocur-1 and -3 decreased the degranulation levels and inhibited cytokine secretion clearly indicates that they have a general effect on mast cell exocytosis. As these results were achieved using concentrations that had no effect on STAT3 target genes, but did affect mitochondrial function, we can conclude that inhibiting mitochondrial STAT3 is sufficient to reduce mast cell degranulation and that these results are not due to enhanced uptake of curcumin. Furthermore, Reddy et al showed that 10 μmol L⁻¹ of non-targeted curcumin, a concentration we showed to have no effect on mast cell degranulation, does not accumulate in the mitochondria, further confirming our theory that inhibition of mitochondrial STAT3 is sufficient to negatively modulate mast cell function. Interestingly, Hox et al³² recently showed that inhibition of STAT3 with C188–9, a STAT3 small molecule, does not affect mast cell degranulation in mouse mast cells. In addition, Qin et al³³ showed that JSI124, a small STAT3 inhibitor which inhibits STAT3 DNA binding and STAT3-mediated gene transcription, failed to effect mast cell degranulation in RBL cells. Most likely, these results are due to these inhibitors' weak effect on mitochondrial STAT3, again validating the role of mitochondrial STAT in mast cell degranulation.

Having established the importance of mitocur-1 and -3 in inhibiting mast cell exocytosis, we investigated their effect in vivo. In an acute systemic anaphylaxis model in mice, a marked decrease in histamine secretion was observed with both inhibitors and this decrease was greater than that seen with non-targeted curcumin (Figure 5A). Allergic asthma involves airway obstruction and inflammation that is partially reversible with medication.³⁴ Eosinophils, whose numbers in asthma increase after exposure to specific allergens,³⁵ have long been recognized as the major inflammatory cells involved in the pathobiology of both childhood-onset, allergic asthma and adult-onset, non-allergic asthma.³⁶ The allergic response is often manifested as a dual reaction, one part of which is an early-phase response mainly involving mast cell degranulation, which is followed by a late-phase response with more sustained

inflammation. This late response involves the recruitment of other effector cells, such as TH2 lymphocytes, eosinophils.^{37,38} For the in vivo experiments, we decided to continue with mitocur-1 alone because it showed higher potency (data not shown). To determine the effect mitocur-1 on allergic asthma, we assessed airway inflammation in BALF and the bronchial submucosa after methacholine challenge in OVA or saline-treated mice in the presence or absence of mitocur-1. Mitocur-1 significantly decreased the inflammation state of BALF and the bronchial submucosa compared to saline. Furthermore, mitocur-1 specifically decreased eosinophilia compared to control in BALF, showing a decrease in the early-phase response (Figure 6D). It is important to note the possibility of an additive or even synergistic physiological effect of mitocur-1 due to the inhibition of mitochondrial STAT3 in other immune cells including eosinophils. In addition, mitocur-1 significantly decreased the secretion of IL-13 (Figure 6E), which plays a key role in the pathogenesis of asthma, as it increases goblet cell differentiation, activation of fibroblasts, elevation of bronchial hyperresponsiveness, and the switching of B-cell antibody production from IgM to IgE.²⁶ As mast cells initiate the early phase of the allergic response which leads to sustained inflammation and our results show reduced inflammation and MCPT-1 levels after mitocur-1 treatment (Figure 6A-E), we can conclude that modulation of mast cell function by mitocur-1 is sufficient to reduce inflammation in an ovalbumin mouse model of asthma. Therefore, the development of mitochondrial STAT3 inhibitors could serve as a potential treatment for allergy-associated diseases.

A major concern about the use of mitochondrial STAT3 inhibition as an intervention for allergy is the potential effect on other cells besides mast cells. As shown in this study, a moderate inhibition of mitochondrial STAT3 activity is enough to provide effective treatment for allergic disease, while not affecting the total ATP content. Other cells known to express mitochondrial STAT3 are cardiomyocytes and spermatozoa.^{39,40} High levels of Stattic (50 $\mu\text{mol L}^{-1}$), a known STAT3 inhibitor, impaired sperm functions such as motility in vitro, while a lower concentration (25 $\mu\text{mol L}^{-1}$) that reduced mast cell degranulation by 90%, did not affect this function.⁴⁰ Similarly, a high concentration (100 $\mu\text{mol L}^{-1}$) of Stattic reduced ATP levels in cardiomyocytes by only 32%.⁴¹ These studies did not discriminate between the effects of STAT3 transcriptional inhibition and STAT3's role in the mitochondria. In addition, in other studies, non-targeted curcumin was administered to mice at higher concentrations (100–300 mg/kg) for up to 30 days without any toxic effects.^{42,43} Therefore, mitocur-1, which is based on curcumin, and was effective when injected at a dose range of 3–7 mg/kg (Figure 5 and 6) should be safe. Further study is needed to determine the formulation of mitocur-1, its suitable administration and its pharmacokinetics to determine the optimal working concentration and possible side effects.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

T.E. and I.S. are equal contributors to this work, designed, performed experiments, analyzed data, and wrote the paper; N.L. performed experiments with CBMC; M.A. performed experiments for the asthma model; O.G. performed experiments; N.B. and F.L.S. and E.R. designed experiments, analyzed data, and wrote the paper.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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Supplementary Figure 1- The effect of mitochondrial STAT3 inhibitors on Bcl-2 mRNA levels and cell viability:

RBL (A) and BMMC (B) cells were treated with mitocur-1 or mitocur-3 (3, 6, 12 or 25 μ M) for 3 h; DMSO was used as control. The levels of β -actin and Bcl-2 were determined by qReal-Time PCR. (C) RBL cells were treated with DMSO, mitocur-1 or mitocur-3 (3, 6, 12 or 25 μ M) for 3 h, and then the cells were washed with PBS and incubated with 0.05% Trypan blue; stained cells are indicated by arrows. (n =3; results represent means $\pm$ SEMs; *p<0.05 compared to DMSO group; all other N.S).

Supplementary Figure 2- Densitometry of the phosphor-727-STAT3 blots shown in Figure2:

This figure represents the densitometry of the blots presented in Figure 2 (D) and (E) showing phospho-727-STAT3 levels relative to β -actin in RBL (A) and BMMC (B). (BMMC n=3, RBL n=4; results represent mean $\pm$ SEM, n=5 *p<0.05).

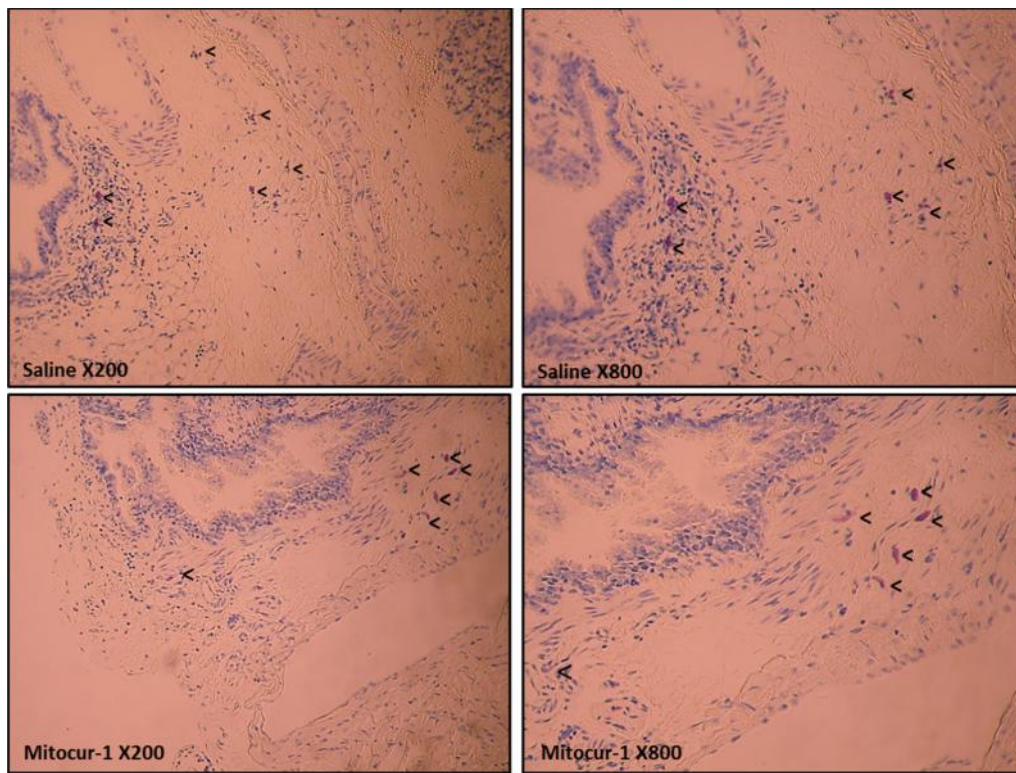
Supplementary Figure 3- Dose response of Mitocur-1 and Mitocur-3 in a degranulation assay in RBL cells:

IgE sensitized RBL cells were incubated with DMSO or (3, 6, or 12 μ M) of either mitocur-1 or -3 for 3 h followed by 30 min DNP-BSA challenge, β -hexosaminidase release was then measured. (n=3; results represent mean $\pm$ SEM, n=5 *p<0.05).

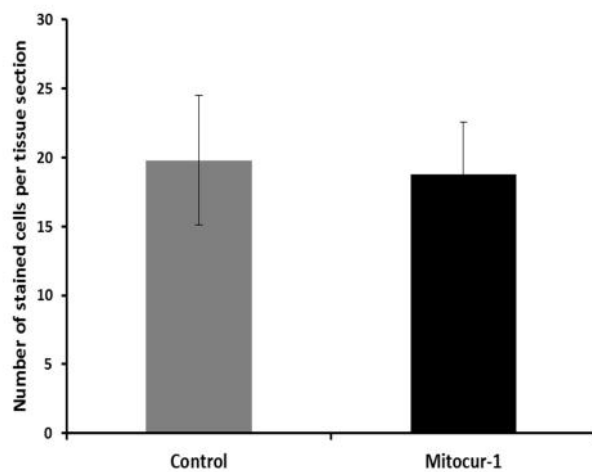
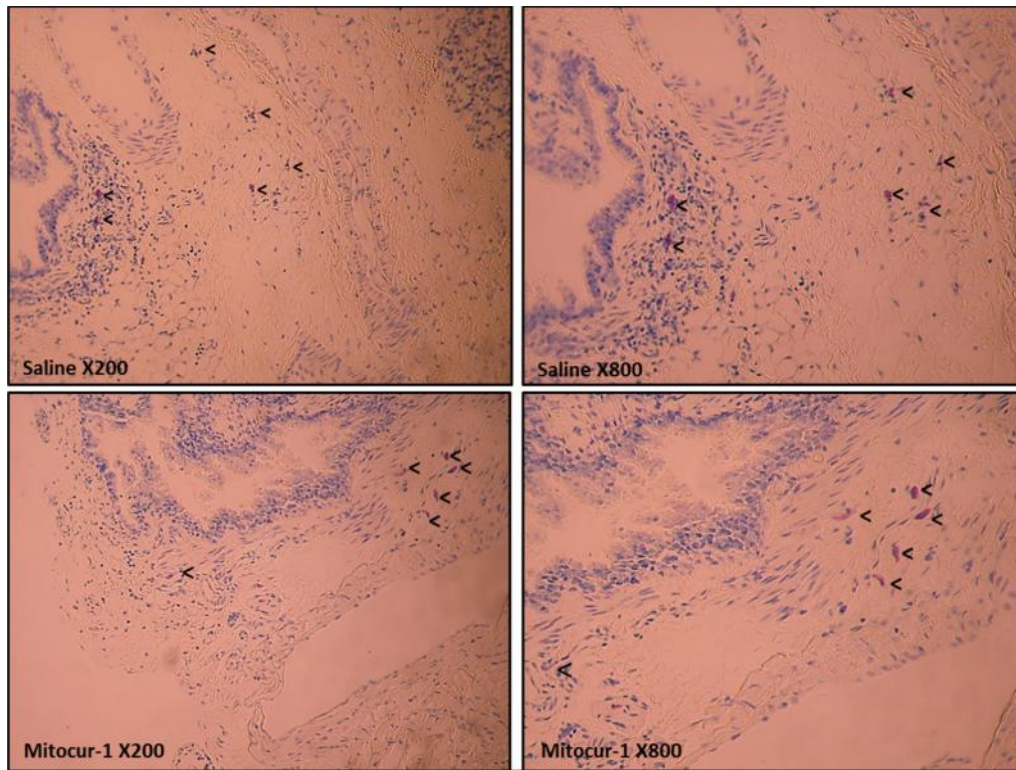
Supplementary Figure 4- The effect of Mitocur-1 and Mitocur-3 on passive systemic IgE-Ag-mediated anaphylaxis:

Mice were injected (i.p) once a day for 3 days with 7 mg/kg of mitocur-1 or 20 mg/kg of curcumin and saline for the control group. The mice were sensitized with IgE anti-DNP through injection to the tail vein. 24 h later, the mice were challenged with DNP-BSA. After 1.5 min of the challenge, blood was collected, and histamine levels were measured by using competitive ELISA (n(Control)=3; n(Mitocur1)=3, n(Curcumin)=3; results represent mean $\pm$ SEM; *p<0.05 compared to control group.)

A



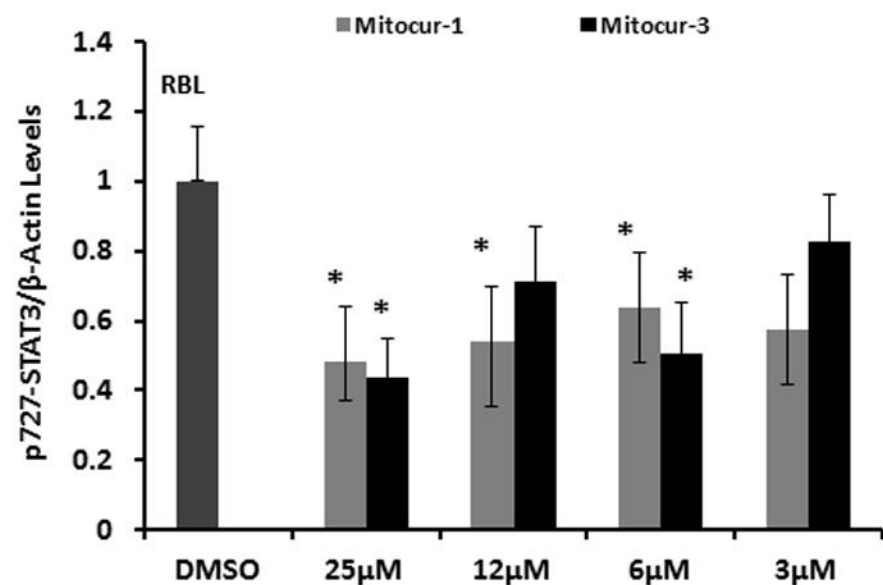
B



Supplementary Figure 5- The effect of Mitocur-1 and Mitocur-3 on the number of mast cells in the lung tissue:

(A) Lung tissue sections from mice treated with saline or with mitocur-1 and stained with Toluidine blue (n=3) (B) Toluidine blue stained mast cell number counted in stained tissue sections (results represent mean $\pm$ SEM n=3; N.S).

A



B

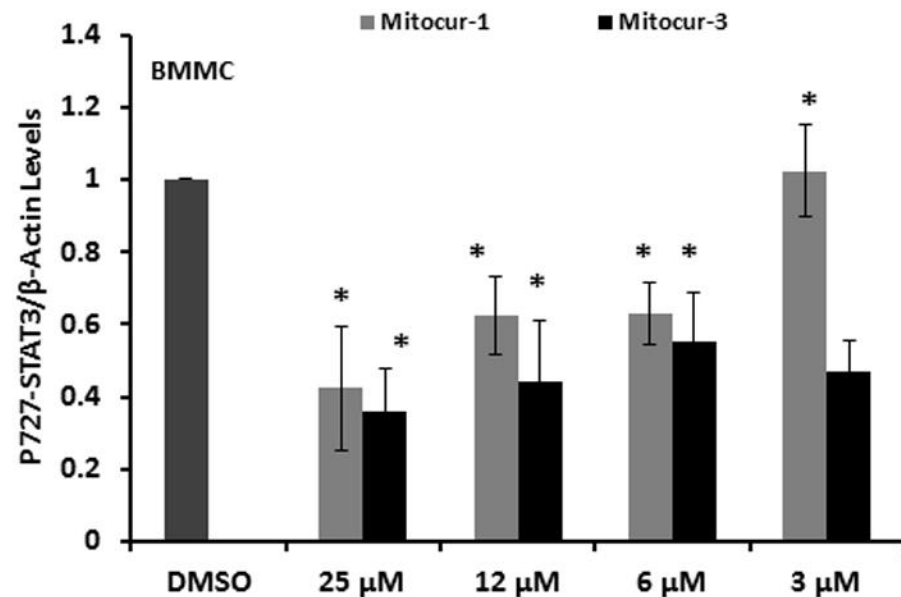


Figure S.2

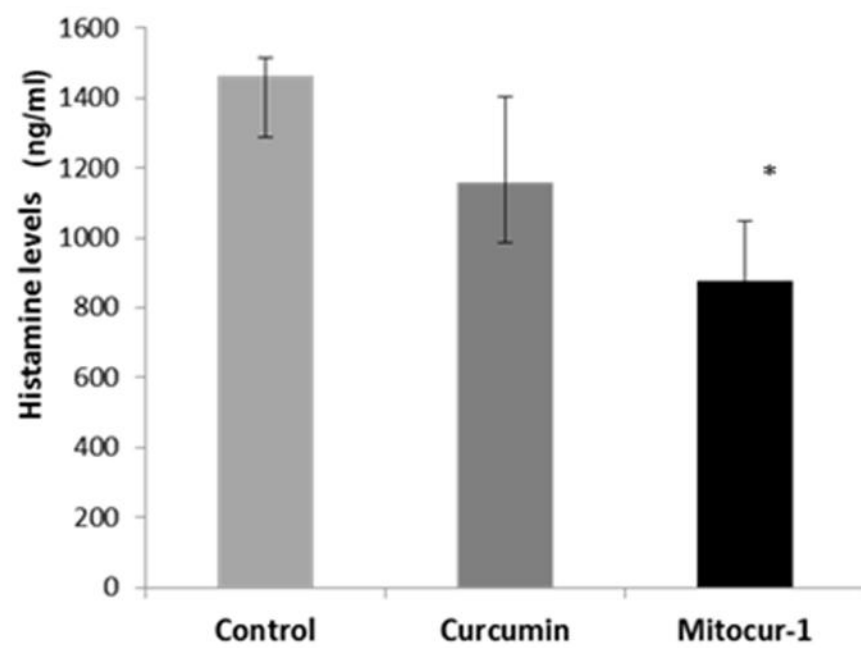


Figure S.4

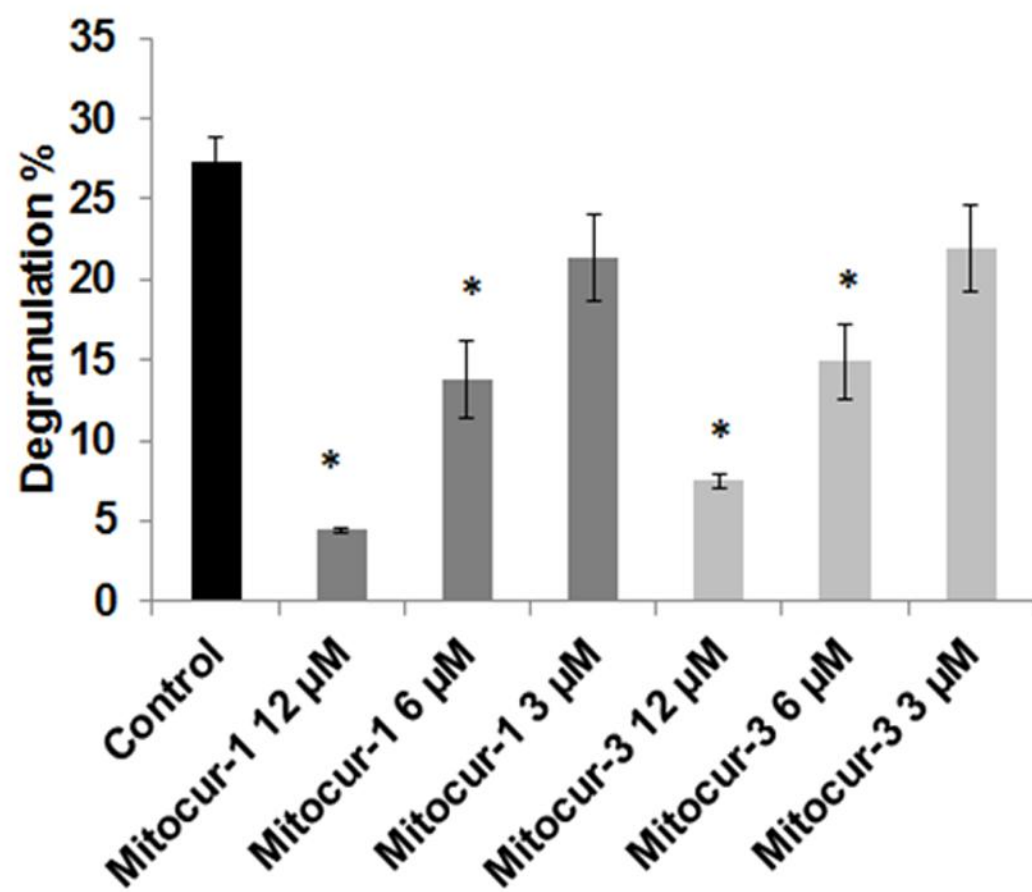


Figure S.3

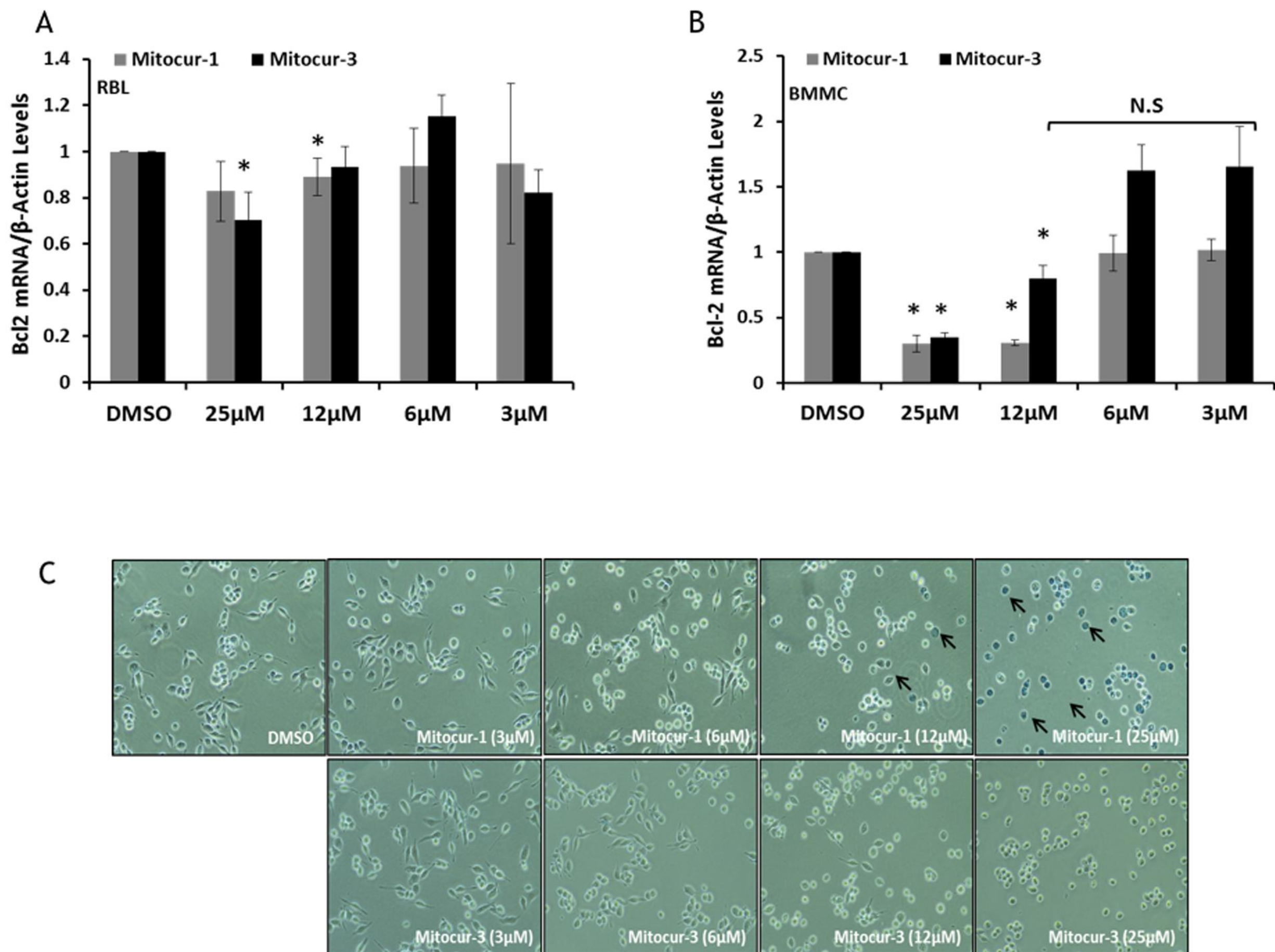


Figure S.1