

Mitochondrial STAT3 plays a major role in IgE-antigen-mediated mast cell exocytosis

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Background: The involvement of mitochondrial oxidative phosphorylation (OXPHOS) in mast cell exocytosis was recently suggested by the finding that mitochondria translocate to exocytosis sites upon mast cell activation. In parallel, mitochondrial signal transducer and activator of transcription 3 (STAT3) was found to be involved in ATP production. However, the regulation of mitochondrial STAT3 function and its connection to mast cell exocytosis is unknown.

Objective: We sought to explore the role played by mitochondrial STAT3 in mast cell exocytosis.

Methods: Experiments were performed *in vitro* with human and mouse mast cells and rat basophilic leukemia (RBL) cells and *in vivo* in mice. OXPHOS activity was measured after immunologic activation. The expression of STAT3, extracellular signal-regulated kinase 1/2, and protein inhibitor of activated STAT3 in the mitochondria during mast cell activation was determined, as was the effect of STAT3 inhibition on OXPHOS activity and mast cell function.

Results: Here we show that mitochondrial STAT3 is essential for immunologically mediated degranulation of human and mouse mast cells and RBL cells. Additionally, in IgE-antigen-activated RBL cells, mitochondrial STAT3 was phosphorylated on serine 727 in an extracellular signal-regulated kinase 1/2-dependent

manner, which was followed by induction of OXPHOS activity. Furthermore, the endogenous inhibitor of STAT3, protein inhibitor of activated STAT3, was found to inhibit OXPHOS activity in the mitochondria, resulting in inhibition of mast cell degranulation. Moreover, mice injected with Stattic, a STAT3 inhibitor, had a significant decrease in histamine secretion. **Conclusion:** These results provide the first evidence of a regulatory role for mitochondrial STAT3 in mast cell functions, and therefore mitochondrial STAT3 could serve as a new target for the manipulation of allergic diseases. (J Allergy Clin Immunol 2014;■■■:■■■-■■■.)

Key words: Allergy, mast cells, degranulation, cytokines, PIAS3, STAT3, mitochondria

The role played by mitochondria in mast cell activation has recently been described. Studies in a human mast cell line showed that degranulation and TNF- α secretion require mitochondrial translocation into exocytosis sites.^{1,2}

Adequate ATP levels in mast cells have been shown to be essential for degranulation and cytokine secretion.³⁻⁵ It was observed that depletion of glucose from mast cell culture medium resulted in a rapid decrease in ATP levels followed by a reduction in histamine secretion, whereas supplementation of glucose to the medium induced a time-dependent increase in ATP levels, which correlated with the recovery in histamine secretion.³ However, the role played by mitochondrial ATP produced by oxidative phosphorylation (OXPHOS) during mast cell degranulation remains elusive and, to our knowledge, has not yet been reported in the literature.

Recently, it has been shown in a variety of cell types that signal transducer and activator of transcription 3 (STAT3) has a noncanonical role in mitochondria, whereby it regulates OXPHOS by modulating mitochondrial electron transport chain activity.⁶⁻⁸ STAT3-null cells were shown to have 50% less total ATP content and decreased activity of electron transfer chain complexes.⁶⁻⁸ Mitochondrial STAT3 function required serine 727 phosphorylation.⁶ Interestingly, we have previously reported that STAT3 undergoes phosphorylation on serine 727 during mast cell activation⁹; however, the significance of this phosphorylation is not fully understood.

The extracellular signal-regulated kinases 1 and 2 (ERK1/2) are known to phosphorylate STAT3 on the serine 727 residue in a variety of cells¹⁰⁻¹² and to be activated after IgE-antigen stimuli.^{13,14} However, ERK1/2 expression in mitochondria has just begun to be explored. ERK1/2 was found in the mitochondria of cardiomyocytes, where it plays a role in protection from ischemia reperfusion,¹⁵ and in neuronal cells.^{16,17} Furthermore, U0126, an ERK1/2 pathway inhibitor, was shown to inhibit ATP production in macrophages.¹⁸ Thus in the present work we

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Abbreviations used

BMMC:	Bone marrow–derived mast cell
CBMC:	Cord blood–derived mast cell
DMSO:	Dimethyl sulfoxide
DNP:	Dinitrophenyl albumin
ERK1/2:	Extracellular signal-regulated kinase 1 and 2
OXPHOS:	Oxidative phosphorylation
PIAS3:	Protein inhibitor of activated STAT3
RBL:	Rat basophilic leukemia
SDH:	Succinate dehydrogenase
siRNA:	Small interfering RNA
STAT3:	Signal transducer and activator of transcription 3

explored the correlation and interplay between mitochondrial ERK1/2-mediated STAT3 phosphorylation and OXPHOS in activated mast cells.

Phosphorylation of STAT3 on serine 727 after mast cell activation is followed by its association with the protein inhibitor of activated STAT3 (PIAS3), which inhibits its transcriptional activity.^{9,19} PIAS3, the main inhibitor of STAT3, is a multifunctional protein that plays a significant role in the modulation of additional key transcription factors, such as microphthalmia transcription factor,²⁰ nuclear factor κ B,²¹ Smads,²² and estrogen receptor.²³ Therefore we wanted to determine whether the inhibition of STAT3 activity by PIAS3 also occurs in the mitochondria, with a subsequent effect on OXPHOS.

In the present work we explored the potential involvement of STAT3 in mitochondrial ATP production and in exocytosis of immunologically activated mast cells and showed that mitochondrial STAT3 serves as a key regulator of mast cell exocytosis.

METHODS

The methods used in this study are described in the [Methods](#) section in this article's Online Repository at www.jacionline.org.

RESULTS**Mitochondrial ATP and mast cell exocytosis**

Glycolysis was prevented and β -hexosaminidase release was measured to determine whether mitochondrial ATP produced by OXPHOS is involved in mast cell degranulation. Rat basophilic leukemia (RBL) cells and bone marrow–derived mast cells (BMMCs) were cultured for 24 hours in a glucose-free, OXPHOS-dependent medium supplemented with dialyzed serum as an energy source or in complete medium used as a control. These cells were activated by 2 hours of incubation with IgE followed by 30 minutes of incubation with dinitrophenyl albumin (DNP). As shown in [Fig 1, A](#), no significant change in degranulation was observed between these 2 groups, indicating that the major part of the energy for degranulation is derived from mitochondrial ATP. The effect of immunologic activation on mitochondrial ATP levels was determined in immunologically activated RBL cells grown in glucose-free medium ([Fig 1, B](#)). The ATP content was determined after 5 minutes of incubation with DNP to determine its role in the initiation of the exocytosis process. The immunologic trigger caused a significant increase of nearly 40% in the level of ATP in the activated cells.

Effect of STAT3 inhibition on mitochondrial ATP production and oxygen consumption

STAT3 and its serine 727 phosphorylated form were previously reported to regulate mitochondrial ATP production in a variety of cells.^{6–8} Thus next we determined the role, if any, played by mitochondrial STAT3 in mast cell function.

RBL cells and BMMCs were incubated with the STAT3 inhibitor Stattic²⁴ for 20 minutes (60 μ mol/L dissolved in 100% dimethyl sulfoxide [DMSO]), a timeframe that should not allow protein synthesis of STAT3 target genes, to inhibit STAT3 and distinguish between its effect on transcription and that on the modulation of mitochondrial activities. Control cells were treated with the same volume of DMSO alone. The effects of STAT3 inhibition on ATP levels, oxygen consumption, and electron transport chain activity (as indicators of OXPHOS activity) were determined. As shown in [Fig 2, A to C](#), STAT3 inhibition resulted in a significant reduction in ATP levels ($72.0\% \pm 9.0\%$, $P < .05$), oxygen consumption ($18.6\% \pm 8.3\%$, $P < .05$), and complex 2+3 activity ($50.8\% \pm 6.1\%$, $P < .05$) in RBL cells. No significant change in cytochrome c oxidase (complex 4) and succinate dehydrogenase (SDH, complex 2) activities were observed ([Fig 2, C](#)). These results indicate a selective effect of STAT3 inhibition on cytochrome c reductase (complex 3) activity. Furthermore, as shown in [Fig 2, A](#), a similar reduction in ATP levels ($76.4\% \pm 9.3\%$, $P < .05$) was observed in BMMCs treated with Stattic. Next it was determined whether these effects correlated with the inhibition of STAT3 serine 727 phosphorylation. The level of serine 727 phosphorylation in total cell lysate was determined in RBL cells treated with Stattic for 20 minutes and immunologically activated for 5 minutes. As shown in [Fig 2, D](#), a significant reduction in the phosphorylation levels of STAT3 was observed in these activated cells treated with Stattic. Densitometric quantification of this Western blot is shown in [Fig E1](#) in this article's Online Repository at www.jacionline.org. Because reduction in ATP content is also a marker for apoptosis, we determined whether the inhibitory effect of Stattic initiated apoptosis by determining levels of cleaved caspase 3. As shown in [Fig E2](#) in this article's Online Repository at www.jacionline.org, no change in caspase 3 levels was observed in cells treated with Stattic.

Role played by STAT3 in mast cell function

RBL cells were incubated for 20 minutes with 60 μ mol/L Stattic or DMSO alone (control), followed by determination of degranulation levels and TNF- α secretion, to further explore the effect of mitochondrial STAT3 on mast cell function. β -Hexosaminidase release and TNF- α secretion in control cells were $28.9\% \pm 2.7\%$ and 23.3 ± 5.1 pg/mL, respectively ([Fig 3, A and B](#)). Incubation with Stattic completely abolished both mast cell β -hexosaminidase release and TNF- α secretion ([Fig 3, A and B](#), $P < .05$). The degranulation was also totally abolished by 5 minutes of incubation with Stattic (data not shown). This effect on IgE-antigen–mediated β -hexosaminidase release of RBL cells was found to be concentration dependent (see [Fig E3](#) in this article's Online Repository at www.jacionline.org). Stattic treatment of immunologically activated BMMCs achieved similar results ([Fig 3, A and C](#)). Granzyme B secretion was also greatly reduced ([Fig 3, D](#)). Further experiments with an additional STAT3 inhibitor, C188-9,²⁵ were carried out to confirm the selectivity of STAT3 inhibition in this system. As shown in

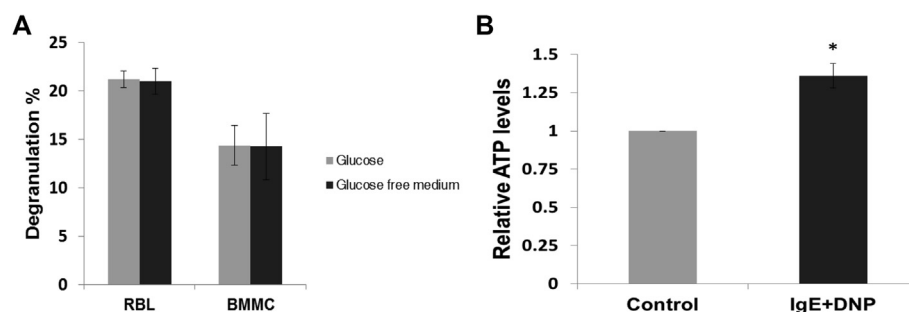


FIG 1. Degranulation and ATP levels of immunologically activated cells grown in glucose-free medium. **A**, BMMCs and RBL cells were grown in glucose-free or complete medium for 24 hours. Cells were sensitized with 100 ng/mL IgE for 2 hours, followed by 30 minutes of stimulation with DNP, and then analyzed for β -hexosaminidase release. Results represent means $\pm$ SEMs ($n = 3$). * $P < .05$. **B**, ATP levels of RBL cells grown in glucose-free medium for 24 hours. Cells were sensitized with 100 ng/mL IgE for 2 hours and then challenged for 5 minutes with DNP, lysed, and analyzed for total cell ATP. Results represent means $\pm$ SEMs ($n = 3$). * $P < .05$.

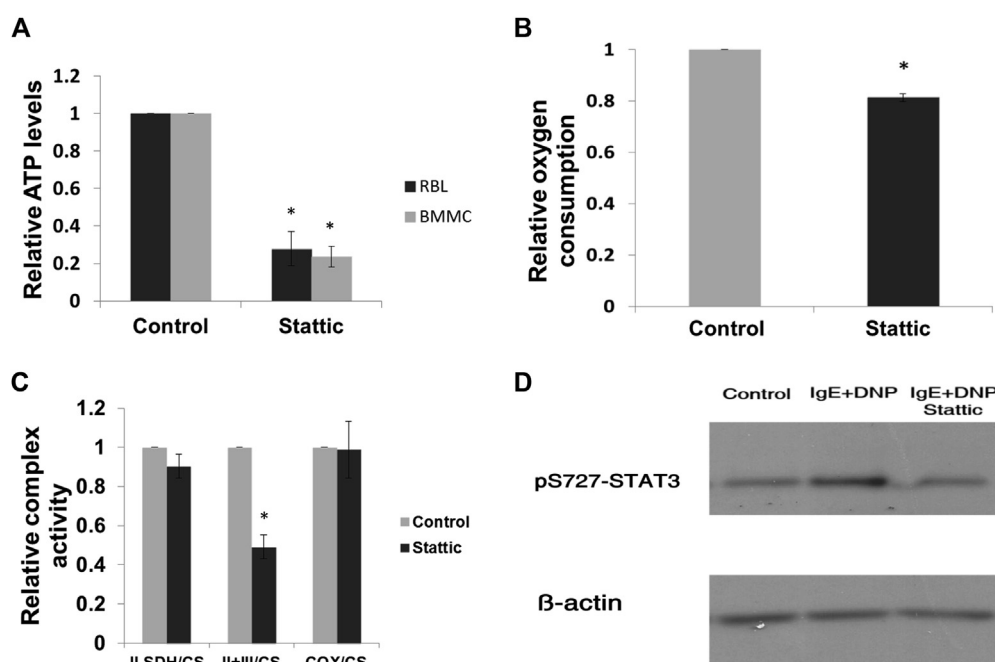


FIG 2. Effect of Stat3 on mitochondrial characteristics. **A**, BMMCs and RBL cells were incubated with 60 μ M of the STAT3 inhibitor Stat3 for 20 minutes. Cells were lysed, and then total cell ATP levels were measured. Results represent means $\pm$ SEMs ($n = 3$). * $P < .05$. **B**, Oxygen consumption analysis. RBL cells were grown in 24-well plates, treated as in Fig 2, A, and then intracellular oxygen consumption was determined with the Seahorse Extracellular Flux Analyzer. Results represent means $\pm$ SEMs ($n = 3$). * $P < .05$. **C**, Electron transport chain activity analysis. RBL cells were incubated with 60 μ M of the STAT3 inhibitor Stat3 for 30 minutes and then analyzed for electron transport chain complex activity. COX, Cytochrome c oxidase; CS, citrate synthase; II+III, complex 2 and 3. Results represent means $\pm$ SEMs ($n = 3$). * $P < .05$. **D**, RBL cells were sensitized with 100 ng/mL IgE for 2 hours, followed by 5 minutes of DNP challenge. Twenty minutes before the addition of DNP, 60 μ M of the STAT3 inhibitor Stat3 was added to the medium. Levels of P-STAT3 727 (pS727-STAT3) and actin control in the cell lysates were determined by using Western blot analysis. One representative of 3 independent experiments is shown.

Fig E4 in this article's Online Repository at www.jacionline.org, degranulation was also abolished in immunologically activated RBL cells treated with 250 μ M C188-9 for 10 minutes.

Mitochondrial STAT3 serine 727 ERK1/2-dependent phosphorylation during mast cell activation

We have previously reported that STAT3 undergoes phosphorylation on serine 727 during mast cell activation.⁹ However, the

biological significance of this phosphorylation in mast cell activation was not fully understood. To determine whether such phosphorylation occurred in the mitochondria after immunologic activation in RBL cells, these cells were sensitized with IgE for 2 hours and then triggered with DNP for 5 minutes, whereas quiescent cells were used as controls. Mitochondria were then isolated from these cells, and the levels of STAT3 serine 727 phosphorylation were determined by means of Western blot analysis. As shown in Fig 4, A, an enhancement in the level of

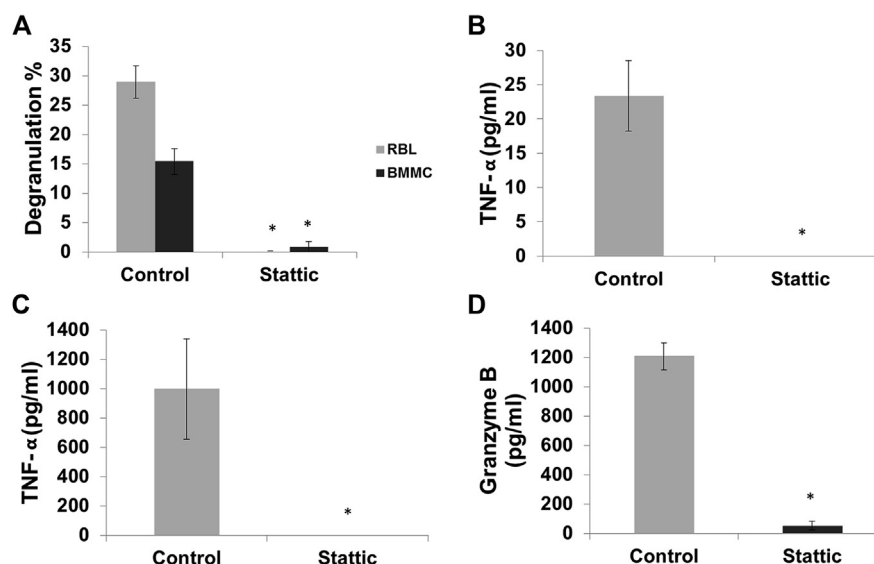


FIG 3. Effect of Stattic on mast cell degranulation. **A** and **B**, BMMCs and RBL cells were sensitized with 100 ng/mL IgE for 2 hours, followed by a 30-minute DNP challenge. Twenty minutes before the addition of DNP-BSA, 60 μ mol/L Stattic was added to the medium. β -Hexosaminidase release and TNF- α secretion were then measured. Results represent means $\pm$ SEMs ($n = 3$). * $P < .05$. **C** and **D**, BMMCs were sensitized with 100 ng/mL IgE for 2 hours, followed by a 30-minute DNP challenge. Twenty minutes before the addition of DNP, 60 μ mol/L Stattic was added to the medium. β -Hexosaminidase release and TNF- α and granzyme B secretion were then measured. Results represent means $\pm$ SEMs ($n = 3$). * $P < .05$.

mitochondrial STAT3 serine 727 phosphorylation was observed in the activated cells. The amount of STAT3 in the mitochondria was determined before and after cell activation to evaluate whether this phosphorylation resulted from STAT3 translocation into the mitochondria. No change in total STAT3 levels was observed (Fig 4, A).

The amount of ERK1/2 in the mitochondria was determined before and after 5 minutes of IgE-DNP activation in RBL cells to determine whether ERK1/2 translocates to the mitochondria upon mast cell activation. As shown in Fig 4, A, an enhancement in the level of mitochondrial ERK1/2 was observed in the activated cells. Cells were treated with the ERK1/2 pathway inhibitor U0126 to further evaluate whether mitochondrial ERK1/2 phosphorylates mitochondrial STAT3 on serine 727. RBL cells were either activated or not for 5 minutes in the presence of 40 μ mol/L U0126 dissolved in DMSO or DMSO alone (control), and total cell levels of phosphorylated STAT3 727 serine were determined by means of Western blot analysis. As shown in Fig 4, B, a significant decrease in STAT3 727 serine phosphorylation was observed in the U0126-treated cells. In a further experiment immunologically activated RBL cells were fractionated into cytosol and mitochondria, and the mitochondrial levels of phosphorylated STAT3 on serine 727 were determined by using Western blot analysis. As shown in Fig 4, C, ERK1/2 inhibition was followed by a respective decrease in STAT3 727 serine phosphorylation. Densitometric quantification of the above Western blots is shown in Fig E5 in this article's Online Repository at www.jacionline.org.

Inhibition of mitochondrial ATP production and oxygen consumption by U0126

U0126 is a well-known inhibitor of mast cell degranulation.²⁶⁻³⁰ To determine whether part of the effect of U0126 on

degranulation is mediated through inhibition of mitochondrial STAT3 serine 727 phosphorylation, we explored the effect of ERK1/2 inhibition by U0126 on degranulation, ATP content, and oxygen consumption in IgE-antigen-stimulated RBL cells. As shown in Fig 5, A, 20 minutes of incubation with 40 μ mol/L U0126 (dissolved in 100% DMSO) resulted in a $33.0\% \pm 0.6\%$ reduction in degranulation compared with that seen in control cells (DMSO alone). Although higher concentrations of U0126 resulted in a more profound reduction in degranulation levels (see Fig E6 in this article's Online Repository at www.jacionline.org), we used the minimum concentration that inhibited mast cell degranulation by more than 30% to ensure specificity. The increase in ATP levels caused by immunologic activation of RBL cells ($12.2\% \pm 1.0\%$ increase, $P < .05$) was reversed by 20 minutes of pretreatment with 40 μ mol/L U0126, resulting in a slight but significant decrease in ATP levels ($3.0\% \pm 0.4\%$ decrease, $P < .05$; Fig 5, B). Furthermore, this inhibitor also caused a statistically significant reduction in oxygen consumption in both quiescent ($9.4\% \pm 2.4\%$ decrease, $P < .05$) and activated ($15.8\% \pm 4.9\%$ decrease, $P < .05$) cells (Fig 5, C). In addition, we determined that the effect of U0126 was not due to apoptosis because no change in caspase 3 levels was observed in cells treated with U0126 (see Fig E7 in this article's Online Repository at www.jacionline.org).

Role played by PIAS3 in mast cell function

As mentioned previously, mast cell activation leads to the phosphorylation of STAT3 on the serine 727 moiety, followed by its association with PIAS3, which inhibits its transcriptional activity.^{9,19} Because PIAS3 is known to be the main endogenous inhibitor of STAT3, we determined whether PIAS3 is localized in the mitochondria as well. Accordingly, RBL cells were

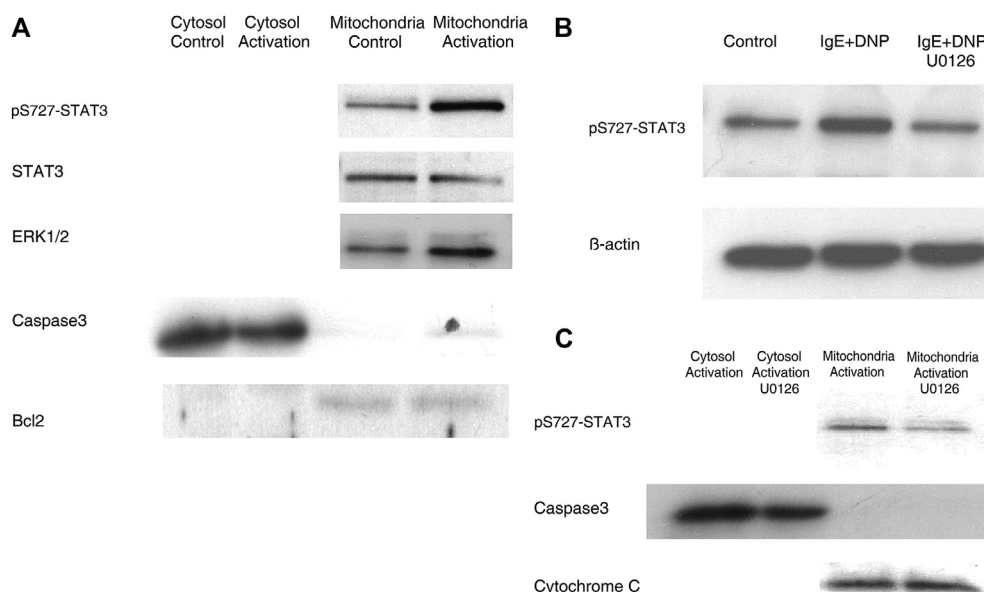


FIG 4. Mitochondrial STAT3 and ERK1/2 signaling in mast cell activation. **A**, RBL cells were sensitized with 100 ng/mL IgE for 2 hours, followed by a 5-minute DNP challenge. The cytosolic and mitochondrial levels of phospho-STAT3 serine 727 (pS727-STAT3), STAT3, ERK1/2, caspase 3, BCL2, and caspase 3 were determined by using Western blot analysis. One representative of 3 independent experiments is shown. **B**, RBL cells were sensitized with 100 ng/mL IgE for 2 hours, followed by a 5-minute DNP challenge. Twenty minutes before the addition of DNP, 40 μ mol/L U0126 was added to the medium. The levels of phospho-STAT3 727 (pS727-STAT3) and actin as a control in the cell lysates were determined by using Western blot analysis. One representative of 3 independent experiments is shown. **C**, RBL cells were treated as in Fig 4, B. The cells were fractionated into cytosol and mitochondria. The levels of phospho-STAT3 serine 727 (pS727-STAT3), caspase 3, and cytochrome C were determined by using Western blot analysis. One representative of 3 independent experiments is shown.

fractionated into cytosol and mitochondria, and additionally, mitoplasts were prepared from the mitochondrial fraction by using detergent treatment. PIAS3 content was determined by means of Western blot analysis. The purity of the cytosolic and mitochondrial fractions and of the mitoplasts was determined by using cytochrome c antibody as an intermembrane space marker, caspase 3 antibody as a cytosol marker, and pyruvate dehydrogenase antibody as a matrix marker. As shown in Fig 6, A, PIAS3 expression was detected in both the cytosolic and mitochondrial fractions and in the mitoplasts. The presence of PIAS3 in the mitoplasts indicated that it is localized within the mitochondria and is not derived from either the contaminating cytoplasm or the mitochondrial intermembrane space. ImageStream imaging flow cytometry was used to further evaluate the expression of PIAS3 in the mitochondria after immunologic activation and to quantitatively assess whether PIAS3 is translocated to the mitochondria. RBL cells were immunologically activated for 20 minutes, and quiescent cells were used as controls. Mitochondria were stained with mitotracker red, PIAS3 antibody was stained with DyLight 650, and cell nuclei were stained with Hoechst. As shown in Fig 6, B, the localization of PIAS3 into the mitochondria increased nearly 3-fold after 20 minutes of activation. Representative images of each treatment are shown.

RBL cells were transfected with a plasmid constructed with PIAS3 fused to a mitochondrial target sequence (PIAS3-mito) or empty plasmid (control) to determine the role played by

mitochondrial PIAS3 in OXPHOS. The translocation of the recombinant PIAS3-mito into the mitochondria was confirmed by means of Western blot analysis. The transfected cells were immunologically activated for 5 minutes, and ATP content and oxygen consumption were determined. As shown in Fig 6, C, transfection of PIAS3-mito resulted in a significant decrease in ATP levels of activated cells compared with those seen in control cells ($15.9\% \pm 1.6\%$ decrease, $P < .05$). Overexpression of PIAS3 targeted to the mitochondria did not change the oxygen consumption in activated cells compared with that seen in activated control cells (empty plasmid; Fig 6, D). RBL cells were transfected with PIAS3-mito or with the empty plasmid to further explore the role played by mitochondrial PIAS3 in degranulation. As shown in Fig 6, E, transfection of PIAS3-mito resulted in a significant decrease in degranulation ($67.5\% \pm 15.0\%$, $P < .05$). Complementary experiments were also performed to confirm that endogenous PIAS3 indeed regulates exocytosis in mast cells. BMDCs were transfected for 48 hours with either small interfering RNA (siRNA) against PIAS3 or with nonrelevant siRNA as a control. As shown in Fig 6, F, silencing of PIAS3 by siRNA led to a significant enhancement in degranulation levels of $66.4\% \pm 8.6\%$ ($P < .05$). The silencing of PIAS3 with its specific siRNA was confirmed by means of Western blot analysis. Similar results were achieved with RBL cells, as shown in Fig E8 in this article's Online Repository at www.jacionline.org.

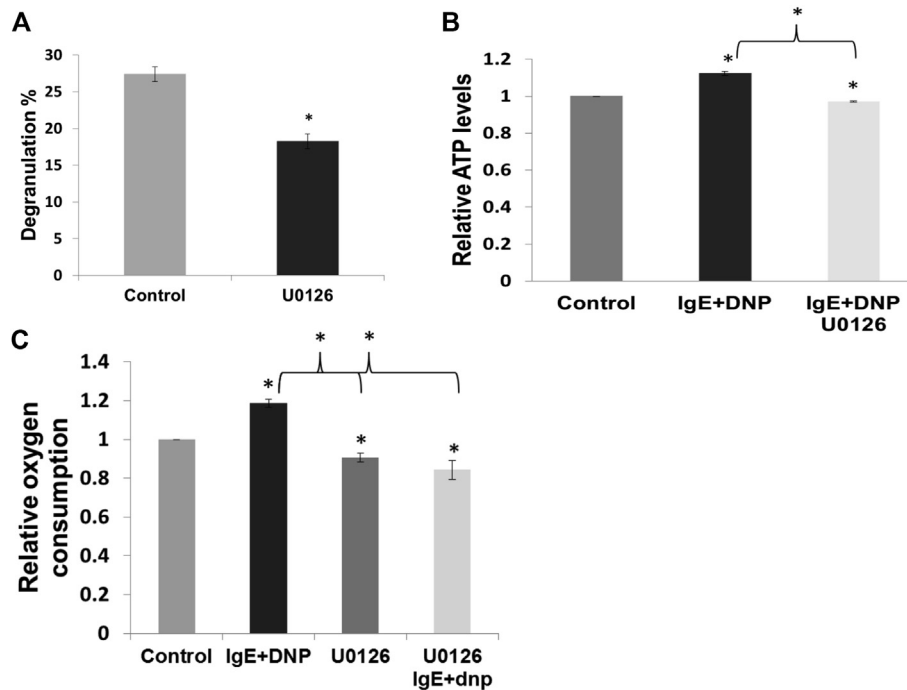


FIG 5. Effect of U0126 on mitochondrial characteristics. **A**, RBL cells were sensitized with 100 ng/mL IgE for 2 hours, followed by a 30-minute DNP challenge. Twenty minutes before the addition of DNP, 40 μ mol/L U0126 was added to the medium. β -Hexosaminidase release was then measured. Results represent means $\pm$ SEMs (n = 3). * P < .05. **B**, RBL cells were sensitized with 100 ng/mL IgE for 2 hours, followed by a 5-minute DNP challenge. Twenty minutes before the addition of DNP, 40 μ mol/L U0126 was added to the medium, and then the cells were lysed and analyzed for total cell ATP. Results represent means $\pm$ SEMs (n = 3). * P < .05. **C**, Oxygen consumption analysis. RBL cells were grown in 24-well plates and treated as in Fig 5, B, and then intracellular oxygen consumption was determined with the Seahorse Extracellular Flux Analyzer. Results represent means $\pm$ SEMs (n = 4). * P < .05.

Mitochondrial STAT3 in human cord blood–derived mast cells

Next we determined whether mitochondrial STAT3 plays a similar role to that described above for murine mast cells in human mast cells. Human cord blood–derived mast cells (CBMCs) were first sensitized with human myeloma IgE. Five minutes before activation with rabbit α -human-IgE, Stat3 or DMSO alone was added, and degranulation levels were determined after 30 minutes by measuring β -hexosaminidase release. As seen in Fig 7, the degranulation seen in control cells (20.9 ± 4.9) was completely abolished (3.5 ± 1.9) by incubation with Stat3.

In vivo experiments

STAT3's role *in vivo* was investigated by evaluating the response to allergic stimulation in Stat3-treated mice. The passive systemic IgE-mediated anaphylaxis model was used. Mice were injected (intravenously) with IgE anti-DNP. Twenty-four hours later, they were injected (intraperitoneally) with Stat3 (80 mg/kg) or corn oil, which was used as a control. Three and a half hours later, the mice were challenged with DNP for 1.5 minutes. Plasma was collected, and histamine levels were measured by using competitive ELISA. Histamine secretion in Stat3-treated mice was 43% less than that in control mice (P < .05, Fig 8). Injection of a lower dose of Stat3 (40 mg/kg) also resulted in a significant decrease (32.4%, P < .05, n = 21)

in histamine secretion after 2½ hours (data not shown). These results demonstrate the critical role of STAT3 in mast cell–dependent allergic reactions.

DISCUSSION

Clearly the mitochondrion plays a critical role in the function of immune-derived cells because T-cell activation requires mitochondrial translocation to the immunologic synapse³¹ and complex 3 activity was found to be essential for their activation.³² Recently, it has also been shown that human mast cells require mitochondrial translocation to exocytosis sites for degranulation and TNF secretion.^{1,2} The metabolic mitochondrial pathway involved in mast cell exocytosis is delineated in the present work for the first time. Although ATP has been shown to be essential for mast cell function,^{3–5} its source, as a result of glycolysis, mitochondrial OXPHOS, or possibly a combination of these events, has not been investigated. Furthermore, the effect of immunologic activation on ATP was previously unclear because ATP levels were measured only before immunologic activation.⁴ In the present study we demonstrated the pivotal role of mast cell mitochondria in ATP production during their immunologic activation. This was based on our observation that mast cell degranulation occurred even without glycolysis. Moreover, the findings that mitochondrial OXPHOS measured both by using ATP production and oxygen consumption were enhanced by immunologic activation strongly support our

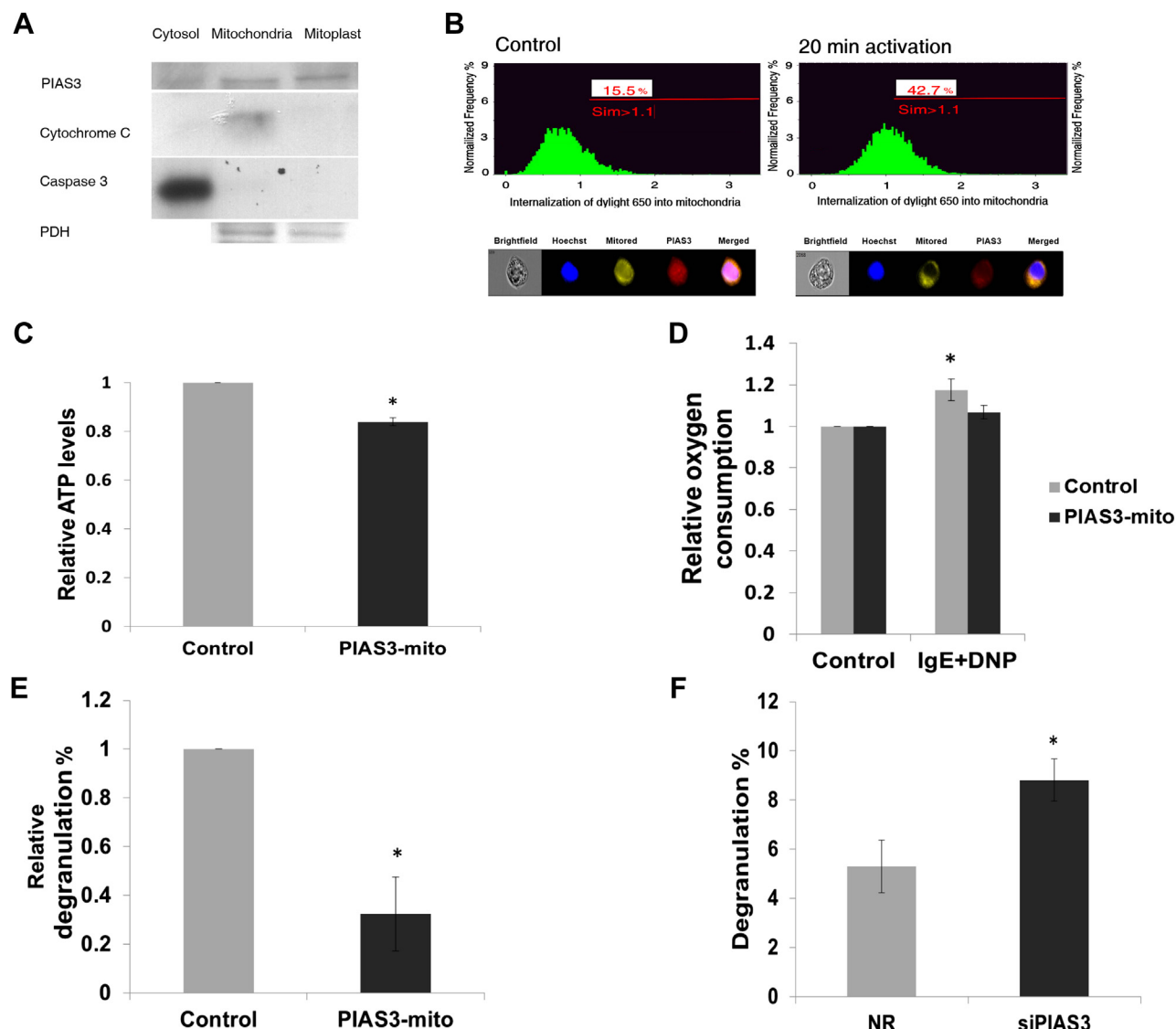


FIG 6. Effect of PIAS3 on mitochondrial characteristics. **A**, PIAS3 mitochondrial localization analysis. RBL cells were fractionated into cytosol and mitochondria. Mitoplasts were prepared by using digitonin treatment. Levels of PIAS3, caspase 3, pyruvate dehydrogenase (PDH), and cytochrome C were determined by using Western blot analysis. One representative of 3 independent experiments is shown. **B**, PIAS3 mitochondrial translocation quantification. RBL cells were activated (or not) with IgE, challenged with DNP for specific times, and then stained for PIAS3 by using anti-PIAS3 antibody followed by DyLight 650-conjugated anti-rabbit antibody together with Hoechst staining to visualize nuclei and mitotracker red to visualize mitochondria. Mitochondrial translocation was evaluated by using ImageStream flow cytometry. For each treatment, 10,000 cells were collected. Positive single cells were gated, and mitochondrial expression of PIAS3 was plotted. The nuclei staining area was omitted to reduce unspecific plotting. Representative images of each treatment are shown. **C**, ATP quantification analysis. RBL cells were transfected with empty plasmid control or MTS-PIAS3 plasmid. Cells were sensitized with 100 ng/mL IgE for 2 hours and then challenged with DNP for 5 minutes. No significant changes between empty plasmid and PIAS3-mito treatments were observed before activation (data not shown). Results represent means $\pm$ SEMs ($n = 5$). * $P < .05$. **D**, Oxygen consumption rate analysis. RBL cells were transfected with empty plasmid control or MTS-PIAS3 plasmid. Cells were sensitized with 100 ng/mL IgE for 2 hours and then challenged with DNP, and oxygen consumption was determined by using the Seahorse Extracellular Flux Analyzer. Results represent means $\pm$ SEMs ($n = 4$). * $P < .05$. **E**, Degranulation assay of RBL cells transfected with empty plasmid control or PIAS3-mito plasmid (relative to empty plasmid). Cells were sensitized with 100 ng/mL IgE for 2 hours, followed by a 30-minute challenge with DNP, and analyzed for β -hexosaminidase release. Results represent means $\pm$ SEMs ($n = 3$). * $P < .05$. **F**, Degranulation assay of BMDCs transfected with nonrelevant (NR) or with PIAS3 siRNA (siPIAS3). Cells were sensitized with 100 ng/mL IgE for 2 hours, followed by a 30-minute challenge with DNP, and analyzed for β -hexosaminidase release. Results represent means $\pm$ SEMs ($n = 3$). * $P < .05$.

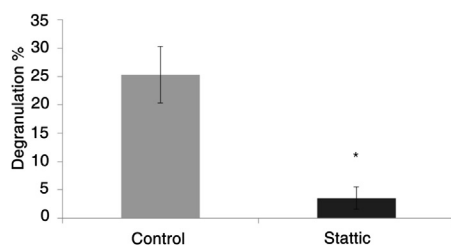


FIG 7. Effect of Stattic on CBMCs. CBMCs were sensitized with 0.3 μ g/mL human myeloma IgE. Five minutes before the addition of 5 μ g/mL rabbit α -human-IgE, 60 μ mol/L Stattic was added to the medium. β -Hexosaminidase release was then measured. Results represent means $\pm$ SEMs (n = 3). * P < .05.

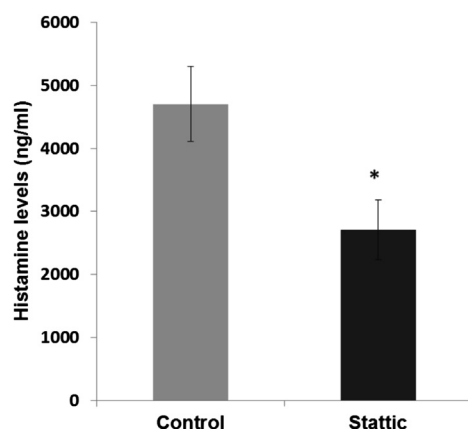


FIG 8. *In vivo* allergic response assay. Mice were sensitized with IgE anti-DNP through injection to the tail vein. Twenty-four hours later, mice were injected with corn oil or Stattic intraperitoneally for 3.5 hours and then challenged with DNP for 1.5 minutes. Plasma was collected, and histamine levels were measured by using competitive ELISA. Results represent means $\pm$ SEMs (n = 8). * P < .05.

hypothesis regarding the key role played by mitochondrial ATP in this physiologic process. The combination of these data indicate an active signaling pathway, which regulates mitochondrial ATP production for the initiation of mast cell exocytosis.

STAT3 is an essential protein in the immune system (reviewed by Yagil et al³³). Here we determined that mitochondrial STAT3 serine 727 phosphorylation occurs in mast cells during immunologic activation. It is important to note that this phosphorylation was not due to STAT3 translocation because the basal levels of mitochondrial STAT3 before and after activation were identical. Of all the serine kinases capable of phosphorylating STAT3, ERK1/2 is the best characterized protein expressed in the mitochondria.¹⁵⁻¹⁷ Furthermore, our results demonstrate that ERK1/2 is the exclusive kinase of STAT3 serine 727 in mast cells because a significant decrease in STAT3 727 serine phosphorylation was observed in U0126-treated cells, even after activation.

For the first time, we have shown ERK1/2 translocation to the mitochondria during mast cell activation. Interestingly, although U0126 is a well-established inhibitor of mast cell function,²⁶⁻³⁰ the possibility that it functions through an effect on mitochondrial activity has not yet been reported. The findings that mitochondrial OXPHOS as measured by both

ATP production and oxygen consumption was decreased by U0126 treatment strongly support our hypothesis that the inhibitory effect of U0126 on degranulation could be mediated through mitochondrial OXPHOS inhibition. The decreased mitochondrial STAT3 serine 727 phosphorylation seen during mast cell activation in cells treated with U0126 suggests the mitochondrion as a target of U0126. Thus the Fc ϵ RI-mediated mitogen-activated protein kinase pathway emerges as a regulator of mitochondrial ATP production during the initiation of mast cell exocytosis and connects a signaling pathway to a metabolic pathway.

Our initial hypothesis was that mitochondrial STAT3 activity is the major mediator of ATP production by OXPHOS during mast cell activation. To prove this, we used Stattic, a small-molecule selective inhibitor of STAT3,^{24,34} and an additional STAT3 inhibitor, C188-9,²⁵ in a timeframe that did not permit nuclear influence and evaluated its effect on mitochondrial activity and mast cell function. In support of the hypothesis, Stattic severely impaired mitochondrial function because both ATP production and oxygen consumption decreased significantly. This interference in mitochondrial activity was mediated through inhibition of complex 3 activity, which correlates with the effect of STAT3 inhibition.⁷ Therefore STAT3 serine 727 phosphorylation in mast cells could serve as a metabolic switch for the mitochondria to initiate ATP production.

After observing the effect of STAT3 inhibition on mitochondrial activity, we hypothesized that STAT3 inhibition would affect mast cell function. On the basis of our observation that mast cell degranulation and cytokine secretion were completely abolished both by Stattic and C188-9 treatment, mitochondrial STAT3 emerges as a key mediator of the onset of exocytosis. It is important to emphasize that this effect is not a result of apoptosis because no caspase 3 cleavage was observed. Furthermore, this effect is selective for STAT3 inhibition because the use of a different STAT3 inhibitor reproduced the degranulation results. In addition, the fact that STAT3 depletion inhibited both degranulation and cytokine secretion clearly indicates that this is a general phenomenon of mast cell exocytosis.

Having established the importance of mitochondrial STAT3 in exocytosis in BMMC and in RBL cells, we found that this role extends to human mast cells because degranulation in IgE-activated human CBMCs was abolished by Stattic. Because this suggests that STAT3 could be a new target for treating allergy, we investigated STAT3 inhibition *in vivo*. In a previously established acute systemic anaphylaxis model, a marked decrease in histamine secretion by Stattic-treated mice compared with that seen in control mice was observed; in other words, Stattic reduced the response to allergic stimulation.

Seeking the endogenous terminator of this signaling, we investigated PIAS3 because it is the main endogenous inhibitor of STAT3. PIAS3 expression in the mitochondria has not previously been studied. Notably, PIAS3 does not contain a consensus mitochondrial target sequence according to the PIAS3 protein accession number (GenBank: EDL85611.1). However, a computer analysis (MitoProt II, version 1.101) found that PIAS3 has a 0.8995 probability of 1 of being present in the mitochondria. We have now confirmed the presence of PIAS3 within the mitochondria and also observed an increase in its protein level after mast cell activation. Furthermore, by targeting PIAS3 to the mitochondria, we have shown that

mitochondrial ATP production during mast cell immunologic activation was abolished. Therefore we conclude that PIAS3 serves as a major regulator of mitochondrial function. When PIAS3 turned off the metabolic ATP production switch, a significant decrease in mast cell degranulation followed. Moreover, PIAS3 silencing by siRNA resulted in enhanced degranulation. These complementary findings indicate that manipulation of PIAS3 translocation into the mitochondria could affect the allergic response.

To conclude, mitochondrial STAT3 emerges as an important regulator of mast cell function. Inhibiting mitochondrial STAT3 either by small molecules or by PIAS3 downregulates mitochondrial activity, which is essential for mast cell function. In a very recent article Siegel et al³⁵ reported on the diminished allergic disease in patients with STAT3 mutations and concluded that future study is required to identify the proximal target of STAT3 in FcεRI signaling. We have now identified mitochondrial function as the proximal target of STAT3 in FcεRI signaling. Together, these findings indicate that STAT3 has a critical role in mast cell function and therefore could emerge as a new target for the manipulation of allergic diseases.

We thank Tzemach Aouizerath for providing technical assistance.

Key messages

- Mitochondrial STAT3 is a key regulator of mast cell exocytosis.
- IgE-antigen activation of mast cells is followed by ERK1/2 mitochondrial translocation, which phosphorylates mitochondrial STAT3 on serine 727.
- Mitochondrial PIAS3 inhibits OXPHOS and mast cell degranulation.

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METHODS

Mice

C3H 6- to 7-week-old male and female wild-type mice 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.

Antibodies

The antibody mouse anti-PIAS3 was purchased from Sigma-Aldrich (St Louis, Mo). Rabbit anti-PIAS3 antibody and goat anti-rabbit IgG H&L (DyLight 650) were purchased from Abcam (Cambridge, Mass). Anti-STAT3, anti-phospho-STAT3 serine 727, anti-phospho-ERK, and anti-cytochrome c antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, Calif). Anti-caspase 3, anti-ERK1/2, and anti-pyruvate dehydrogenase α 1 subunit were purchased from Cell Signaling Technology (Beverly, Mass). Donkey anti-mouse IgG H&L (HRP) and donkey anti-rabbit IgG H&L (HRP) were purchased from Abcam.

Chemical inhibitor treatment

Statice and U0126 were purchased from Sigma-Aldrich. C188-9 was purchased from Merck-Millipore (Billerica, Mass). All inhibitors were dissolved in 100% DMSO (Sigma-Aldrich), according to the manufacturer's instructions.

Cell culture

RBL-2H3 cells were maintained in RPMI 1640 medium, as previously described.^{E1} Bone marrow was isolated from 5- to 6-week-old mice, and BMMCs were cultured in RPMI medium supplemented with 20 ng/mL IL-3 and 20 ng/mL stem cell factor, as previously described.^{E2,E3} Cells were generally grown for a minimum of 4 weeks and used when greater than 90% of the population expressed Fc ϵ RI. BMMCs and RBL cells were sensitized first with anti-DNP IgE mAb (SPE-7, Sigma-Aldrich), washed in PBS and then challenged with DNP (Sigma-Aldrich). IgE antibody was centrifuged (18,000g for 5 minutes) before use to remove aggregates. Cell activation was verified by demonstrating increased phosphorylation of ERK1/2 after the antigen challenge. For glycolysis elimination, cells were maintained in glucose-free RPMI supplemented with 10% dialyzed FCS and 5 mmol/L galactose.

CBMC culture and activation

CBMCs were prepared from umbilical cord blood of healthy donors according to a well-described method.^{E4} Briefly, fresh cord blood diluted in Hank's solution was loaded on Ficoll-Hypaque (Amersham Pharmacia Biotech AB, Uppsala, Sweden) and centrifuged (350g for 25 minutes). Mononuclear cells were washed twice and resuspended in MEM- α medium supplemented with 10% heat-inactivated FBS, 100 U/mL penicillin, 100 μ g/mL streptomycin, and 10 μ g/mL ribonucleosides. All media and supplements were purchased from Biological Industries (Beit Haemek, Israel). CBMC growth media also contained recombinant human stem cell factor (100 ng/mL; PeproTech, Rocky Hill, NJ), human IL-6 (10 ng/mL, PeproTech), and prostaglandin E₂ (0.3 μ mol/L, Sigma-Aldrich). Cells were maintained in a humidified incubator (37°C at 5% CO₂), with media replaced on a weekly basis. After a 6- to 8-week culture period, the CBMCs were examined for maturity by using acidic toluidine blue staining (positive >90%) and for viability by using Trypan blue exclusion (negative >95%) and used thereafter.

For activation experiments, CBMCs were sensitized by the addition of human myeloma IgE (0.3 μ g/mL; Calbiochem-Merck, Schwalbach, Germany) and human IL-4 (0.10 ng/mL, PeproTech) to cell cultures for 4 to 5 days in a humidified incubator (37°C at 5% CO₂). CBMCs (1 $\times$ 10⁷/100 μ L) were washed and resuspended in TG++ buffer (0.2% gelatin, 137 mmol/L NaCl, 12 mmol/L NaHCO₃, 5.5 mmol/L L-glucose, 2 mmol/L KCl, 0.3 mmol/L Na₂HPO₄, 1.8 mmol/L CaCl₂, and 0.9 mmol/L MgCl₂). The cells were then activated by means of incubation with rabbit α -human-IgE (5 μ g/mL; Dako, Glostrup, Denmark) for 30 minutes at 37°C. In inhibition experiments 60 μ mol/L Statice or DMSO alone (control) was added for 5 minutes, and cells were centrifuged, resuspended in new TG++ buffer,

and activated as above. Supernatants and cell pellets were collected for assessment of β -hexosaminidase release, as described below.

Mitochondrial isolation

RBL cells were washed once in cold PBS before homogenization. Cells (9×10^6) were homogenized in buffer A (320 mmol/L sucrose, 5 mmol/L Tris, and 2 mmol/L ethyleneglycol-bis-[β -aminoethylether]-N,N,N',N'-tetraacetic acid, pH 7.4) with 10 strokes of a Teflon Dounce homogenizer. The procedure was performed at 4°C. The homogenate was centrifuged for 3 minutes at 2000g, and the supernatant was kept. This was repeated twice, and the supernatants were combined and then centrifuged for 3 minutes at 2000g to remove the nuclei and cell debris. The supernatant was transferred to a clean tube and centrifuged for 10 minutes at 12,000g to pellet mitochondria, washed twice, resuspended in a small volume of buffer A, and stored at -70°C. The mitochondrial pellet was resuspended in buffer A with the addition of 0.02% (wt/vol) digitonin to prepare mitoplasts. The pellet was resuspended and washed twice in large volumes of digitonin-free buffer A.

Measurements of enzymatic activity

The enzymatic activities of respiratory chain complexes were measured at 37°C by using standard spectrophotometric methods, as previously described.^{E5,E6} Briefly, complex II was measured as SDH based on the succinate-mediated phenazine methosulfate reduction of dichloroindophenol at 600 nm. Complex 2+3 was measured as succinate cytochrome c reductase and after the reduction of oxidized cytochrome c at 550 nm. Complex 4 (cytochrome c oxidase) was measured after the oxidation of reduced cytochrome c at 550 nm.

Citrate synthase, an ubiquitous mitochondrial matrix enzyme serving as a control, was measured in the presence of acetyl-CoA and oxaloacetate by monitoring the liberation of CoASH coupled to 5',5'-dithiobis (2-nitrobenzoic) acid at 412 nm. Activities are presented as a ratio to citrate synthase. Protein concentrations were determined by using the Lowry method and calculated according to a BSA standard curve.

Expression constructs

Mouse PIAS3 was constructed with a mitochondrial target sequence in the pCMV/myc/mito vector (Invitrogen, Carlsbad, Calif).

siRNA

PIAS3 siGENOME SMARTpool siRNA oligomers for rat were purchased from Dharmacon (Thermo Fischer, Waltham, Mass). The PIAS3 TriFECTa RNAi Kit for mouse was purchased from IDT (Integrated DNA Technologies, Jerusalem, Israel).

β -Hexosaminidase release assay (degranulation assay)

RBL cells (1×10^4) or BMMCs (3×10^4) were immunologically activated, as described above. After IgE incubation, the cells were washed 3 times with 5 mL of degranulation buffer (130 mmol/L NaCl, 5 mmol/L KCl, 5.6 mmol/L glucose, 1 mmol/L MgCl₂, 1.2 mmol/L CaCl₂, 10 mmol/L HEPES [pH 7.4], and 0.1% BSA) and resuspended in 200 μ L of the same buffer with DNP for 30 minutes. β -Hexosaminidase release was determined in triplicates in a 96-well plate. Aliquots (20 μ L) of supernatants and cell lysates were incubated for 30 minutes with 100 μ L of substrate solution (1.3 mg/mL *p*-nitrophenyl-b-D-2-acetamido-2-deoxyglucopyranoside in 0.1 mol/L citrate, pH 4.5). The reaction was stopped by the addition of 200 μ L of 0.2 mol/L glycine (pH 10.7). The plate OD was read in an ELISA reader at a wavelength of 405 nm. Percentage release values for each experimental condition were calculated.^{E3}

ATP determination

ATP levels were measured by using the ATPlite luminescence-based assay, according to the manufacturer's instructions (PerkinElmer, Waltham, Mass).

Oxygen consumption

The oxygen consumption rate was measured with an XF24 extracellular flux analyzer (Seahorse Biosciences, North Billerica, Mass).

Gel electrophoresis and Western blots

RBL cells and mitochondrial pellets were lysed by the addition of lysis buffer (50 mmol/L Tris-HCl [pH 7.4], 1% Nonidet P-40, 0.25% Na-deoxycholate, 150 mmol/L NaCl, 1 mmol/L EDTA, and 1 mmol/L phenylmethylsulfonyl fluoride) supplemented with 15 μ L of protease inhibitor (Sigma-Aldrich). Cells and mitochondria were vortexed and incubated on ice for 15 minutes. The lysates were then centrifuged at 14,000g for 15 minutes, and pellets were discarded. The protein concentration of each sample was determined by using Bradford Reagent (Sigma-Aldrich). Proteins were resolved by using 10% to 15% SDS-PAGE under reducing conditions and transferred to polyvinylidene difluoride membranes (Millipore, Billerica, Mass). The membranes were incubated in 5% fat-free milk and TBST (10 mmol/L Tris-HCl [pH 7.4], 150 mmol/L NaCl, and 0.1% Tween 20) for 1 hour and then in 5% BSA and TBST containing various dilutions of primary antibodies for 18 hours at 4°C. The membranes were washed 3 times with TBST for 5 minutes before and after each incubation with secondary antibody. The proteins were detected with the appropriate secondary antibody (1 hour at room temperature) coupled to horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse antibody and visualized by means of chemiluminescence, according to the manufacturer's instructions (Pierce, Cheshire, United Kingdom).

Transfection

Amata Nucleofector technology (Amata, Cologne, Germany) was used to transfect cells. A total of 2×10^6 RBLs or 1.5×10^6 BMMCs were transfected with 3 μ g of the selected siRNA oligonucleotide or scrambled nonrelevant siRNA, according to the manufacturer's protocol. For plasmid transfection, 2 μ g was used. Briefly, the cells were resuspended in 100 μ L of Ingenio solution (Mirus, Madison, Wisconsin), either DNA (plasmid) or RNA was added, and the mixture was transferred to an electroporation cuvette. Electroporation was performed with the T-20 program for RBLs and T-17 for BMMCs.^{E3,E7}

ImageStream flow cytometry

For ImageStream analysis (Amnis, Seattle, Wash), RBL cells were incubated with anti-DNP IgE mAb and 1.5 μ mol/L MitoTracker Red CM-H2XROS (Molecular Probes, Eugene, Ore) for 90 minutes. Cells were washed 3 times in PBS and then challenged with DNP for the indicated times. Cells were washed 3 times in PBS, fixed in 10% paraformaldehyde, permeabilized with PBS 0.1% triton, and blocked in PBS 0.5% BSA. Anti-PIAS3 antibody and goat anti-rabbit IgG H&L were used to stain PIAS3. Hoechst 33258 (Sigma-Aldrich) was used to stain nuclei. Fluorescence imaging was carried out with the ImageStream flow cytometer (Amnis). Data analysis was performed with IDEAS software, version 1.10, with compensation according to the software standards.

An ImageStream instrument automatically acquires up to 6 different spatially registered images (bright-field, dark-field, and 4 fluorescent images)

per cell at rates on the order of thousands of objects per minute by using a digital charge-coupled device camera. The digital imagery obtained is analyzed by using the IDEAS statistical image analysis program, which provides tools for the objective numeric scoring and discrimination of cells based on the characteristics of their imagery. The ability to numerically score large numbers of automatically acquired images is ideally suited to the analysis of mitochondrial translocation within primary immune system cells.

Measurements of secreted mediators

BMMCs (1×10^6) were immunologically activated, as described above. After IgE incubation, cells were washed 3 times in PBS and incubated with DNP (Sigma-Aldrich) for 30 minutes in complete growth medium. Supernatant was collected, and granzyme B and TNF- α content was measured with ELISA kits (eBioscience, San Diego, Calif).

In vivo histamine release assay

Wild-type C3H mice were sensitized with 3 μ g of murine IgE anti-DNP mAb SPE-7 by means of intravenous injection in the tail vein. Twenty-four hours later, mice were treated with 80 mg/kg static dissolved in corn oil or corn oil alone for 3.5 hours, and then mice were challenged with intravenous injection of DNP (250 μ g per mouse). One hundred microliters of blood was collected immediately and added to an equal volume of pre-cooled Alsever solution. Blood was centrifuged at 2000g for 20 minutes, and the plasma supernatant was collected. Plasma histamine levels were determined by using a competitive ELISA (IBL International GmbH, Hamburg, Germany).

Statistical analysis

Either the 1-sample *t* test or the 2-tailed Student *t* test was performed, when appropriate. Data are reported as means $\pm$ SEMs.

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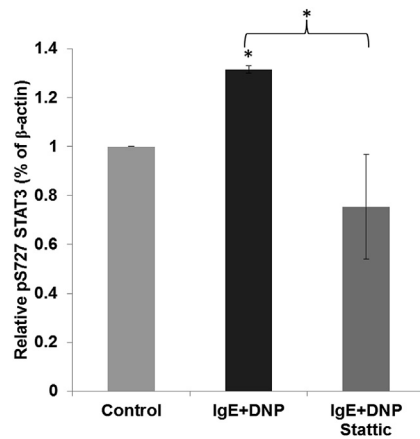


FIG E1. Densitometric quantification of STAT3 serine 727 phosphorylation inhibition by Stattic. The protein bands were quantified in Image J software, and β -actin was used as the internal control. Results represent means $\pm$ SEMs ($n = 3$). * $P < .05$.

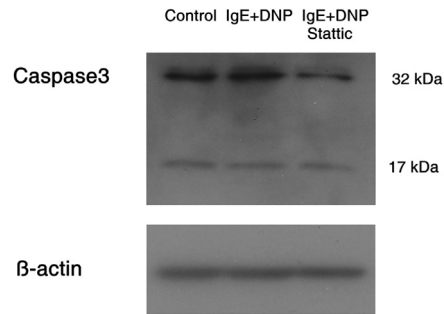


FIG E2. Effect of Stattic on apoptosis in activated RBL cells. RBL cells were sensitized with 100 ng/mL IgE for 2 hours, followed by a 30-minute DNP challenge. Twenty minutes before the addition of DNP, 60 μ mol/L Stattic was added to the medium. Levels of caspase 3 were determined by using Western blot analysis. One representative of 3 independent experiments is shown.

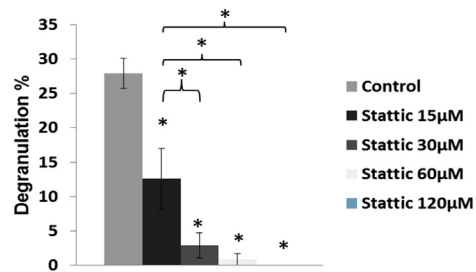


FIG E3. Effect of Stattic on RBL degranulation. RBL cells were sensitized with 100 ng/mL IgE for 2 hours, followed by a 30-minute DNP challenge. Twenty minutes before the addition of DNP, the stated concentrations of the STAT3 inhibitor Stattic were added to the medium. β -Hexosaminidase release was then measured. Results represent means $\pm$ SEMs ($n = 3$). * $P < .05$.

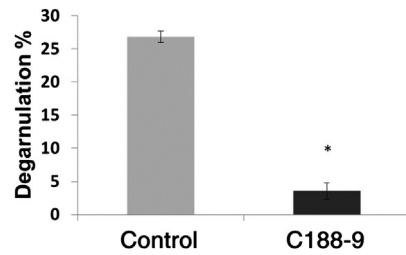


FIG E4. Effect of C188-9 on RBL degranulation. RBL cells were sensitized with 100 ng/mL IgE for 2 hours, followed by a 30-minute DNP challenge. Ten minutes before the addition of DNP, 250 μ mol/L of the STAT3 inhibitor C188-9 was added to the medium. β -Hexosaminidase release was then measured. Results represent means $\pm$ SEMs ($n = 3$). * $P < .05$.

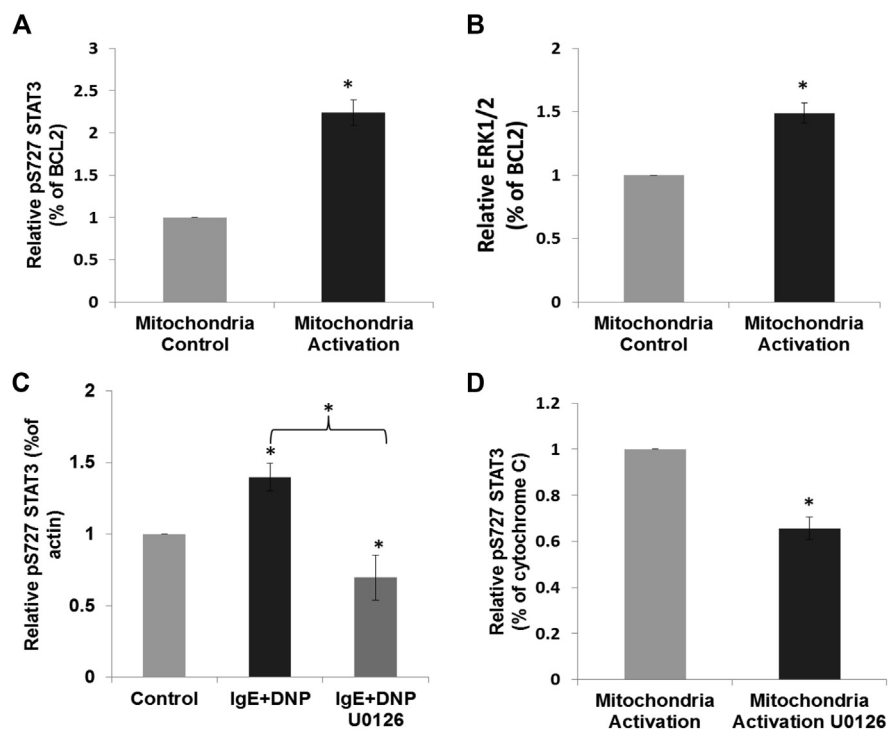


FIG E5. Densitometric quantification of mitochondrial STAT3 serine 727 ERK1/2-dependent phosphorylation during RBL activation. The protein bands were quantified in Image J software. **A**, Mitochondrial STAT serine 727 phosphorylation. **B**, Mitochondrial ERK1/2 expression. **C**, Total STAT serine 727 phosphorylation. **D**, Mitochondrial STAT serine 727 phosphorylation.

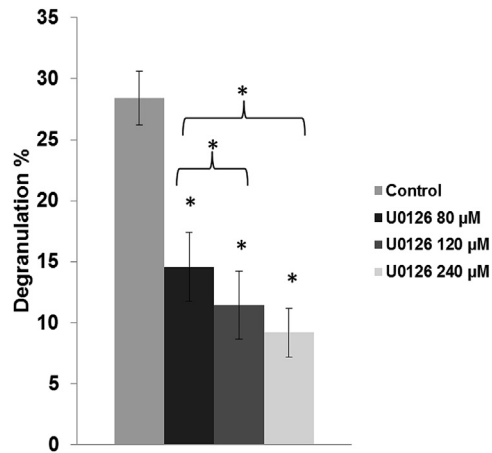


FIG E6. Effect of U0126 on RBL degranulation. RBL cells were sensitized with 100 ng/mL IgE for 2 hours, followed by a 30-minute DNP challenge. Twenty minutes before the addition of DNP, the stated concentrations of U0126 were added to the medium. β -Hexosaminidase release was then measured. Results represent means $\pm$ SEMs ($n = 3$). * $P < .05$.

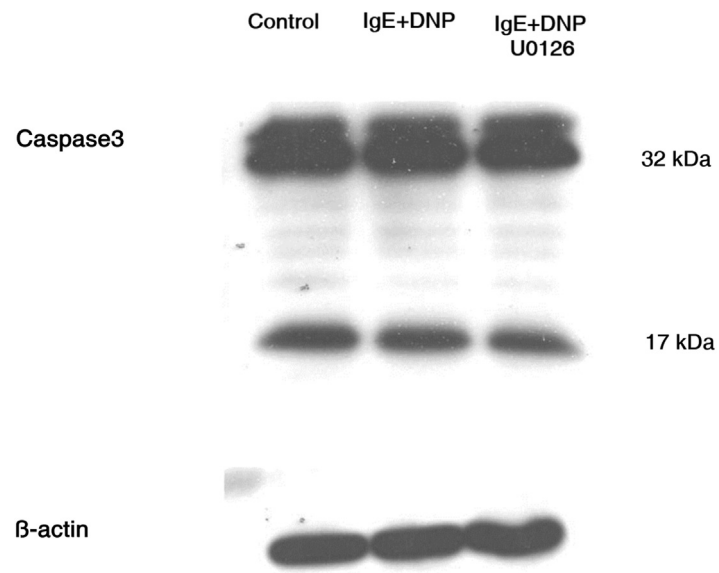


FIG E7. Effect of U0126 on apoptosis in activated RBL cells. RBL cells were sensitized with 100 ng/mL IgE for 2 hours, followed by a 5-minute DNP challenge. Twenty minutes before the addition of DNP, 40 μ mol/L U0126 was added to the medium. Levels of caspase 3 were determined by using Western blot analysis. One representative of 3 independent experiments is shown.

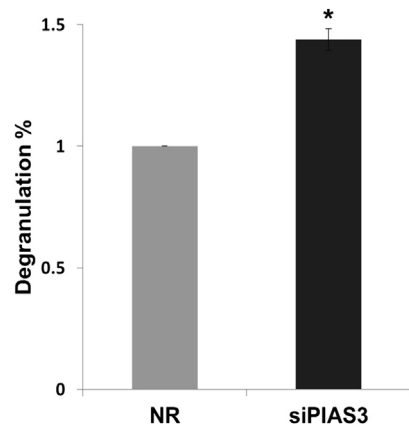


FIG E8. Effect of PIAS3 on RBL degranulation: degranulation assay of RBL cells transfected with nonrelevant or PIAS3 siRNA. Cells were sensitized with 100 ng/mL IgE for 2 hours, followed by a 30-minute challenge with DNP, and β -hexosaminidase release was measured. Results represent means $\pm$ SEMs ($n = 3$). * $P < .05$.